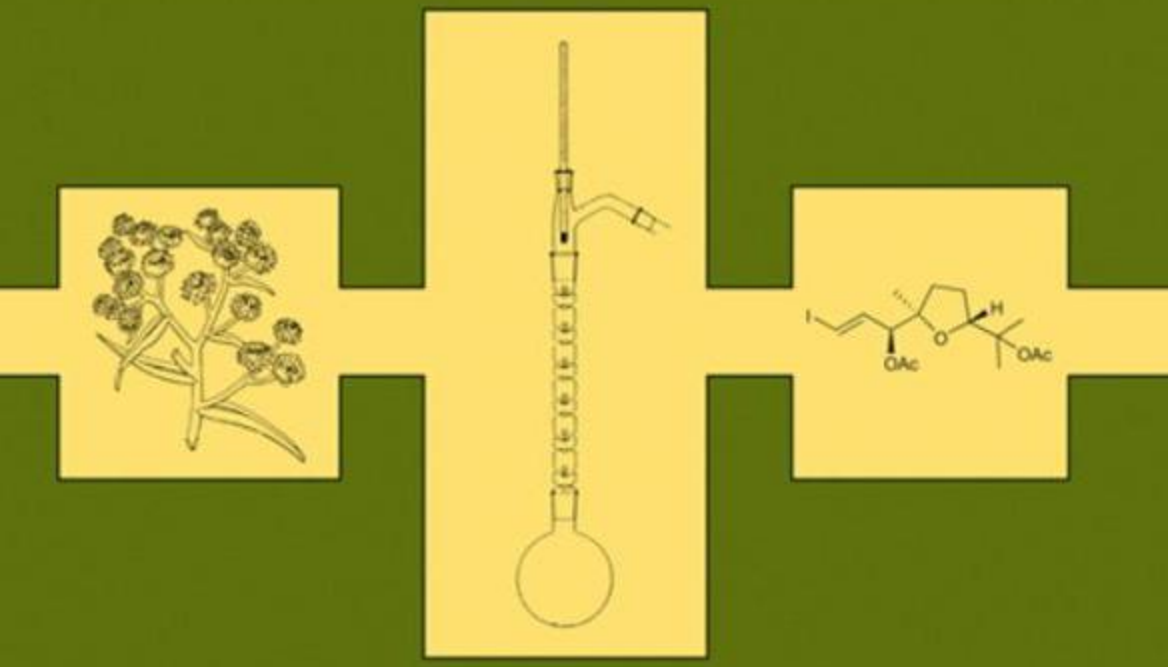
Natural Products Chemistry

Atta-ur-Rahman, FRS
Editor



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Bioactive Natural Products (Part O)

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PREFACE

This volume of "Studies in Natural Product Chemistry" represents the 35th of this series which I initiated, the first volume of which was published in 1988. It also represents the 14th volume devoted to bioactive natural products. The first seven reviews cover interesting recent developments in the field of bioactive marine natural products. The article by Little and coworker describes synthetic approaches to thyrsiferol and its analogues along with their biological activities. Marine invertebrates such as ascidians, sponges etc. are an important source of bioactive secondary metabolites. Ueda and coworker describe the isolation, structure elucidation, bioactivity and synthetic approaches to bioactive metabolites from marine invertebrates from Okinawan waters. Another article by Martinez and coworkers describes recent developments on antiviral products from marine sources, particularly from invertebrates such as sponges, tunicates, bryozoans and molluscs as well as from marine bacteria and cyanobacteria. Kalinin and coworkers present a comprehensive review on triterpene glycosides from sea cucumbers, including their functions and biological activities. The article by Liu and coworker focuses on new compounds with anti-tumor activity, enzyme inhibitors, anti-virus and other bioactive metabolites from marine microorganism including fungi, bacteria, actinomycetes and cyanobacteria reported between 2000 and 2005. The review by Maier is concerned with biological activities of sulfated glycosides from echinoderms. It particularly focuses on the structural characteristics and biological properties of saponins isolated from starfishes and sea cucumbers in the last five years with special reference to the structural elucidation and evaluation of antifungal, cytotoxic and antiviral properties. Another interesting review by Turk and coworkers is concerned with the synthesis, biological activity and potential uses of 3-akylpyridinium and 3-alkylpyridine compounds from marine sponges.

Novel Domino reactions involving acid-catalyzed intermolecular cyclization have been used as a viable synthetic tool for the stereospecific formation of different classes of polycyclic natural products. This is discussed in the review by Bhar and coworker by using these reactions for the synthesis of bioactive diterpenoids and alkaloids. About 1/3rd of all the diseases worldwide are due to infectious diseases. There have been therefore constant efforts to discover new anti-microbial compounds that have a broad range of activities especially against multidrug-resistant strains of microbes. The article by Mahady and coworkers focuses on medicinal plants and phytochemicals active against a wide range of gram-positive and gram-negative bacteria. The potential of medicinal plants of the Anthemideae tribe, both as potential antimicrobial crude drugs as well as sources for natural compounds that act as new anti-infectious agents, is described in the review by Martinez and coworkers. The article by Maurya reviews compounds with antiosteoporotic activity. Rodrigues and coworkers have presented an interesting review of plants with possible anxiolytic and/or hypnotic effects.

Another article by Daffre and coworkers reviews recent developments in the field of bioactive natural peptides including their characterization and biological activities. Many cyclic lipopeptide antibiotics have been discovered, mainly from microorganisms, algae and plants that often exhibit interesting and useful biological activities. The article by Hashizume and coworker describes the chemistry, biological activities and pharmacology of natural cyclic lipopeptides. A large number of *Salvia* diterpenoids have exhibited interesting biological activities e.g. anti-tuberculous, antitumor, antimicrobial, antibacterial, antileishmanial and antispasmolytic activities. This is discussed in the review by

Kabouche and coworkers. Finally Rezanka and coauthor present a comprehensive review on biologically active compounds of semi-metals such as boron, silicon, arsenic, selenium and tellurium.

It is hoped that this volume will be another useful addition to this Series and be of considerable interest to a large number of scientists working on bioactive compounds with potential use in medicine.

We would like to express our thanks to Mr. Shamsher Ali for his assistance in the preparation of the index. We are also grateful to Mr. Wasim Ahmad for composing and typing and to Mr. Mahmood Alam for the editorial assistance.

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SYNTHETIC EFFORTS TOWARD, AND BIOLOGICAL ACTIVITY OF, THYRSIFEROL AND STRUCTURALLY-RELATED ANALOGUES

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ABSTRACT: Marine natural products have played an important role in the discovery of novel biologically active compounds. Thyrsiferol was originally isolated off the coast of New Zealand from a marine red algae of the genus *Laurencia*. Several analogues having similar structural features have been identified and their biological properties investigated and identified. The array of activities is significant and includes: cytotoxic, anti-viral, and anti-tumor activity, the specific inhibition of protein serine/threonine phosphatase 2A, and apoptotic cell death in human leukemic T- and B-cell lines. Therefore, it is of interest to design and develop methods that would make these compounds accessible in the laboratory through synthetic organic chemistry. This review will discuss the biological properties and synthetic endeavors that have been used to access thyrsiferol and related analogues.

THYRSIFEROL AND ANALOGUES

Introduction

The marine ecosystem has revealed a multitude of bioactive natural products [1]. These marine-derived compounds are found primarily among soft corals, sponges, algae, and bacteria. Their therapeutic properties include significant activities in antitumor, anti-inflammatory, analgesia, allergy, and anti-viral assays. Although there is no single marine-derived natural product that has become a pharmaceutical drug as of 2004, a large number of compounds of marine origin are currently undergoing clinical trials. Bryostatin I (phase II), ecteinascidin 743 (phase II/III), and discodermolide (phase I), for example, are currently under clinical investigation for the treatment of cancer [2].

Hence, marine natural products represent a valuable foundation for the discovery of novel biologically active compounds. The potential therapeutic applications provided by these molecules along with their unique structural features have encouraged substantial scientific interest and investigations.

Squalene-derived polyethers encompass a unique class of marine natural products displaying a broad array of bioactivities [3]. These triterpenoids have been isolated primarily from *Laurencia*, a red alga found in several geographic locations. The next section of this chapter will serve to introduce the reader to the isolation, characterization, structural features, and pharmacological profiles of marine polyoxygenated triterpenoid ethers isolated from *Laurencia*.

Isolation and Characterization of Triterpenoids from *Laurencia*

Thyrsiferol [**1**, Fig. (**1**)] was isolated from the red algae *Laurencia thyrsifera* that was collected off the coast of New Zealand. It constitutes the first example of a triterpenoid squalene-derived polyether of marine origin containing a dioxabicyclo[4.4.0]decane B-C framework. The unique framework of thyrsiferol consists of a central *trans*-fused pyranopyran unit, an appended cyclic bromo ether and an aliphatic side chain connecting the central unit to a *trans*-tetrahydrofuran ring. Isolation and spectroscopic characterization of thyrsiferol was first carried out by Munro *et al* in 1978 [4]. An x-ray crystallographic analysis of its C_{18}-acetate derivative established its chemical structure and assigned each of the relative stereocenters. The absolute stereochemistry was not determined at the time of the isolation, but it was elucidated a few years later when venustatriol (**2**) was characterized [5].

The X-ray crystallographic analysis of thyrsiferol 18-acetate (**5**) revealed a strained tetrahydropyran ring C in a twist-boat conformation so as to avoid 1,3-diaxial interactions between the methyl groups at C_{10} and C_{15}. Initial biological studies of the natural product by Munro *et al* did not reveal any significant pharmacological activity [4].

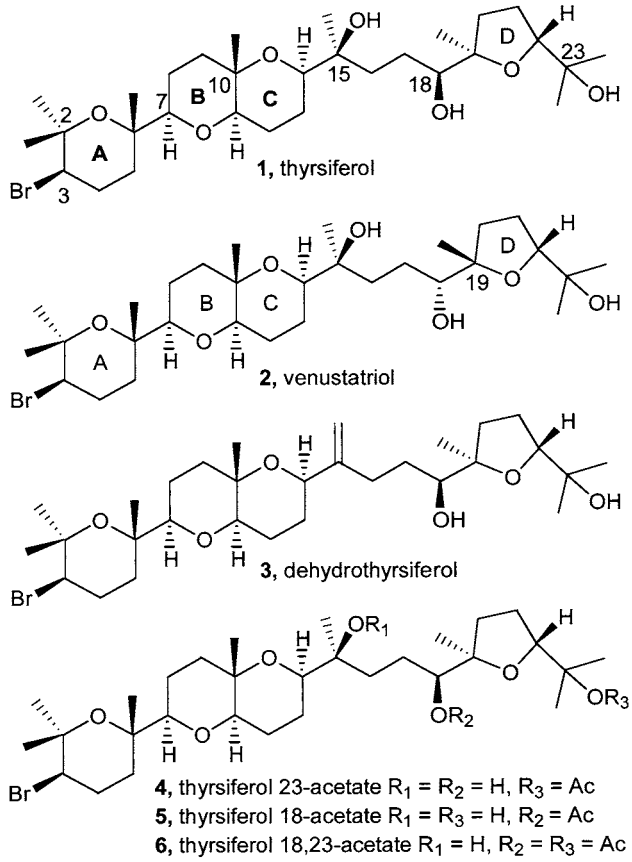


Fig. (1). Structure of thyrsiferol and analogues

Dehydrothyrsiferol (**3**), an analogue of thyrsiferol (**1**), was the next marine metabolite discovered from *Laurencia.* In an effort to assess halogen-based secondary metabolite synthesis, Norte and co-workers isolated compound **3** in 1984 as a white crystalline solid [6]. This natural product was found in *Laurencia pinnatifida* located on the Cannary Islands of Spain. Its chemical structure was verified via chemical transformation into thyrsiferol (**1**).

A year later, the crude extracts of *Laurencia obtusa,* obtained off the coast of Japan, were shown to exhibit a strong cytotoxic activity against P-388 cells. Purification of the crude material and structural elucidation revealed the structure of thyrsiferol 23-acetate (**4**) [7].

Treatment of **4** with K_2CO_3 in MeOH yielded a product whose spectroscopic data were identical to thyrsiferol (**1**).

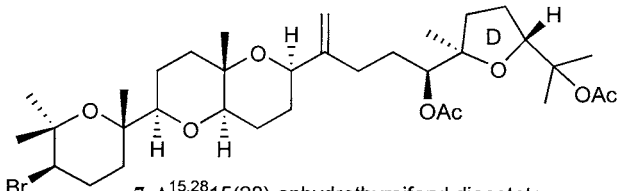
Compound **4** displayed a remarkable *in vitro* cytotoxic activity (ED_{50} of 0.3 ng/mL) against the P-388 cancer cell line as compared to structurally similar derivatives (Table 1) [7].

Table 1. Cytotoxicity against P-388 cancer cell line

Compound	ED_{50} (ng/mL)
thyrsiferyl 23-acetate (**4**)	0.3
thyrsiferol (**1**)	10
thyrsiferyl 18-acetate (**5**)	300
thyrsiferyl 18,23-acetate (**6**)	520

In 1986, venustatriol (**2**) was isolated from the red algae *Laurencia venusta* [5]. This compound was shown to possess many of the same structural features as thyrsiferol (**1**), with the exception of the stereocenters at C_{18} and C_{19} as indicated in Fig. (**1**). At the time of its isolation, venustatriol (**2**) was reported to display anti-viral activity against vesicular stomatitis virus (VSV) and herpes simplex virus type 1 (HSV-1). The absolute configuration of venustatriol (**2**) was verified by x-ray crystallography, an experiment that facilitated the assignment of the absolute stereochemistry of thyrsiferol (**1**).

Similar bromine-containing polyethers continued to be isolated in the ensuing years. From *L. obtusa* for instance, compounds 15(28)-anhydrothyrsiferyl diacetate (**7**), 15(16)-anhydrothyrsiferyl diacetate (**8**), and magireols A (**9**), B (**10**), and C (**11**) were discovered, Fig. (**2**) [8].



7, $\Delta^{15,28}$15(28)-anhydrothyrsiferyl diacetate

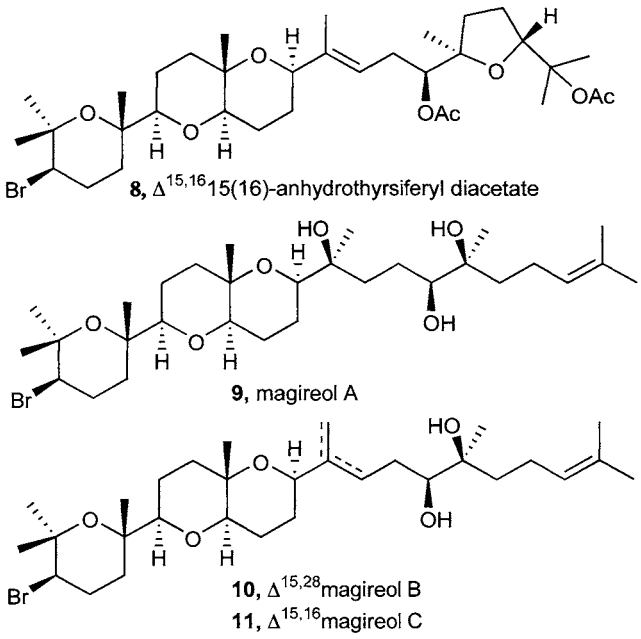
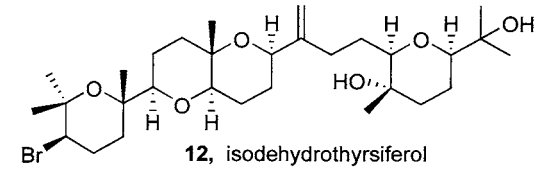


Fig. (2). Anhydrothyrsiferyl and magireol.

In 1996, Norte and co-workers elucidated the structures of two new antitumor polyoxygenated squalene derivatives that were isolated from the acetone extracts of *Laurencia viridis* [9]. Isodehydrothyrsiferol [**12,** Fig. (**3**)] possesses an appended tetrahydropyran unit instead of the common tetrahydrofuran moiety observed in previous chemical structures.

10-*epi*-Dehydrothyrsiferol (**13**) was identified through spectroscopic analysis. The ROESY NMR experiments revealed a correlation among the hydrogens at C_7 and at C_{11}, and between C_{14} and C_{27}, thus establishing that the two central pyran rings must be *cis*- rather than *trans*-fused [9]. The novelty of these two molecules may prove helpful in assessing possible biosynthetic pathways by which these natural products are generated.



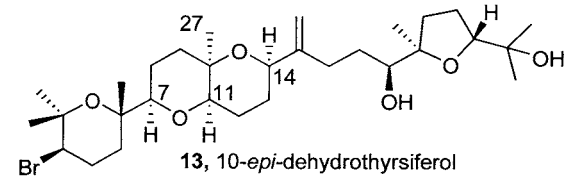
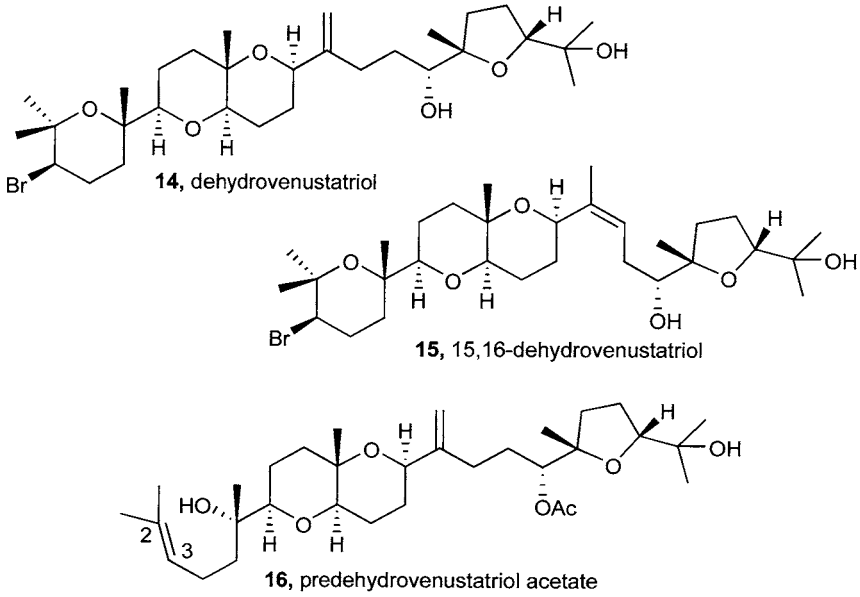


Fig. (3). Isodehydrothyrsiferol and 10-*epi*-dehydrothyrsiferol.

From the same *L. viridis* algae, Norte's research group was able to isolate five new polyether triterpenes structurally related to both thyrsiferol (**1**) and venustatriol (**2**) [10]. These new compounds include: dehydrovenustatriol (**14**), 15,16-dehydrovenustatriol (**15**), predehydrovenustatriol acetate (**16**), 16-hydroxydehydrothyrsiferol (**17**) and 10-*epi*-15,16-dehydrothyrsiferol [**18**, Fig. (**4**)].



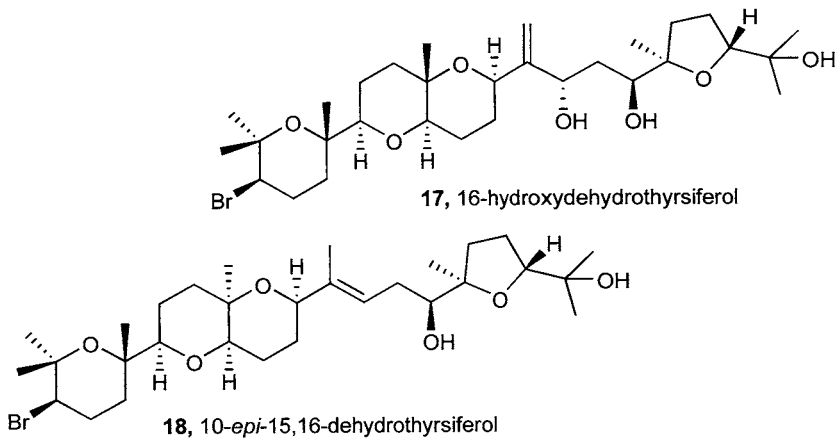
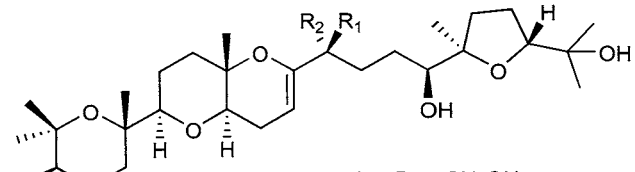
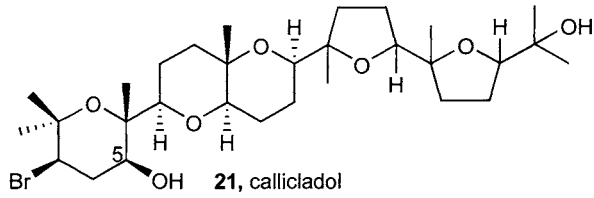


Fig. (4). Structure of five new metabolites.

Among the marine natural products shown in Fig. (**4**), predehydrovenustatriol acetate (**16**) presents an attractive structural feature in regards to its biogenesis. The absence of the A-ring and the presence of the trisubstituted double bond between C_2 and C_3 points to a step-wise biosynthetic pathway; this subject is discussed in the next section of this Chapter. *Laurencia viridis* has also been the source of thyrsenol A (**19**) and thyrsenol B [**20**, Fig. (**5**)]. Both of these compounds display an unusual enol ether moiety in the C-ring [11].

Callicladol [**21**, Fig. (**5**)] was found in *Laurencia calliclada,* a Vietnamese species isolated from the red seaweed class [12]. This compound constitutes the first example of a halogenated squalene-derived polyether from the genus *Laurencia* that contains an hydroxyl group at C_5.



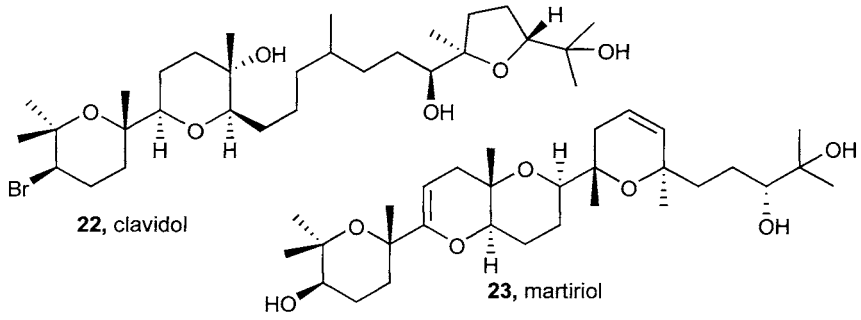


21, callicladol

Fig. (5). Thyrsenol A and B and callicladol structures.

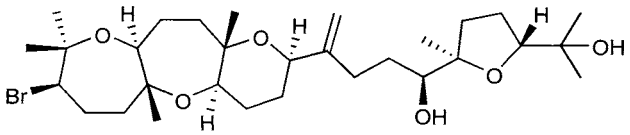
In 2001 and 2002, additional squalene-derived triterpenes from *L. viridis* were isolated and characterized, Fig. (**6**) [13,14]. The compounds show some divergence from the common backbone structures previously observed. For example, clavidol (**22**) lacks the C-ring, dioxepandehydrothyrsiferol (**24**) contains a framework with two *trans*-fused 7-membered ether rings, and lactodehydrothyrsiferol (**25**) contains a lactone in place of the A-ring bromoether.

Naturally occurring thyrsiferol analogues continue to be an inspiration for many synthetic, biosynthetic, and structure-activity relationship investigations. An overview of the efforts directed toward the biosynthesis of these natural products along with the biological activity displayed by each of the aforementioned compounds is addressed in the next section.



22, clavidol

23, martiriol



24, dioxepandehydrothyrsiferol

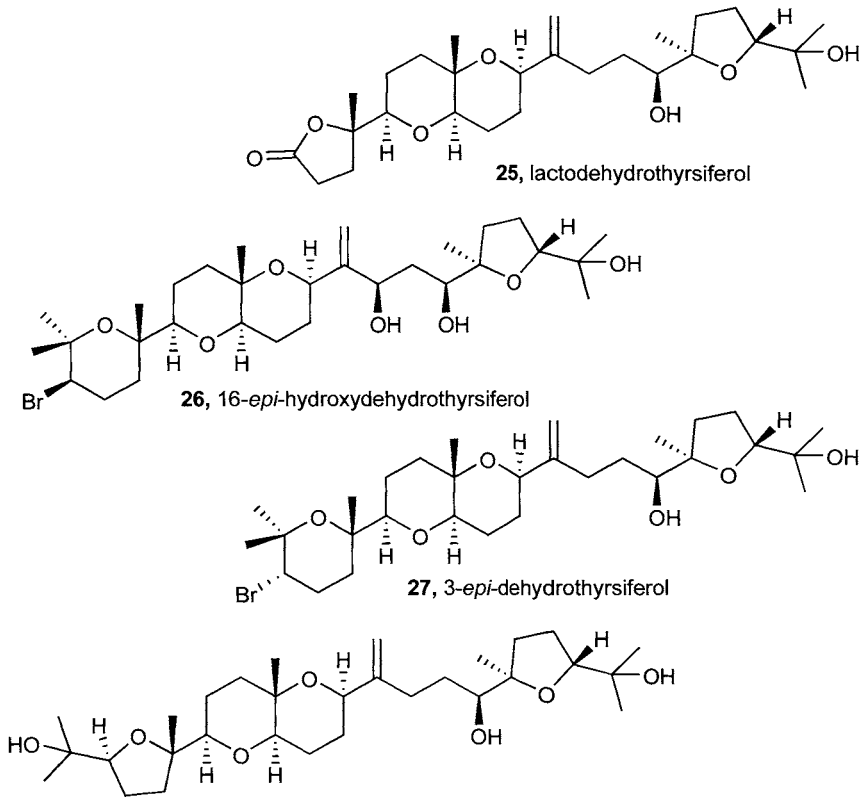
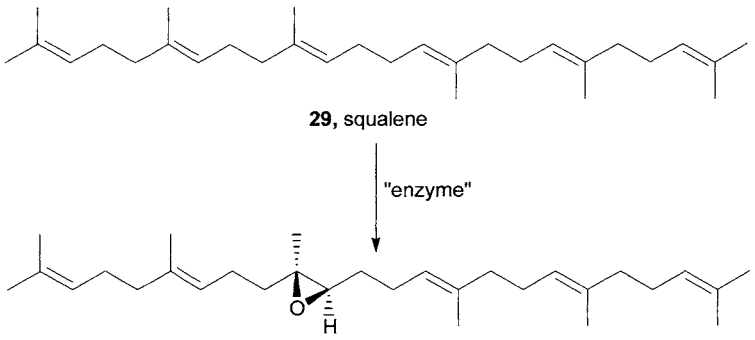


Fig. (6). Additional metabolites.

Biosynthesis for Compounds Isolated from *Laurencia*

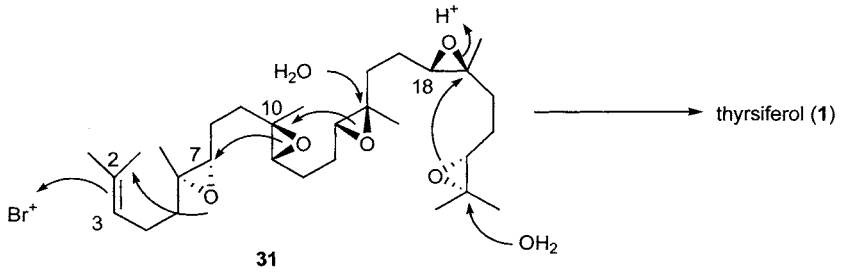
Based on the structural diversity of the many congeners originating from *Laurencia*, the elucidation of a biosynthetic mechanism for the likely development of these metabolites is of much interest. The isolation and characterization of monoepoxide **30** (Scheme 1) from *Laurencia okamurai* [15] has allowed investigators to postulate that this compound (**30**) may be a common precursor for the biosynthesis of all other secondary metabolites derived from squalene [3]. Its absolute stereochemistry was verified via asymmetric synthesis utilizing a Sharpless asymmetric epoxidation [16] of *trans, trans*-farnesol,

followed by other chemical transformations to convert the adduct to **30**. Both synthetic and natural products displayed identical spectral characteristics and optical rotations.



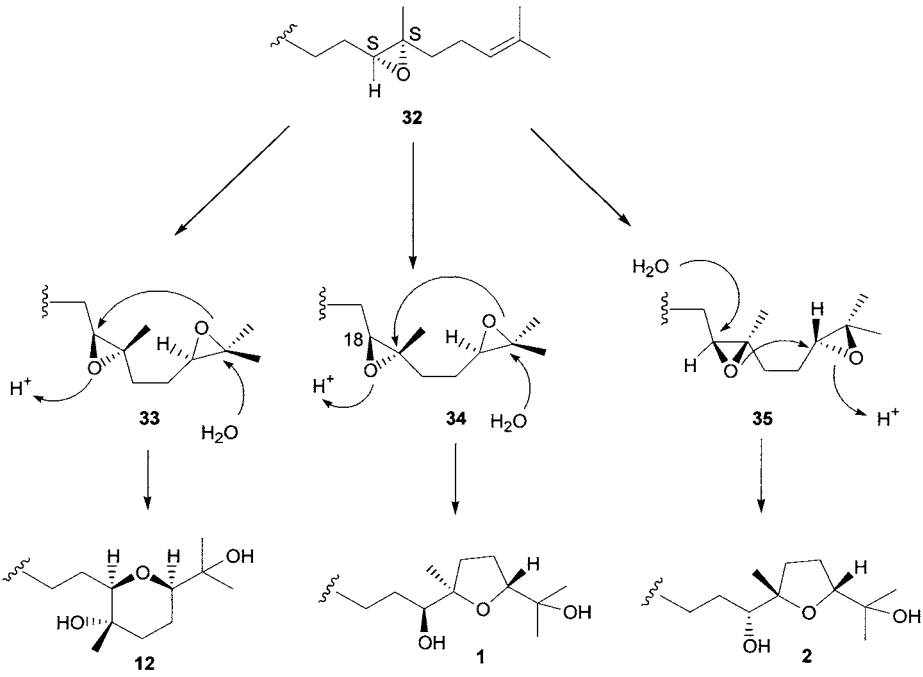
Scheme (1). First postulated biosynthetic step.

It was originally proposed that both thyrsiferol (**1**) and venustatriol (**2**) could originate from a concerted two-stage biosynthetic process involving a common squalene penta-epoxide precursor **31** (presumably originating from the monoepoxide **1-30**) [5,17]. The first step would require an attack of bromonium ion at the C_2-C_3 double bond, causing the concerted cyclization of the three remaining epoxides to take place at C_6-C_7, C_{10}-C_{11}, and C_{14}-C_{15}, thereby producing rings A, B, and C in the manner suggested in Scheme 2. The characterization of predehydrovenustatriol [**16**, Fig. (**4**)] lacking the A-ring may be indicative of a cyclization process that is sequential rather than concerted.



Scheme (2). Possible sequence for the biosynthesis of thyrsiferol (**1**).

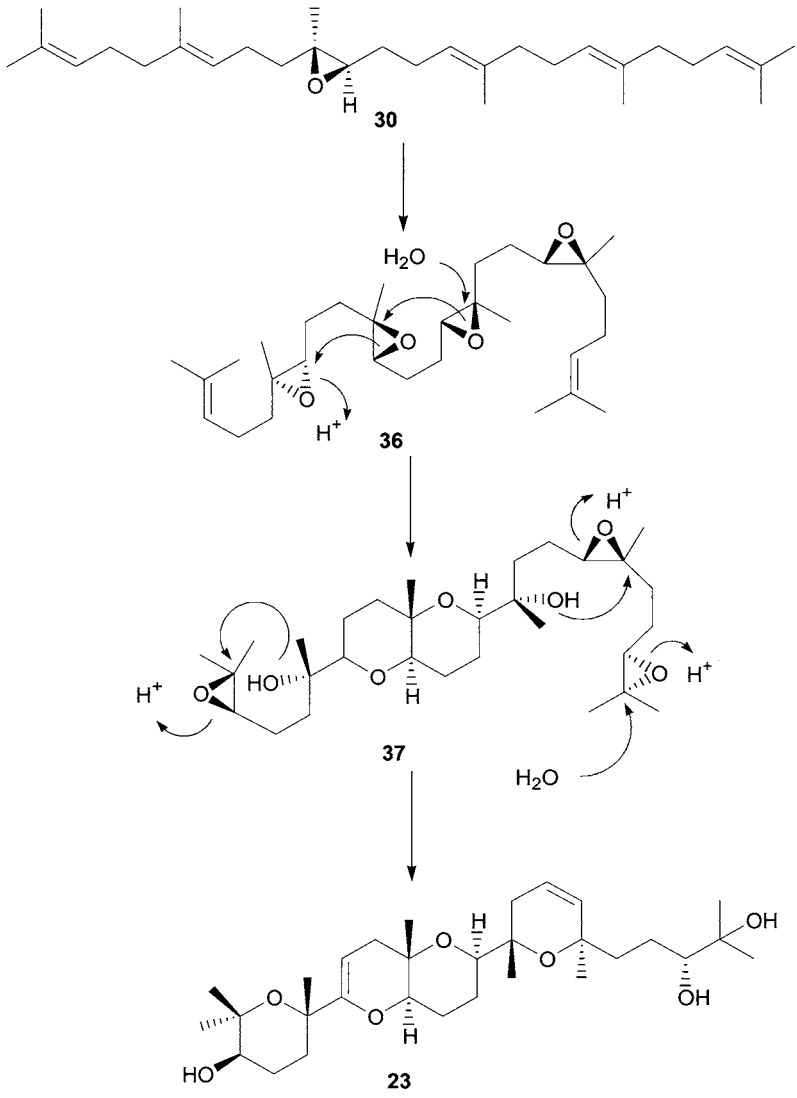
The second step could then be the furan D-ring formation, either by protonation of the C_{18}-C_{19} (**34**) followed by cyclization to afford thyrsiferol (**1**) or protonation of the C_{22}-C_{23} (**35**) and cyclization in the case of venustatriol (**2**) as illustrated in Scheme 3. The construction of the D-ring of isodehydrothyrsiferol [**12**, Fig. (**3**)] was proposed via the protonation of the C_{18}-C_{19} epoxide, followed by cyclization at C_{18}, to yield the tetrahydropyran ring. These mechanisms account for the different ring sizes and stereochemistries observed around ring D for these compounds [3].



Scheme (3). Biosynthesis of D-ring.

The biogenesis of compounds that are somewhat dissimilar from thyrsiferol (such as **23**) has also been investigated. Norte *et al* postulated the biosynthetic pathway shown in Scheme 4 for compound **23** [13]. Starting with monoepoxide **30**, formation of a tetra-epoxide intermediate gives compound **36**. Cyclization of the BC ring gives the intermediate **37** and a series of subsequent cyclizations affords the final

structure **23**. Biosynthetic pathways leading to clavidol (**22**), lactodehydrothyrsiferol (**25**), and dioxepandehydrothyrsiferol (**24**) have also been proposed [12,13].



Scheme 4. Proposed biosynthesis of **23**.

The discovery of the polyethers discussed in this section has undoubtedly allowed investigators to gain further insights into the biosynthetic paths by which nature chooses to synthesize these compounds. Of equal if not greater significance is the information that these natural products can provide regarding their interactions with the human organism at the cellular level. The ultimate goal would be to utilize this knowledge of marine-derived natural products toward the treatment of human diseases. The next section includes a survey of the pharmacological potential of this marine-derived family of compounds.

Biological Properties for Polyethers from *Laurencia*

To explore the possibility of using these natural triterpenoid ethers as the basis for the development of potent therapeutic agents, it is necessary to investigate the essential structural features responsible for eliciting given biological responses. Structure-activity relationship studies are an effective means of accomplishing this. Norte's laboratory has published a comprehensive report evaluating the cytotoxic activity of several of the polyethers obtained from *Laurencia* (Table 2) [3a,18].

In this work, cytotoxic effects were evaluated with the following cultured tumor cell lines: P-388 (suspension culture of a lymphoid neoplasm from a DBA/2 mouse), A-549 (monolayer culture of a human lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma) and MEL-28 (monolayer culture of a human melanoma). Potent and selective activity against P-388 cells was observed for most of the compounds investigated, with special attention directed to **1, 3, 4, 12, 14**, and **20**. These results established the fact that small chemical changes in molecular structure can greatly affect cytotoxicity.

Table 2. Cytotoxic activity of marine polyethers

	IC_{50} values							
	P-388		A-549		HT-29		MEL-28	
Comp	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM
1	0.01	0.016	10.0	16.53	10.0	16.53	-	-
3	0.01	0.017	2.5	4.26	2.5	4.26	5	8.52

4	0.0003	0.00047	-	-	-	-	-	-
5	0.30	0.47	-	-	-	-	-	-
6	0.52	0.75	-	-	-	-	-	-
7	0.05	0.074	-	-	-	-	-	-
8	0.10	0.148	-	-	-	-	-	-
9	0.03	0.052	-	-	-	-	-	-
10	0.03	0.052	-	-	-	-	-	-
11	0.03	0.052	-	-	-	-	-	-
12	0.01	0.017	2.5	4.26	2.5	4.26	2.5	4.26
13	1.00	1.70	5.0	8.52	5.0	8.52	5.0	8.52
14	0.01	0.017	2.5	4.26	2.5	4.26	2.5	4.26
15	0.25	0.43	2.5	4.26	2.5	4.26	2.5	4.26
16	1.20	2.18	2.5	4.54	5.0	9.09	2.5	4.54
17	0.50	0.85	2.5	4.26	1.2	2.04	2.5	1.99
18	0.50	0.85	2.5	4.26	1.2	2.04	2.5	4.26
19	0.25	0.40	>1.0	>1.62	>1.0	>1.62	>1.0	>1.62
20	0.01	0.016	>1.0	>1.62	>1.0	>1.62	>1.0	>1.62
21	1.75	2.89	-	-	-	-	-	-

Through molecular simplification, Norte *et al* determined that the presence of the flexible side chain was essential for the cytotoxic activity. It was further postulated that the conformation of the C_{14}-C_{19} side chain was at least partially responsible for the variation in biological responses among the analogues. In order to verify this hypothesis, the stable conformations for all the compounds were determined using calculations that involved a multiconformer search using the Monte Carlo program in Macromodel and MM2 force fields [18].

The Norte laboratory study concluded that the most potent compounds (**1**, **4**, **12**, **14** and **20**) adopted similar spatial orientations of the flexible side chain [see **3** and **20**, Fig. (**7**)], whereas compounds with reduced potencies adopted conformations where the side chain was directed toward the opposite direction [see **13**, Fig. (**7**), the least potent of the series].

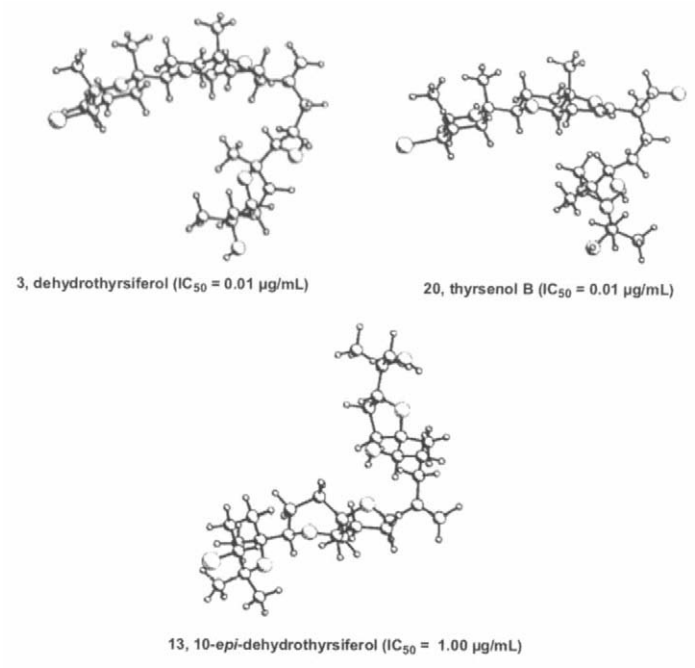


Fig. (7). Spatial orientation of compounds **3**, **20**, and **13**.

Dehydrothyrsiferol (**3**, DT) and thyrsiferyl 23-acetate (**4**, TF-23A) have gained special attention due to their appealing and potent pharmacological profiles. Norte and co-workers investigated the mechanism of growth inhibition by DT in MDR^+ human epidermoid cancer cell lines [19]. DT was found to circumvent multidrug resistance mediated by P-glycoprotein and to display a novel pattern of growth inhibition when compared to doxorubicin (a compound known to intercalate into the DNA helix) and colchicine (a microtubule disrupting agent).

In a separate study, Souto characterized the features of growth inhibition caused by DT in human BCA (breast cancer) cells [20]. The cell lines examined included T47D, ZR-75 and Hs578T. The respective IC_{50} values for cytotoxicity were determined to be 7.9, 9.4, and 11.1 µg/mL. The studies allowed the authors to conclude that apoptosis was induced in estrogen dependent and independent breast

cancer cells, making DT an interesting candidate for antitumor drug development and possible treatment.

As indicated in Table 2, TF-23A (**4**) is the more potent of the analogues, and it has attracted the most attention from both the biological and synthetic perspectives. TF-23A exhibits a strong cytotoxic property (ED_{50} of 0.3 ng/ml) against P-388 in vitro cell lines, which is suggestive of a powerful cell growth inhibitor [7]. This feature invited further exploration of its promising therapeutic properties.

A study conducted by Matsuzawa *et al* published in 1994 showed TF-23A to display very potent and specific inhibition of serine/threonine protein phosphatase 2A (PP2A); IC_{50} values of 4-16 μM, depending on the concentration of the enzyme were measured.[21] Protein phosphorylation is a well-recognized means for the regulation of protein function. While protein kinases are responsible for the phosphorylation event, protein phosphatases are accountable for reversing the process. The discovery of several protein kinase inhibitors has permitted the examination of specific protein kinase functions. On the other hand, protein phosphatase inhibitors are much less common [22]. Elucidation of TF-23A's activity could therefore allow investigators to identify particular protein phosphatases involved in specific signal transduction pathways of the cell.

Among other members of the family of protein phosphatases (PP1, PP2B, PP2C) and protein tyrosine phosphatases, TF-23A displayed no affect in activity in concentrations of up to 1 mM. Such a unique property makes TF-23A a novel probe for elucidating the regulation of cellular processes by PP2A. Other compounds displaying similar inhibitory activities include okadaic acid, microcystin LR, calyculin A and tautomcin [22]. However, only TF-23A displays specific PP2A inhibition.

Based on these findings, Souto *et al* broadened the scope of the investigation by analyzing the PP2A inhibitory activity of the several marine polyethers described in the previous section [23]. This was an effort to probe the necessary structural features for the activity (Table 3).

Table 3. PP2A inhibitory activity of thyrsiferol analogues

Compound	Concentrations			
	1 □M	10 □M	100 □M	250 □M
3	8.2	12.2	25.2	37.7
12	0	6.2	74.7	81.3
14	7.5	23.7	66.8	95.3
17	41.1	93.4	100	100
18	17.9	41.9	99.5	100
19	15.2	31.8	89.6	97.8
20	52.8	93.2	99.1	100
22	43.6	45.9	57.8	74.5
24	18.9	20.4	59.7	66.5
25	33.7	40.1	49.4	63.2
26	42.2	45.6	65.1	79.3
28	0	0	40.4	55.4

The structure-activity relationship study concluded that neither modification of the size of the ring or stereochemistry in rings A, B, and D, nor the absence of ring C, alter the PP2A inhibitory activity when compared to that of dehydrothyrsiferol (**3**). The most potent analogues were compounds **17** and **20**, suggesting that the presence of a hydroxyl unit at C_{15} or C_{16} is crucial for a potent inhibitory affect. These conclusions offer insights for the rational development of analogues designed to target specific PP2A inhibition.

Matsuzawa *et al* discovered that TF-23A induces apoptosis in various leukemic T- and B-cell lines under serum-deprived conditions [24]. Furthermore, this study demonstrated that the TF-23A-induced apoptosis did not occur through the inhibition of PP2A [25]. TF-23A was labile and gradually lost the PP2A inhibitory activity during storage at 4 °C. However, the degraded TF-23A still showed the apoptosis-inducing activity and therefore the two events were assumed to be unrelated. The molecular mechanism by which PP2A inhibition occurs remains to be elucidated.

The structural complexity associated with the secondary metabolites from *Laurencia* coupled with introductory work on their

biosynthesis and remarkable biological diversity have led several synthetic organic research laboratories, including ours, to endeavor to complete the total synthesis of these fascinating molecules. As will be discussed shortly, the information gained from the postulated biosynthetic mechanism has inspired some research groups to incorporate a biomimetic approach into their synthetic strategies. Others have chosen to use the challenges associated with the construction of this family of natural products as a foundation for the development of novel chemical methodologies. The compounds thyrsiferol (**1**) and venustatriol (**2**) have gained the most attention and are the focus of the next portion of this chapter.

PUBLISHED SYNTHESIS OF THYRSIFEROL AND VENUSTATRIOL

Introduction

The construction of thyrsiferol (**1**) and venustatriol (**2**) from readily available commercial materials is clearly a challenging venture. Thyrsiferol's molecular architecture is characterized by its highly oxygenated skeleton, containing 10 stereocenters, 4 of which are quaternary.

Any successful strategy must address a number of challenges including, for example, the regio- and stereoselective synthesis of the bromotetrahydropyran A ring, a challenge that has been extensively investigated but not completely solved. Additional challenges include the stereoselective construction of the central BC-pyranopyran unit, and the connection of ring C to the flexible side chain consisting of carbons C_{15}-C_{23}. This central unit, in particular ring C, is known to adopt a chair-twist boat conformation [4], a feature that needs to be taken into consideration when designing a synthetic scheme. Lastly, the stereoselective assembly of the *trans*-tetrahydrofuran D ring poses additional difficulties.

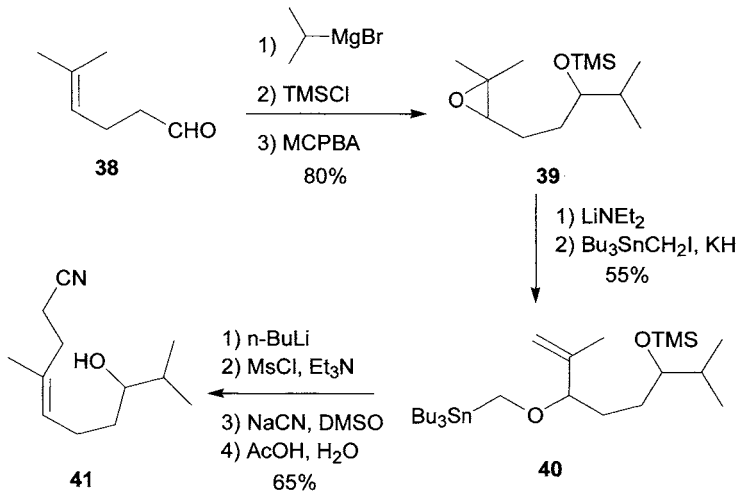
Researchers have been attracted by both the biological profiles and the above-mentioned synthetic challenges associated with the construction of **1** and **2**. This section highlights the synthetic endeavors of Broka [26], Shirahama [27], Corey [28], Forsyth [29] McDonald

[30] and Little [31] addressing the key reactions associated with each approach.

Broka's Approach Toward the ABC Framework

Broka's strategy toward the synthesis of the ABC framework of thyrsiferol (**1**) and venustatriol (**2**) consisted of iterative mercuricyclizations for the assembly of both B- and C-rings, followed by an installation of the A-ring via a bromonium ion induced cyclization [26].

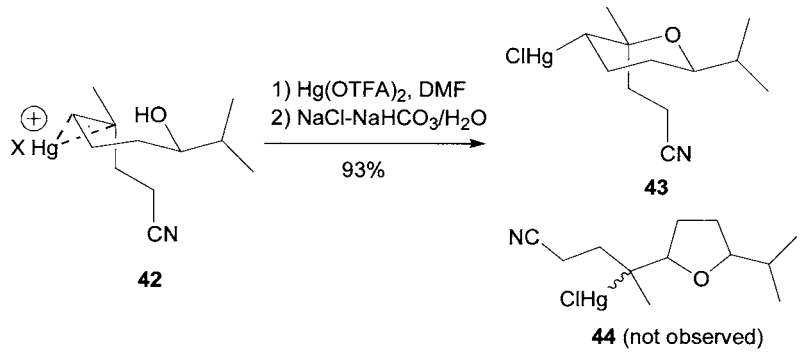
The researchers chose to synthesize model substrate **41** in order to investigate the mercuric ion promoted reaction to form the tetrahydropyran C-ring (Scheme 5). The synthesis of **41** begins with aldehyde **38** [32] as illustrated in Scheme 5. The most notable step in this sequence is the [2,3]-sigmatropic rearrangement (conversion of **40** to **41**) to afford only one geometric isomer [33]. The double bond stereochemistry of the Z-homoallylic nitrile **41** also plays a crucial role to the successful implementation of the strategy.



Scheme 5. Broka's synthesis of nitrile **41**.

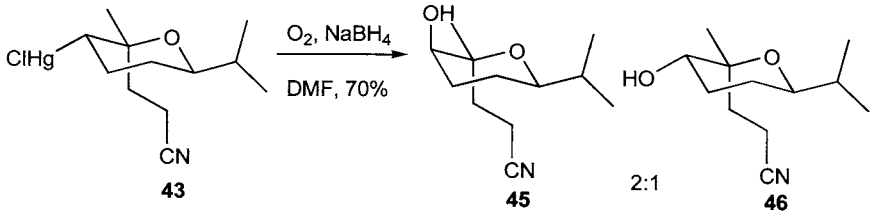
With substrate **41** in hand, the mercuricyclization step was explored under a variety of reaction conditions (Scheme 6). The optimal set of

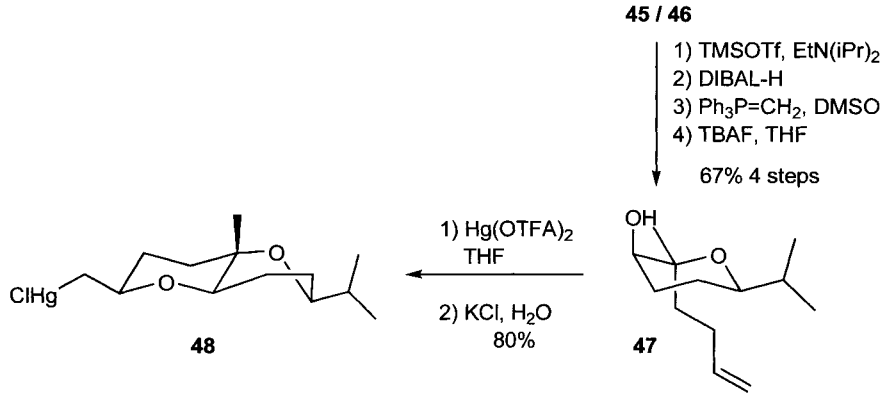
reagents included $Hg(OTFA)_2$ in DMF, furnishing product **43** in 93% yield as the only observed isomer [34]. It was speculated that the oxymercuration step was reversible due to the presence of trifluoroacetic acid [from $Hg(OTFA)_2$] and therefore that the reaction proceeded to afford the thermodynamically preferred pyran product **43**. The pseudoequatorially oriented isopropyl group in the chair-like representation (**42**) is presumably responsible for the observed stereoselectivity.



Scheme 6. Broka's oxymercuration reaction.

In contrast, when the reaction was performed with $Hg(OAc)_2$ (leading to the weak acid, AcOH), the reaction was presumed to be irreversible and kinetically controlled, giving a 1:1 mixture of compounds **43** and **44**. In support of this argument is the fact that when cyclizations were performed with $Hg(OTFA)_2$ in the presence of excess HgO (to consume the TFA produced) a 1:1 mixture of regioisomers was also observed.

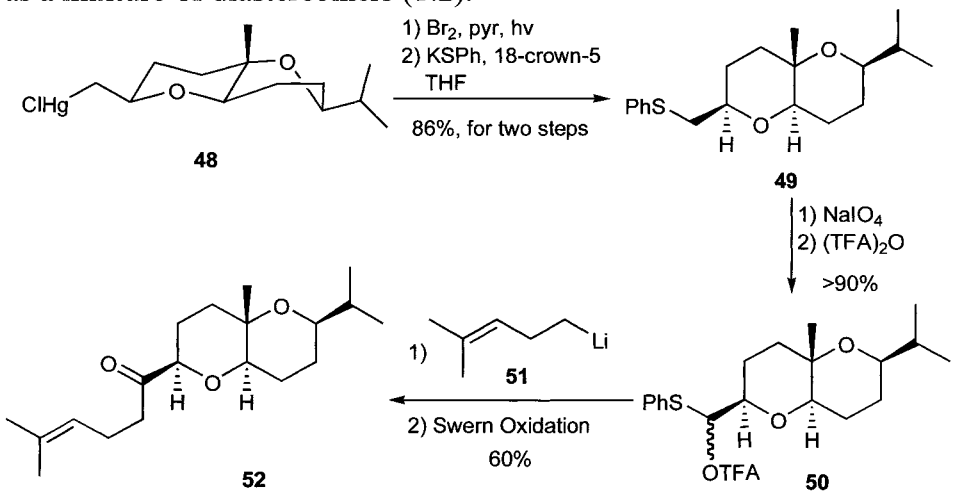




Scheme 7. Broka's synthesis of the B-ring.

Four straightforward transformations converted **45** to the terminal alkene **47** (Scheme 7). Exposure of this substrate to $Hg(OTFA)_2$ furnished compound **48** in excellent yield. Other sources of electrophiles (NBS, PhSCl) provided disappointing results [35].

Upon treatment of **48** with Br_2 under photochemical conditions [36], the primary bromide was generated and subsequently displaced with thiophenoxide to give product **49** (Scheme 8). Periodate oxidation of **49** followed by a Pummerer rearrangement [37] furnished adduct **50** as a mixture of diastereomers (1:2).

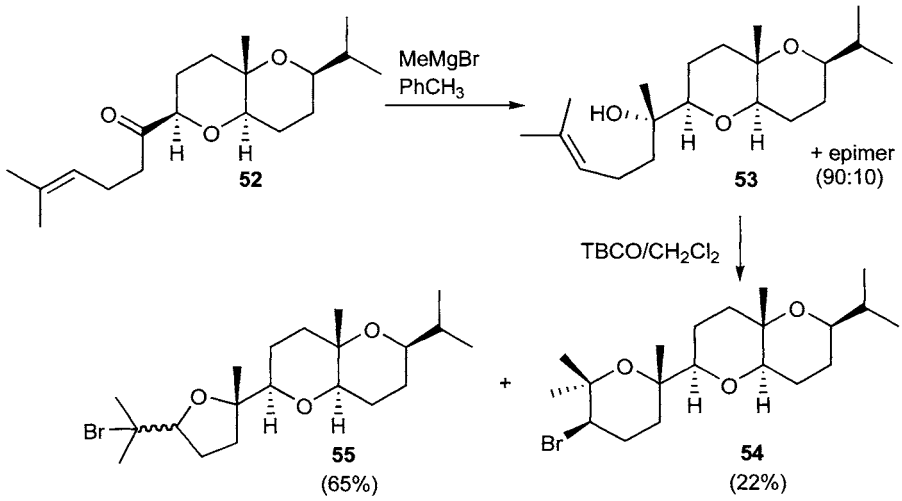


Scheme 8. Broka's addition of side chain

Compound **50** acts as a masked aldehyde; its reaction with excess organolithium reagent **51** [38] followed by oxidation under Swern conditions, furnished pyranopyran **52**. Presumably, the excess organolithium reagent **51** reveals the aldehyde *in situ*. Further 1,2-addition of the organolithium reagent yielded the corresponding alcohol, which upon oxidation gave ketone **52**.

Chelation-controlled reaction of **52** with MeMgBr allowed the isolation of product **53** with a *d.r.* of 90:10 (Scheme 9) [39]. The only step remaining to form ring A was the electrophilic cyclization. Several reagents and reaction conditions were investigated, including TBCO [40], Br_2, NBS, $Hg(OAc)_2$, $Hg(OTFA)_2$, the best conditions proving to be TBCO in dichloromethane to give a 3:1 mixture of bromotetrahydrofuran (**55**) and bromotetrahydropyran (**54**) in 65% and 22% yield respectively. The tetrahydrofuran by-product **55** could be reconverted to the starting material (**52**) by exposure to Zn/AcOH and recycled.

Although the completion of the natural product synthesis has not been published by the authors, their work comprises a very detailed synthetic investigation concerning the assembly of the ABC framework. It also served to familiarize the chemical community as to the challenges associated with the synthesis of these natural products.

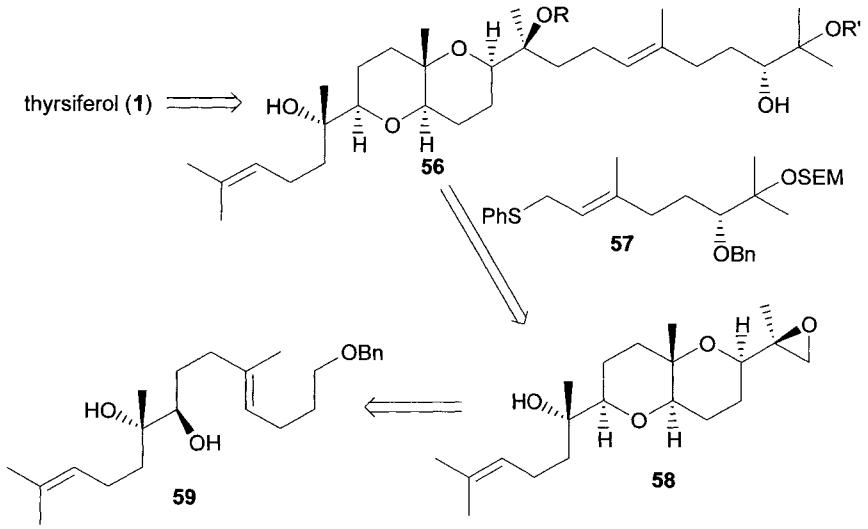


Scheme 9. Broka's ABC framework.

Shirahama's Total Synthesis of Thyrsiferol and Venustatriol

The first total synthesis of thyrsiferol (**1**) and venustatriol (**2**) was reported by Shirahama *et al.* Their approach was based upon the biogenesis shown in Schemes 2 and 3 for the construction of polyethers [27].

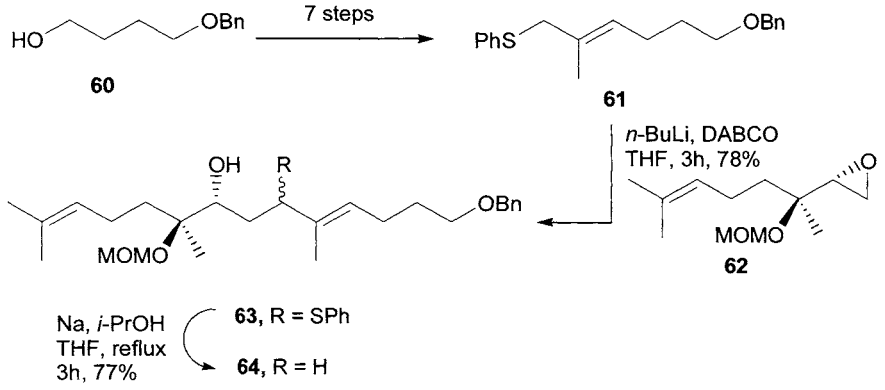
The authors opted to install the bromotetrahydropyran A-ring last due to its possible instability under radical, strongly basic, and/or acidic conditions. The D-ring was envisioned to arise from a stereoselective epoxidation followed by cyclization to afford the tetrahydrofuran framework. Key to achieving this plan was accessibility to structure **56** (Scheme 10). This fragment in turn was envisioned to be assembled by coupling the anion derived from **57** with epoxide **58**. Compound **58** could presumably be accessed via stereoselective cyclizations from diol **59**.



Scheme 10. Shirahama's retrosynthesis

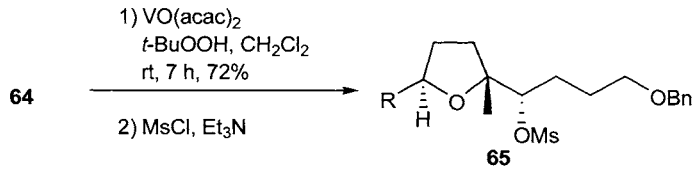
Shirahama's synthesis commenced with 4-(benzyloxy) 1-butanol (**60**, Scheme 11) [27]. In 7 steps intermediate **61** was obtained. This product was coupled with epoxide **62** [41] (derived from linalool) via the generation of the lithium anion of **61** [42]. In this fashion,

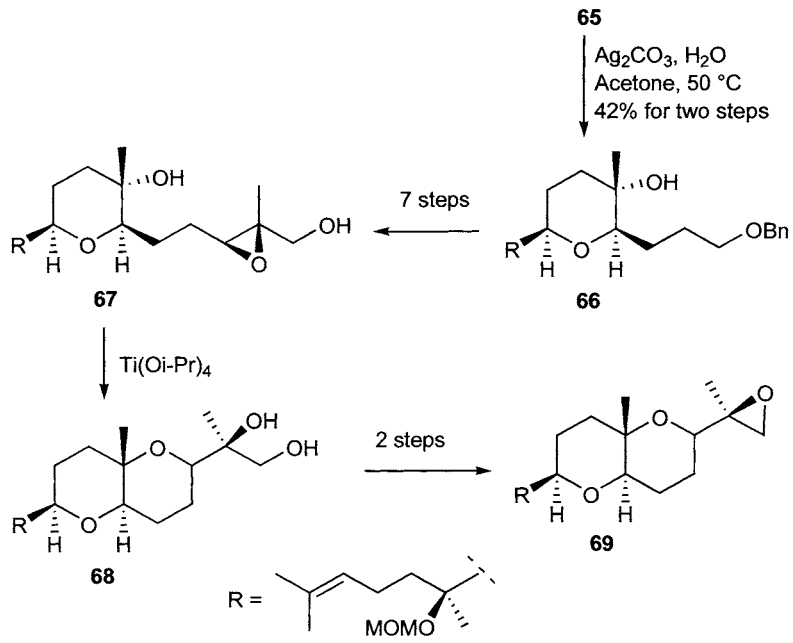
compound **63** was obtained in 78% yield. Reduction of the phenylthio group under Bouveault-Blanc conditions [43] gave intermediate **64**.



Scheme 11. Shirahama's synthesis of alcohol **64**.

With compound **64** available, vanadyl acetylacetonate catalyzed epoxidation [44] accompanied by simultaneous cyclization, afforded the corresponding tetrahydrofuran and its diastereomer in a 4:1 ratio (Scheme 12). Ring expansion of the corresponding mesylate **65** with silver (I) carbonate afforded compound **66** in a 42% yield for the two steps [45]. Extension of the side chain in six steps, followed by an asymmetric epoxidation, gave product **67** stereoselectively. The cyclization of **67** with titanium tetraisopropoxide in a manner consistent with model studies [27d], afforded bicyclic ether **68** in 65% yield. Transformation to the epoxide under standard conditions afforded fragment **69** ready to be coupled with the D-ring side chain.





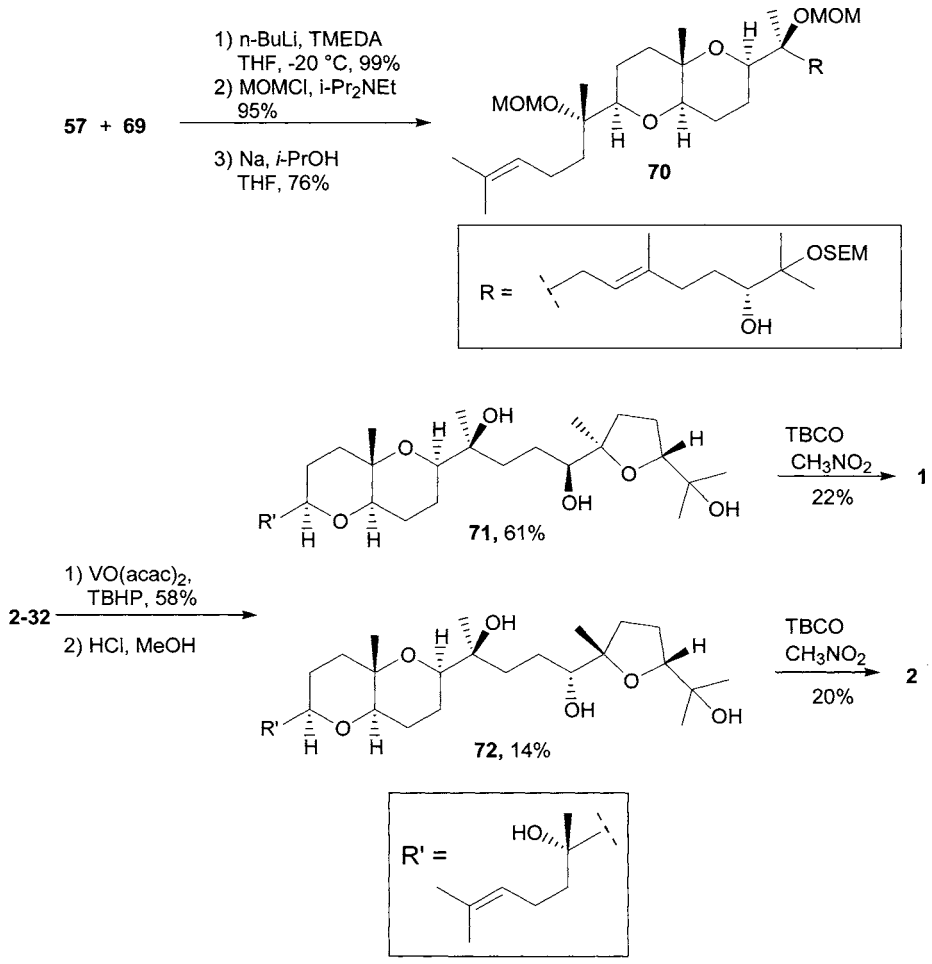
Scheme 12. Shirahama's ring expansion and cyclization.

The coupling reaction of epoxide **69** with the anion derived from the allyl sulfide **57**, a system derived from geraniol, was accomplished in a 99% yield by employing 4 equivalents of the anion (Scheme 13). Protection of the alcohol as the MOM ether (95%), followed by debenzylation and removal of the phenylthio group under Birch conditions (76%) gave diene **70**. Vanadium catalyzed oxidation and spontaneous cyclization of **70** under acidic conditions afforded a diastereomeric mixture of tetraols **71** and **72** in 61% and 14% yields, respectively.

Following the chromatographic separation of the tetraols, treatment of compound **71** with TBCO in nitromethane resulted in the bromonium ion induced cyclization to form the tetrahydropyran unit of ring A. The isolated yield of thyrsiferol for this last step was 22%. Similarly, compound **72** was converted to venustatriol in 20% yield.

The main features of the Shirahama synthesis include the tetrahydrofuran ring expansion to afford the ring B and his incorporation of stereoselective epoxide cyclizations in accord with the

postulated biosynthetic pathway. The total synthesis pursued by Shirahama proceeded in a total of 38 steps with 26 steps for the longest linear sequence.

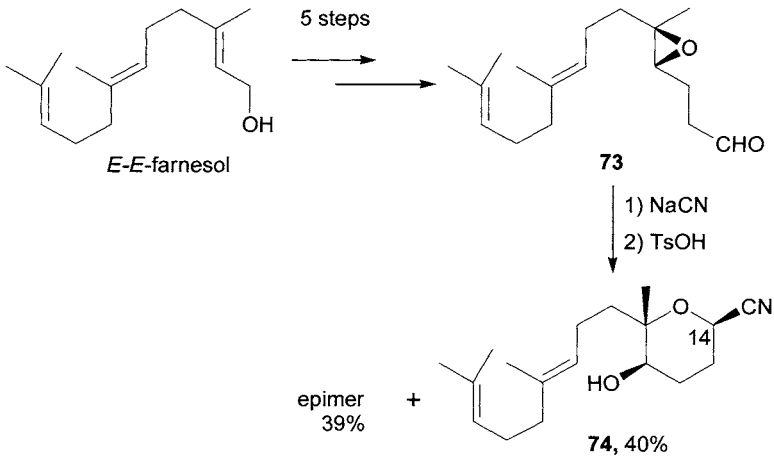


Scheme 13. Shirahama's completion of thyrsiferol and venustatriol.

Corey's Total Synthesis of Venustatriol

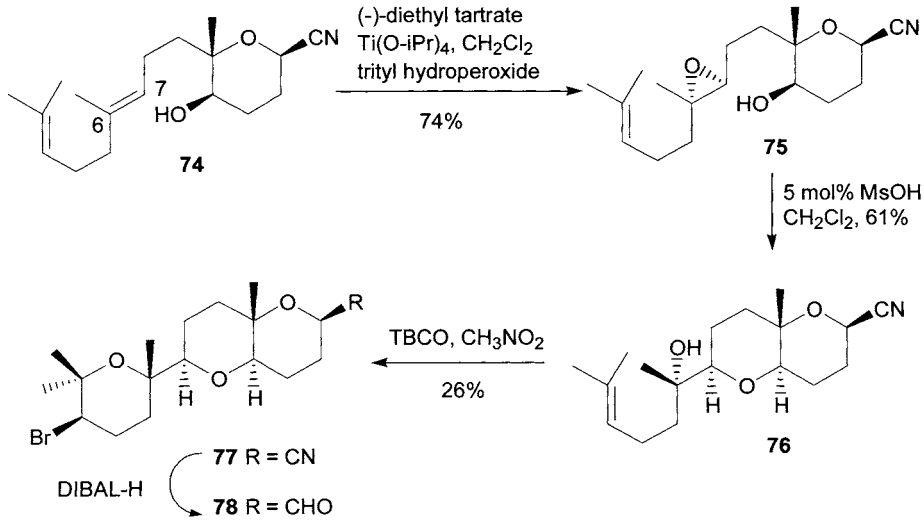
The second enantioselective total synthesis of venustatriol was published by E. J. Corey in 1988 [46].

Starting with *E,E*-farnesol, a Sharpless asymmetric epoxidation [16] followed by four convenient functional group transformations afforded aldehyde **73** (Scheme 14). Reaction of **73** with 2 equivalents of sodium cyanide gave a mixture of cyanohydrins (1:1) which upon treatment with 5 mol % of tosic acid underwent cyclization to give cyano ether **74** (40%) and the C_{14}-epimer (39%). The epimer could be treated with potassium hexamethyldisilazane to afford more of the desired product (**74**) [46].



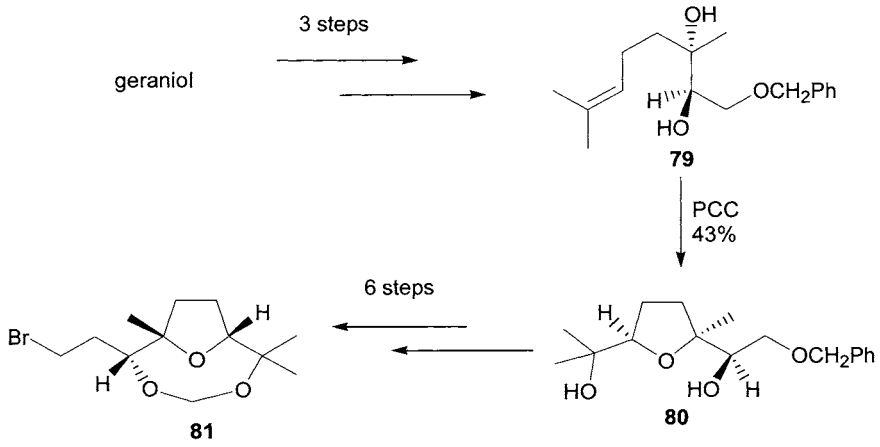
Scheme 14. Corey's synthesis of C-ring.

Epoxidation of the C_6-C_7 double bond of **74** in a stereoselective fashion (Scheme 15),[47] followed by cyclization to form the tetrahydropyran unit by using a catalytic amount of methanesulfonic acid gave compound **76**. This product was exposed to TBCO to give the desired (*R*)-3-bromotetrahydropyran **77** in 26% yield (along with its epimer and the corresponding tetrahydrofurans). Other conditions for the brominative cyclization [solvents: CH_3CN, THF, DMF, and pyridine or reagents: $Br_2C(CN_2)$] were less effective. Conversion of the cyano group to the aldehyde functionality with DIBAL-H afforded compound **78**.



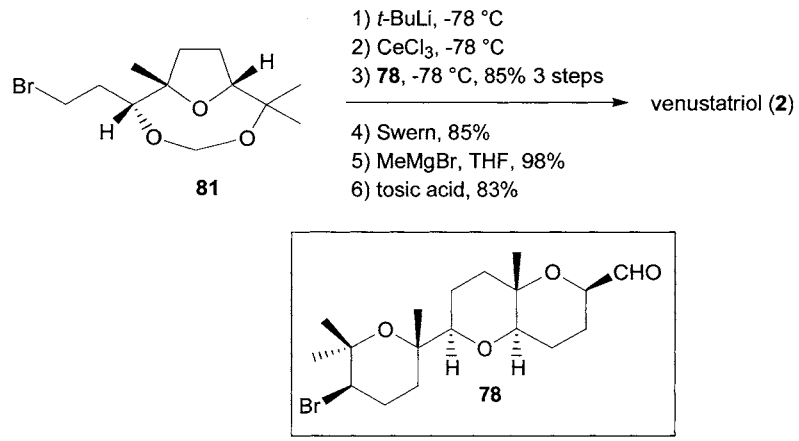
Scheme 15. Corey's synthesis of B-ring.

The coupling partner **81** was assembled from geraniol in a 10 steps reaction sequence (Scheme 16). A noteworthy step was the PCC oxidation of **79** to give the *cis*-2,5-disubstituted-tetrahydrofuran (**80**) stereoselectively [48].



Scheme 16. Synthesis of the D-ring.

The union of fragments **78** and **81** was performed by treatment of **81** with *t*-BuLi to induce a bromine-lithium exchange, followed by transmetallation to the cerium reagent [49] and finally the addition of aldehyde **78** (Scheme 17). The coupled product was obtained in 85% yield as a diastereomeric mixture of alcohols. Swern oxidation [50] and reaction with MeMgBr at –78 °C provided the tertiary alcohol stereoselectively in 98% yield. A final deprotection with tosic acid gave venustatriol (**2**).

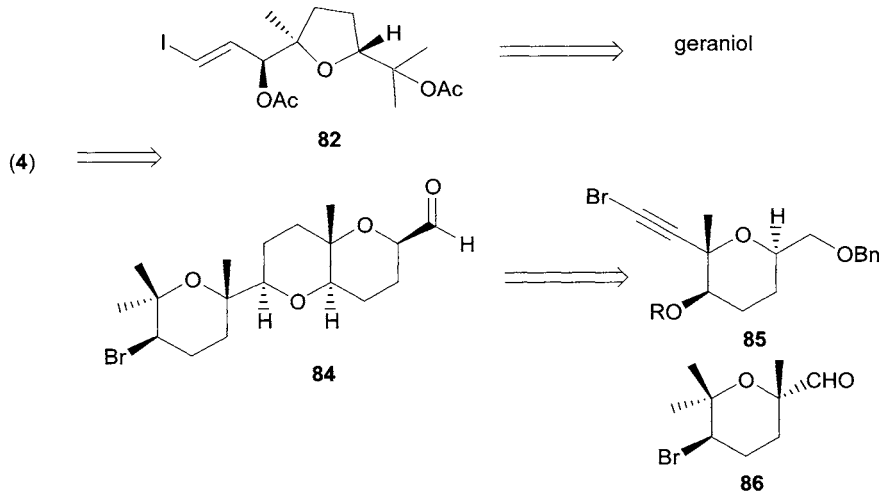


Scheme 17. Corey's last steps.

Notable features concerning this work include the trishomoallylic epoxidation and the conversion of **78** to **80** by means of a PCC oxidation. The total synthesis proceeded in a total of 26 steps with 13 steps for the longest linear sequence.

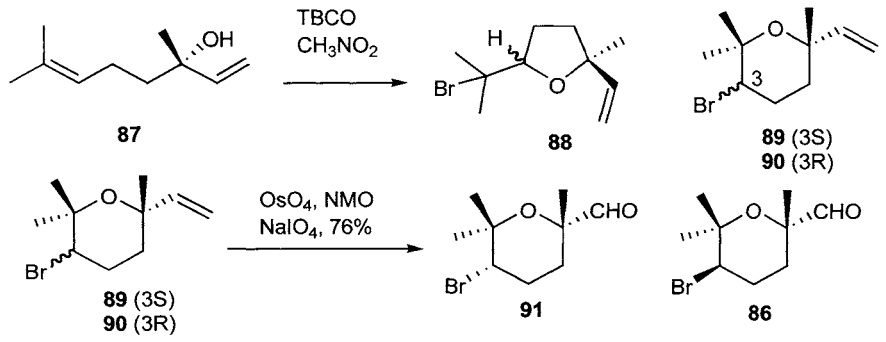
Forsyth's Total Synthesis of Thyrsiferyl 23-Acetate (4)

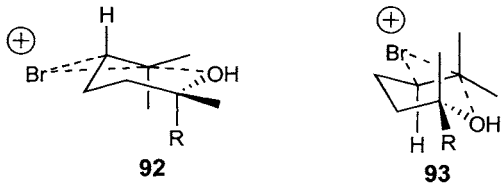
The most recent total synthesis of thyrsiferol 23-acetate (**4**) was reported by Forsyth and co-workers [29]. Their work focused upon a convergent assembly of the three fragments: **82**, **85**, and **86**, illustrated in Scheme 18, offering both efficiency and practicality.



Scheme 18. Forsyth's Retrosynthesis.

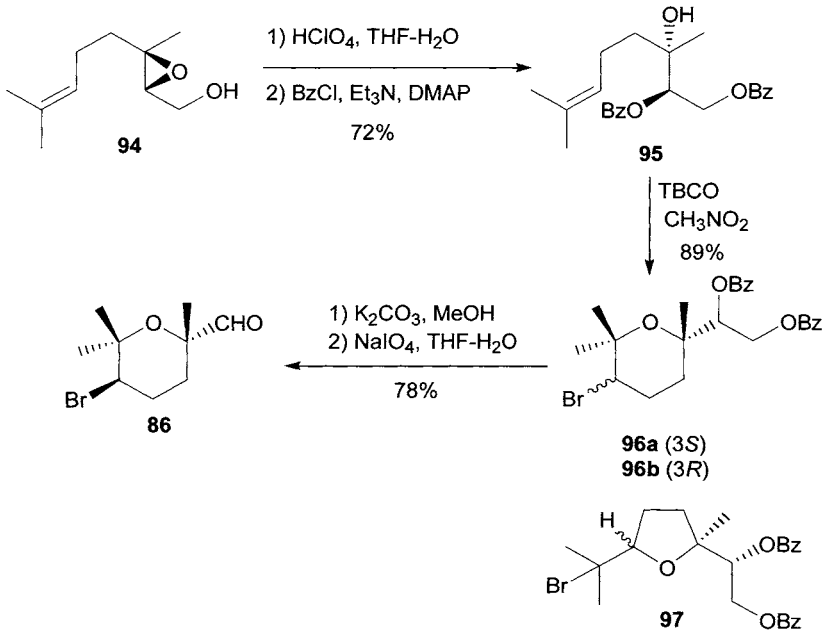
In their strategy, three methods were investigated for the synthesis of aldehyde **86**. Initially, linalool was selected as the starting material (Scheme 19). Cyclization with TBCO afforded a mixture of tetrahydropyrans (**89** and **90**, 10:6.9) and tetrahydrofurans (**88**) in 56% and 24% yields respectively. The preference for the stereoselective formation of pyran **89** over the desired compound **90** was postulated to be associated with the chair transition states shown in Scheme 19. Presumably, the diaxial interaction between the C_1 methyl group and the vinyl substituent R in intermediate **92** is less severe than those between the two methyl groups in intermediate **93**, leading to the undesired tetrahydropyran **89** as the major product [51].





Scheme 19. Forsyth's attempt to assemble the A-ring.

Dihydroxylation of the terminal alkene of **90**, followed by oxidative cleavage to the aldehyde allowed the authors to isolate compound **86** in an enantiomerically pure form. However, the low yielding (24%) bromoetherification step led the authors to search for a more efficient method.



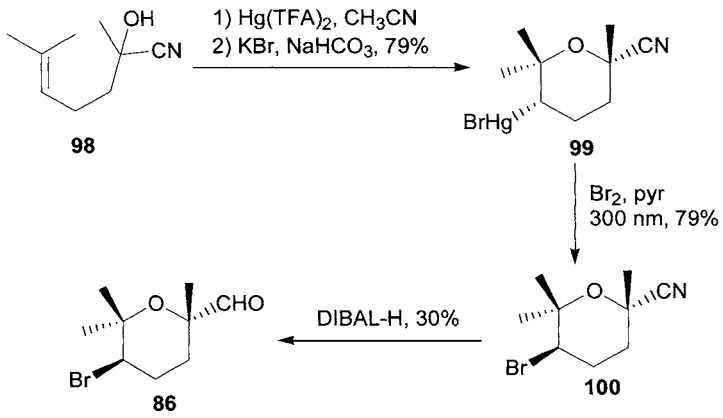
Scheme 20. Second attempt to construct the A-ring.

The second strategy was to implement the bromoetherification at a later stage in an effort to increase the stereoselectivity for the bromonium ion cyclization. Thus, compound **95** was synthesized from

geraniol in three steps (Scheme 20). The cyclization of alcohol **95** with TBCO afforded the tetrahydropyrans (**96a** and **96b**, 2.4:6.9) and tetrahydrofurans in an 89% combined yield. The choice of Bz as the protecting group functions to increase the stereoselectivity by placing a bulky group in the equatorial position of the chair-like transition state. Deprotection of the benzoate, followed by oxidative cleavage of the resulting diols afforded aldehyde **86**.

Oxymercuration of cyanohydrin **98** was examined as a third option (Scheme 21) [52]. Treatment of this cyanohydrin under Hg(II) trifluoroacetate-mediated brominative conditions afforded tetrahydropyran **99**. Inversion of configuration at C_3 by allowing **99** to react with Br_2 under photolytic conditions afforded pyran **100**. DIBAL-H reduction of the cyano group provided aldehyde **86** in racemic form. This method was later abandoned due to the difficulties in obtaining or synthesizing an enantiomerically pure starting material (**98**).

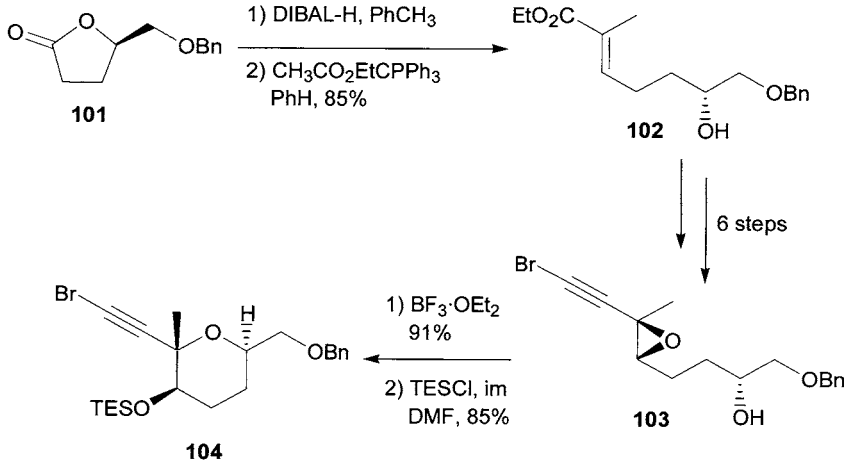
The most practical sequence for the assembly of compound **86** proved to be the route illustrated in Scheme 20; it used five steps and began from (2*R*, 3*R*)-epoxy geraniol in a 16% overall yield.



Scheme 21. Third attempt to assemble **86**; oxymercuration.

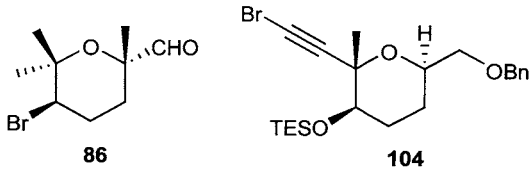
The coupling partner (**104**) for the aldehyde **86** was synthesized as shown in Scheme 22. Starting with lactone **101** [53], a reduction with DIBAL-H to afford the lactol and extension of the carbon framework furnished compound **102**. In six steps, the researchers were able to

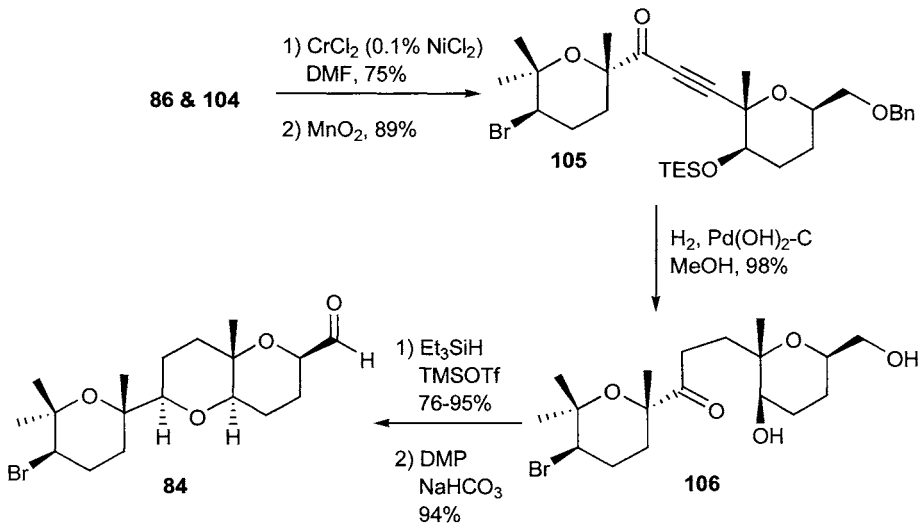
access epoxy alcohol **103**. Lewis acid-catalyzed cyclization with $BF_3{\cdot}OEt_2$ provided the C-ring of **104** stereoselectively; protection of the alcohol with the TES group afforded **104** in 11 steps and 22% overall yield from **101**.



Scheme 22. C-ring synthesis.

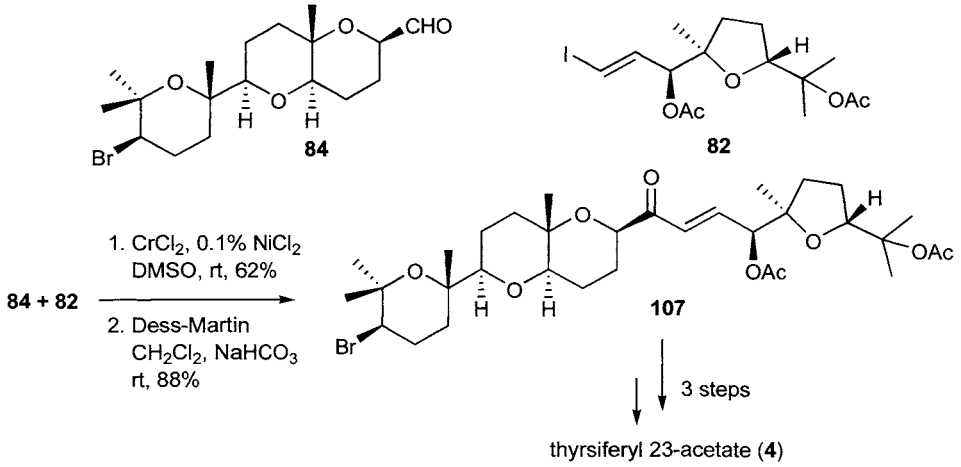
In order to join rings A and C, the organochromium reaction [54] illustrated in Scheme 23 was used to furnish ketone **105**, it being obtained after oxidation of the diastereomeric alcohol mixture with MnO_2. Reduction of the alkyne and deprotection of both the Bn and TES units gave the corresponding keto-alcohol **106**. Reductive cyclization with Et_3SiH and TMSOTf [55] followed by oxidation of the primary alcohol afforded adduct **84** in good yields. In this fashion, an efficient and convergent route to the ABC ring system was achieved.





Scheme 23. Forsyth's ABC framework.

Coupling of vinyl iodide **82** (a substrate derived from geraniol) with aldehyde **84** was accomplished via a Nozaki-Hiyama-Kishi [56] coupling reaction to give **107** after oxidation (Scheme 24). Three straightforward transformations allowed the authors to isolate thyrsiferol (**1**) and thyrsiferol 23-acetate (**4**) in a total of 36 steps and 24 steps for the longest linear sequence.

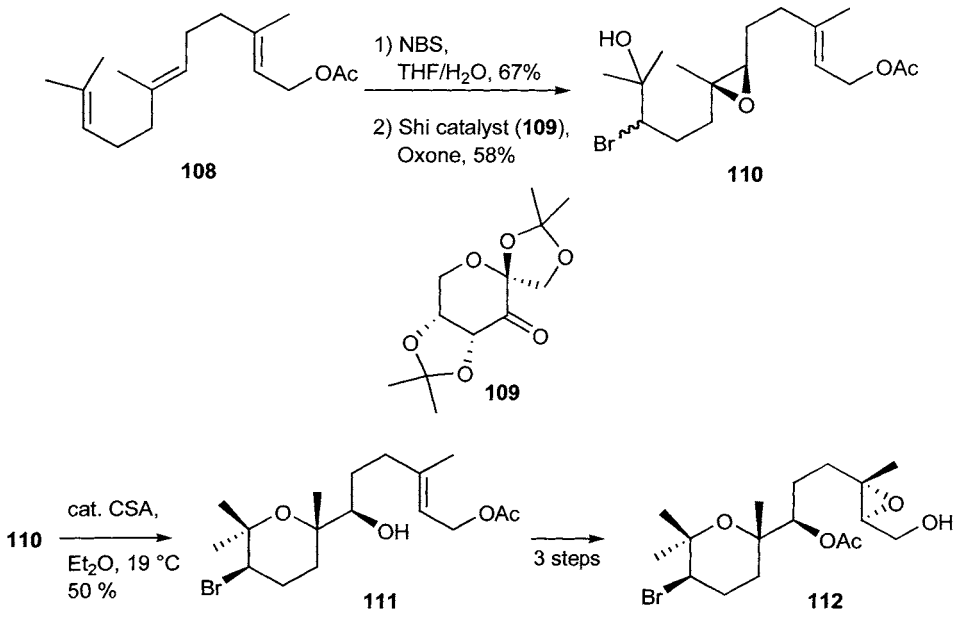


Scheme 24. Final steps of Forsyth's total synthesis.

McDonald's approach toward the ABC framework.

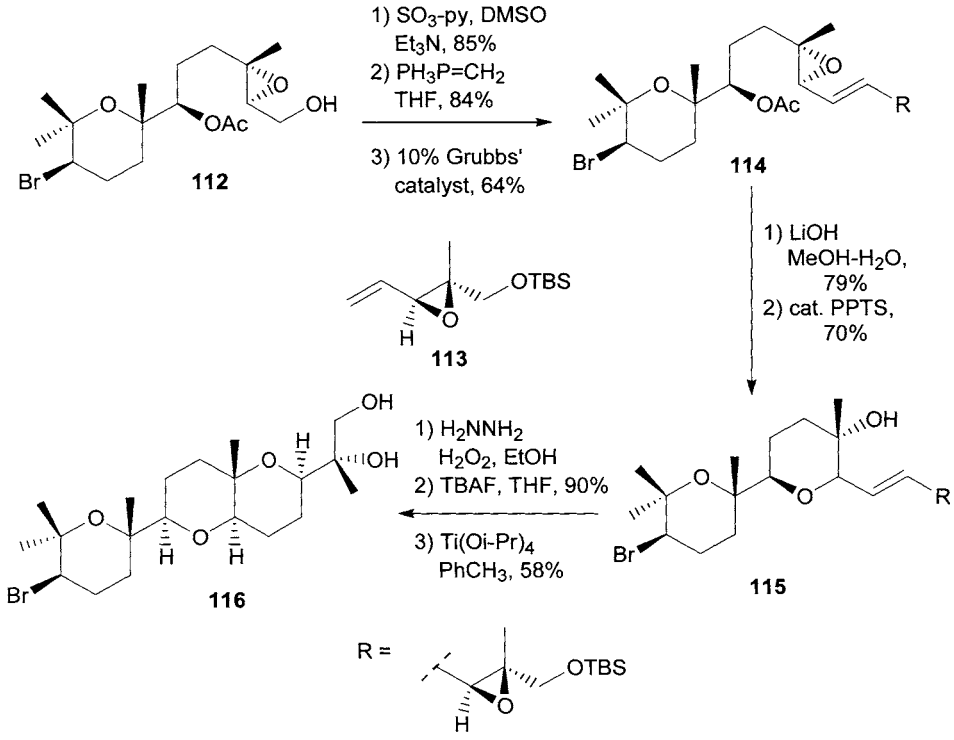
McDonald and co-workers have published a partial synthesis of thyrsiferol (**1**) and venustatriol (**2**) [30]. They focused upon the regio- and stereoselective oxacyclizations [57] to approach the ABC framework, a structural feature that is common to both natural products.

Implementation of the plan commenced with farnesyl acetate (**108**). Bromohydrin formation to give a racemic mixture (Scheme 25) [58], followed by Shi epoxidation [59] in a regio- and enantioselective manner, afforded compound **110**. Acid-catalyzed cyclization of **110** proceeded to afford the requisite tetrahydropyran framework. Under carefully controlled conditions, kinetic resolution was achieved; a yield of 50% was observed for the cyclization of **110** to the halogenated tetrahydropyran **111**. This strategy constitutes the highest yielding option discussed so far for the formation of the A-ring for this family of natural products. The synthesis continues with epoxidation of the C-C π bond, and protecting group transformations to furnish epoxyalcohol **112**.



Scheme 25. McDonald's approach for the A-ring.

A 2-step elaboration of the alcohol (**112**, Scheme 26) to a terminal alkene set the stage for a cross-coupling metathesis [60] reaction with compound **113** [61] in the presence of Grubbs' catalyst. The cross-coupling reaction proceeded to provide the diepoxy-*trans*-alkene **114** (44%) along with the homodimer side product.



Scheme 26. McDonald's scheme to assess compound **116**.

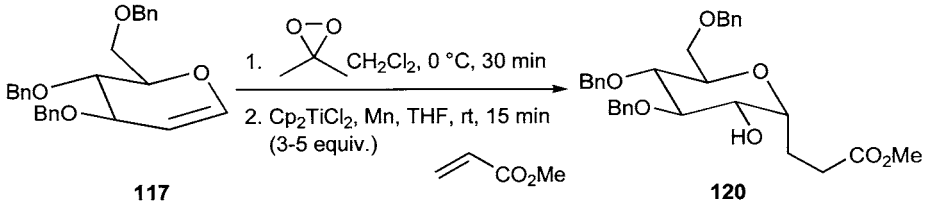
With **114** in hand, deprotection of the acetate unit, followed by acid-induced cyclization, furnished epoxy-alcohol **115** in good yields. The authors used another acid-catalyzed cyclization to form the C-ring of the desired fragment **116** in a regio- and stereoselective manner. Thus, functional group manipulation of **115** and cyclization upon exposure to $Ti(O\text{-}iPr)_4$ [62] afforded compound **116** in 58% yield. This synthesis proceeded in 14 steps from farnesyl acetate (**108**) with 1.5%

overall yield. The completion of the natural product synthesis using this set of reaction conditions remains to be published.

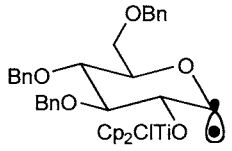
Little-Nishiguchi approach to thyrsiferol

Our interests in the total synthesis of thyrsiferol (**1**) and potential analogues evolved as a venture aimed at implementing a novel and straightforward means by which to convert anhydrosugars to α-*C*-glycosides by using titanocene monochloride (Cp_2TiCl). The application of this reagent in organic chemistry was pioneered by RajanBabu and Nugent [63].

In 2002, our laboratory reported the stereoselective synthesis of α-*C*-glycosides from glycals [64]. Central to these studies was the generation of an anomeric radical from 1,2-anhydrosugars using in situ generated Cp_2TiCl. In the presence of a suitable trapping agent, the radical could be trapped to furnish the α-C-glycosides in good yields (Scheme 27). Thus, tri-*O*-benzyl glycal (**117**) was treated with DMDO to afford epoxide (**118**) stereoselectively [65]. By exposing the epoxide to Cp_2TiCl in the presence of an electron deficient alkene, the corresponding α-*C*-glycosides were isolated in yields ranging from 56% to 61% for the two steps (presumably via the anomeric radical **119**).

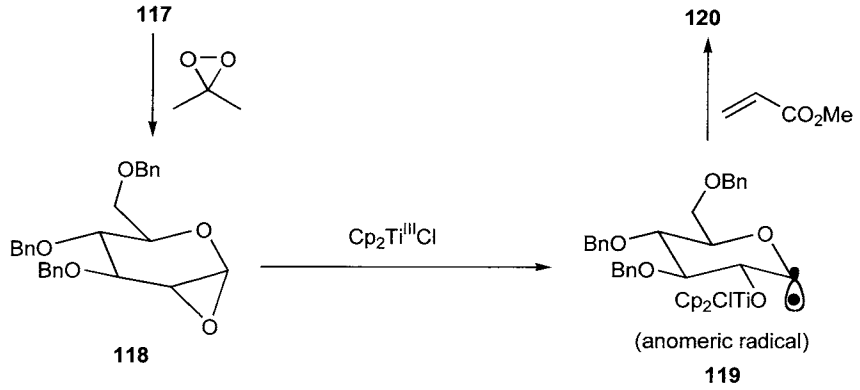


117 **120**



(anomeric radical)

119

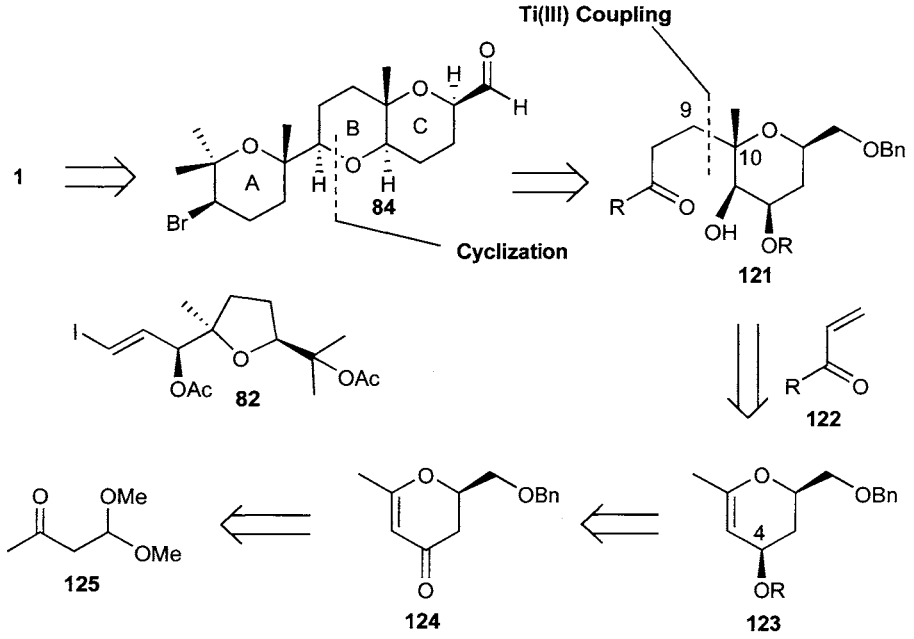


Scheme 27. □-*C*-Glycoside methodology.

Among the reactive trapping agents, only □,β-unsaturated esters and nitriles that reduced with potentials in the range of –2.7 to –2.9 V proved to be effective; other alkenes whose redox potential fell outside the range of –2.7 to –2.9 V (vs. $Ag/AgNO_3$) window were unreactive.

We were curious to determine whether the chemistry could be extended to more elaborate coupling partners with an eventual goal being to apply it to the total synthesis of natural products containing the pyranopyran framework. Hence, the construction of synthetically useful □-*C*-glycosides and their conversion into cyclic ethers became of interest to us.

We selected the natural product thyrsiferol as an ideal target to test our ideas. Its total synthesis was envisioned to proceed as illustrated in the Scheme 28. The successful coupling between aldehyde **84** and vinyl iodide **82** via a Nozaki-Hiyama-Kishi (NHK) reaction [66] had been demonstrated previously [29]. We therefore sought to model our final steps after precedence presented by Forsyth for the union of these two fragments. The focus of our synthetic strategy centered around the stereoselective synthesis of the ABC framework (**84**) of thyrsiferol (**1**) as a scaffold to validate the scope of the Cp_2TiCl reaction with epoxides toward the assembly of □-*C*-glycosides and cyclic ethers.



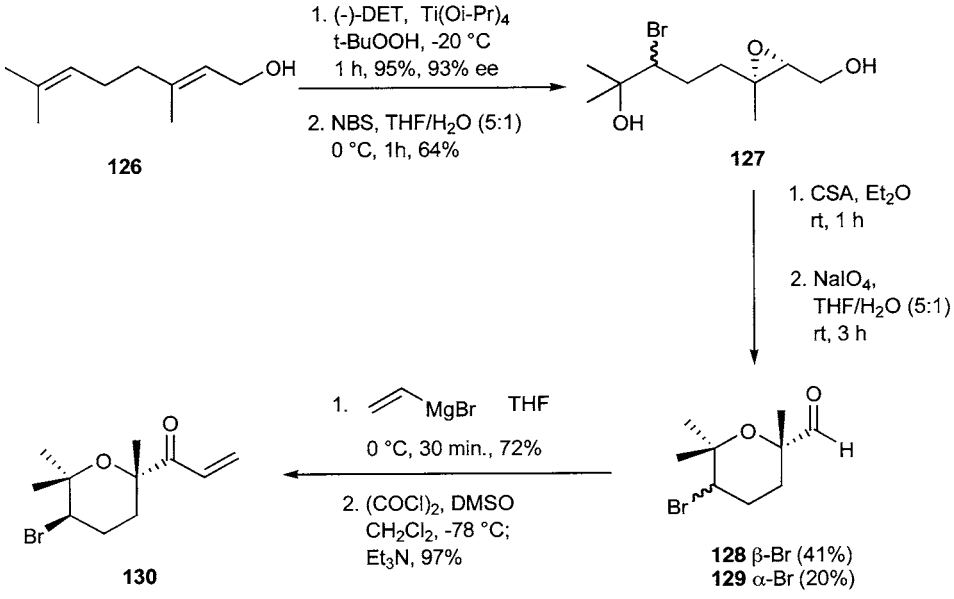
Scheme 28. Retrosynthetic analysis of thyrsiferol.

Retrosynthetic cleavage of the bond indicated in structure **84** reveals keto-alcohol **121** as a possible precursor, it containing a fully substituted carbon centered at C_{10} and a free hydroxyl group at C_{11}. Simplification of this intermediate by breaking the C_9-C_{10} bond, discloses fragments **122** and **123**. Their stereoselective coupling was envisioned to be accomplished via the titanium^{III}-mediated reaction. In the forward direction, reaction of the epoxide derived from **123** with Cp_2TiCl would regioselectively generate the more stable anomeric radical, which in the presence of the electron deficient alkene **122**, would afford product **121**.

The origin of dihydropyran **123** can be traced to the known dihydropyranone **124**, derived from commercially available acetylacetaldehyde dimethylacetal **125**. Although the oxygen at C_4 of dihydropyran **123** is not present in the natural product, its role was to facilitate a stereocontrolled epoxidation reaction as well as the stereoselective formation of the □-*C*-glycoside. Its presence also

offered the opportunity for analogue generation. Given the number and variety of reactions describing deoxygenation procedures [67] the successful removal of the oxygen at C_4 of **123** at later stages of the synthesis was not of immediate concern.

The successful implementation of this strategy involves the synthesis of a suitable trapping agent. Hence, trapping agent **130** was synthesized in the manner outlined in Scheme 29 via the cyclization of the bromohydrin epoxide **127** under similar conditions to those described by McDonald and co-workers [30].



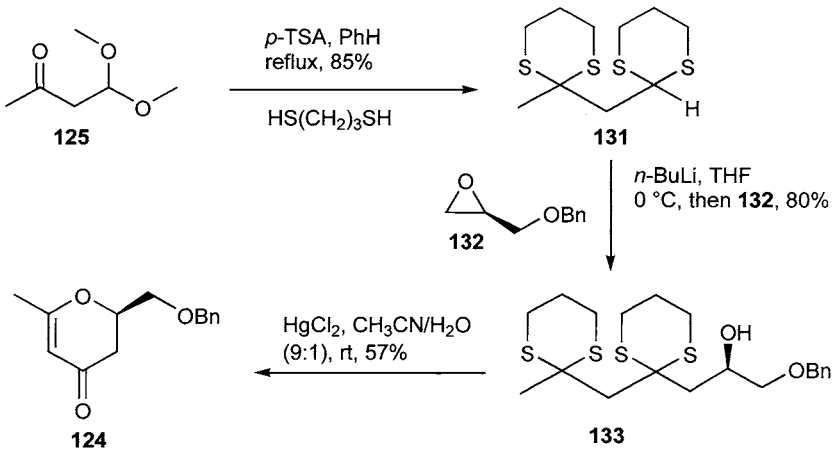
Scheme 29. Synthesis of trapping agent **130**.

Starting with geraniol (**126**), Sharpless asymmetric epoxidation [68] proceeded in 95% yield and with 93% *ee* (see Scheme 29) [69]. Hydrobromination of the alkene was achieved under standard conditions using NBS in THF and water (5:1) to afford a 64% yield of diastereomeric bromo alcohols (**127**). Cyclization to the tetrahydropyran diol was accomplished by treating **127** with camphorsulphonic acid in diethyl ether, followed by diol cleavage with sodium periodate to give a mixture of aldehydes **128** and **129**. Upon

separation of the two isomers, the desired []-isomer (**128**) was isolated as the major product in 41% yield over the two steps. Although the same kinetic resolution reported by McDonald [30] was not observed with our substrate, the straightforward separation of the diastereomeric aldehydes **128** and **129** rendered this methodology practical.

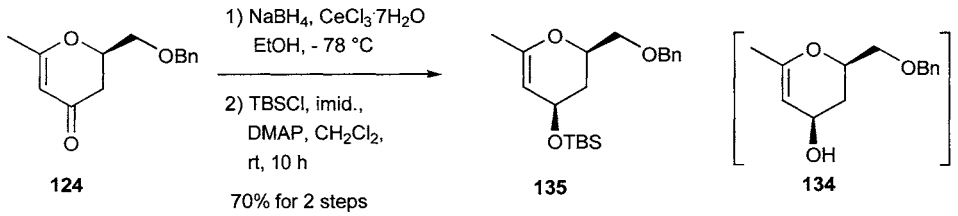
Treatment of aldehyde **128** with vinyl magnesium bromide followed by oxidation of the diastereomeric mixture of alcohols under Swern conditions delivered compound **130** in an overall yield of 17% for the 6 steps.

The synthesis of the coupling partner (enone **124**) began in the manner reported by Noda [70] (Scheme 30). Thus, starting with commercially available acetylacetaldehyde dimethylacetal (**125**), the dithiane protection of both the ketone and the dimethyl acetal units under acidic conditions afforded compound **131** in 86% yield as a white crystalline solid. Deprotonation with *n*-BuLi, followed by the addition of *R*-benzyl glycidyl ether (**132**) provided an 80% yield of the hydroxyl ether **133**. Deprotection of the dithiane moiety with $HgCl_2$ in acetonitrile and water revealed the two carbonyl units, which underwent spontaneous cyclization to the enone **124**. Spectroscopic data for this compound were in complete agreement with the published data [70].



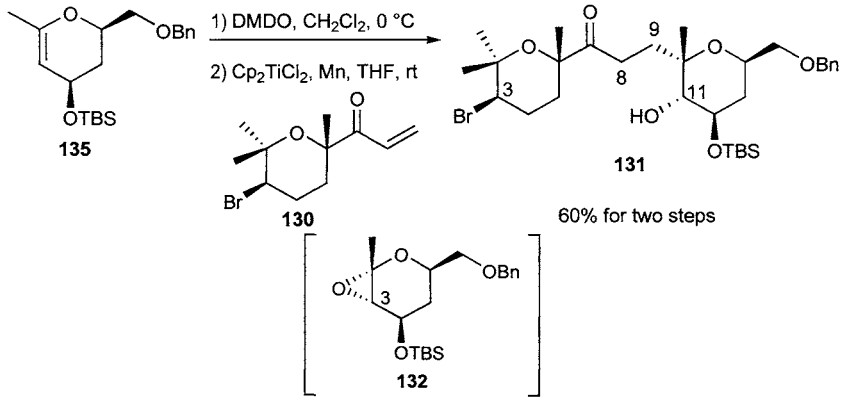
Scheme 30. Synthesis of dihydropyranone.

Luche's conditions [71] for the reduction of **124** delivered the unstable allylic alcohol (**134**), which was quickly treated with TBSCl and Et_3N to afford **135** in 70% yield for the two steps (Scheme 31). This 5-step reaction sequence from **125** proved to be usable in sizable quantity (~20 g) and it also offered the advantage of starting with a simple and commercially available chiral moiety (S-glycidol).



Scheme 31. Dihydropyran synthesis.

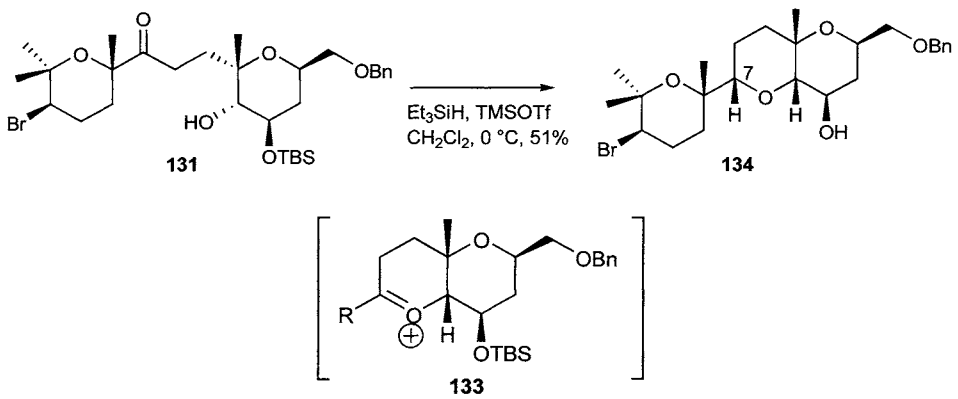
With compound **135** available, its epoxidation was investigated. Treatment of **135** with a solution of dimethyldioxirane afforded a single isomer and our initial attempts to couple this product with the bromopyran **130** in the presence of Cp_2TiCl provided disappointingly low yields (10-15%) of **131**. We suspected that the titanium^{III}-induced ring opening of **132** was at fault, but quickly discovered that our difficulties could be traced to the increased lability of the methyl-substituted epoxide **132**, relative to **118**.



Scheme 32. The titanium^{III}-mediated coupling reaction.

Presumably, traces of water present in the dimethyldioxirane solutions were promoting the opening of the epoxide and hindering the radical chemistry from taking place. The most effective and reproducible way to obviate the problem was to ensure that the DMDO solution was used immediately after drying with freshly activated, crushed 4Å molecular sieves; both the epoxidation and trapping reactions were also run in the presence of the molecular sieves. Additionally, upon isolation of the epoxide **132** via evaporation of the solvent, the epoxide had to be used immediately in the trapping reaction. Once these modifications were made, the yields for the isolated product rose significantly to 50-60% for the two steps. Compound **131**, fully substituted at the anomeric center and with a free hydroxyl unit at C_{11}, was obtained [31].

Delighted by the success of this reaction sequence, the synthesis proceeded as follows. Treatment of **131** with triethylsilane and trimethylsilyl triflate in dichloromethane, under conditions developed by Olah and Prakash, afforded product **134** (Scheme 33) [72]. This compound was a white crystalline solid and therefore an x-ray structure was obtained, Fig. (**8**).



Scheme 33. Reductive cyclization of **131**.

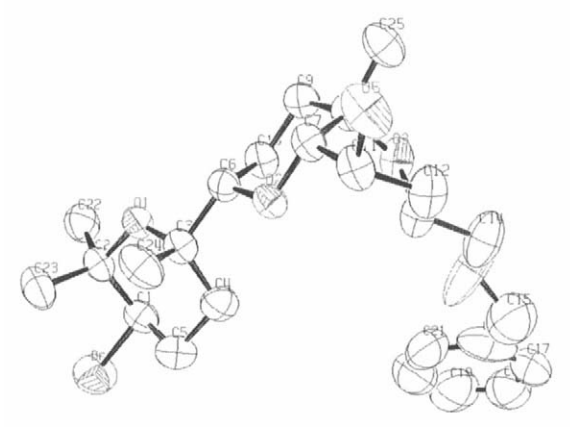
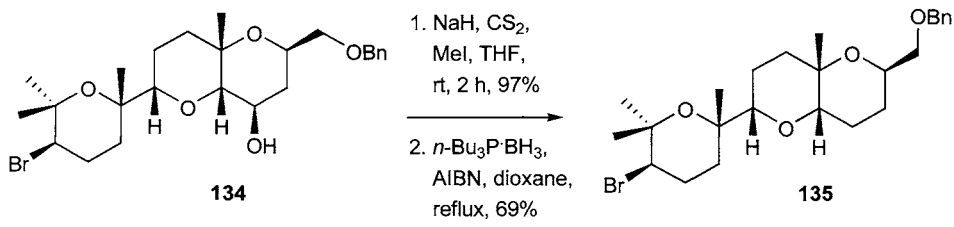
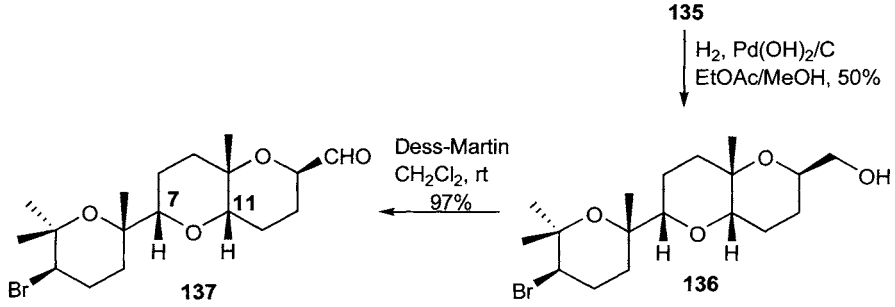


Fig. (8). X-ray structure of **134**.

Analysis of the x-ray results revealed that all of the stereocenters for compound **134** were as depicted in Scheme 33. The BC-rings were shown to adopt a chair/twist boat conformation to avoid 1,3-diaxial interactions between the methyl group and the benzyloxy unit. A similar conformation was reported for the C-ring in the case of the *trans*-fused pyranopyran framework [4]. The configuration of the newly formed stereocenter at C_7 in structure **134** is undoubtedly the consequence of a stereoelectronically controlled addition to the cyclic oxonium intermediate, **133**.

Efforts to remove the hydroxyl group from C_{12} of **134**, or to effect deoxygenation at earlier stages of the sequence were problematic. Eventually, we discovered that an elegant method developed by Barton and co-workers to handle deoxygenation in a hindered environment could be used. Of added value is the fact that the chemistry, though reductive, does not touch the potentially labile C-Br bond that is found in the A-ring [73].





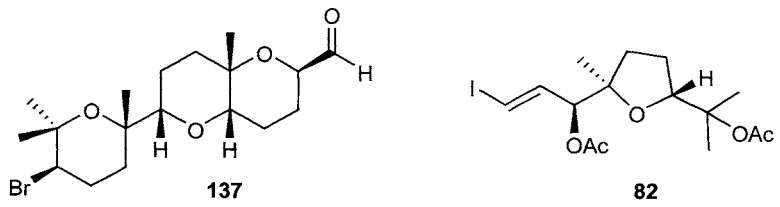
Scheme 34. Xanthate synthesis and deoxygenation.

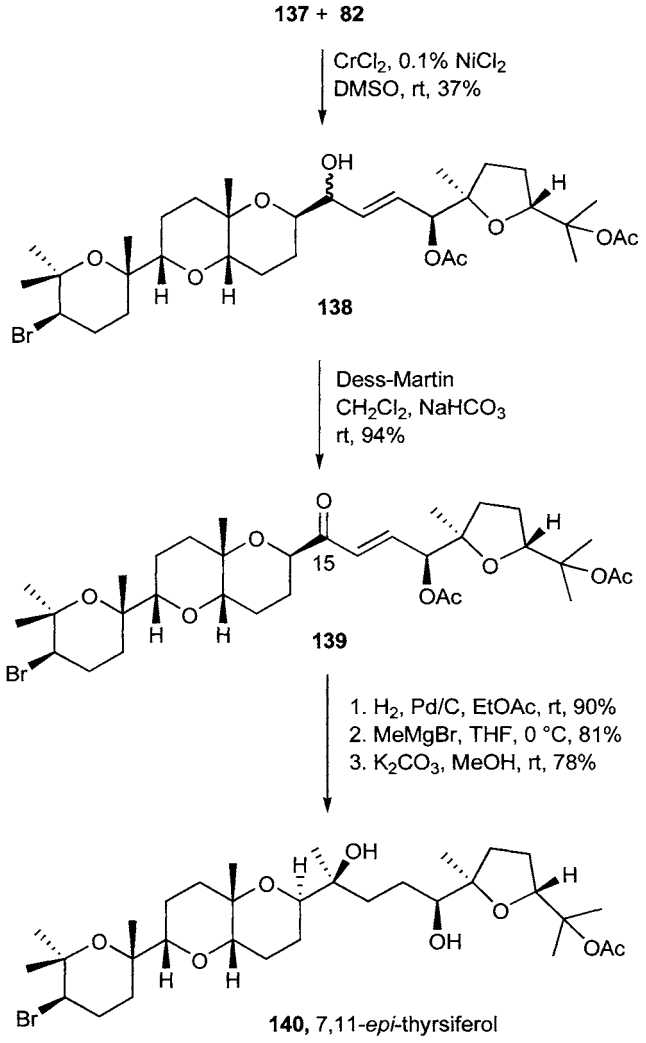
The xanthate required to implement the transformation was synthesized by first treating alcohol **134** with NaH and CS_2, followed by the addition of MeI to give a 98% yield of the desired product (Scheme 34). Reaction of the xanthate with tributyl phosphine-borane complex and AIBN in refluxing dioxane gave the reduced product **135** in a 69% yield.

Upon deprotection of the benzyl group under hydrogenolysis conditions, and oxidation of the resulting primary alcohol to the corresponding aldehyde with Dess-Martin periodinane [74], compound **137** was obtained. Aldehyde **137** is the coupling partner for the NHK reaction with the vinyl iodide **82**.

The synthesis of vinyl iodide **82** followed the report of Forsyth and co-workers [29]. Their reaction conditions proved efficient [75].

With substrates **82** and **137** in hand, the stage was set for the Nozaki-Hiyama-Kishi reaction; it was performed using $CrCl_2$ and 0.1% $NiCl_2$ in dry DMSO. Under these mild and chemoselective conditions, a diastereomeric mixture of alcohols **138** was obtained in a 37% yield. Further optimization of these reaction conditions was not investigated due to the small quantity of substrate available.





Scheme 35. NHK reaction.

Four more steps were necessary to get to the final product. The overall sequence required a total of 32 steps and 17 steps for the longest linear sequence. The successful completion of the synthesis of 7,11-*epi*-thyrsiferol (**140**) gives us confidence that the synthetic methods will be applicable in other complex settings as well. Our progress toward

the total synthesis of thyrsiferol (**1**) using the same methodology is in progress.

Pharmacological Studies for 7,11-*epi*-Thyrsiferol (140)

The completion of the total synthesis of 7,11-*epi*-thyrsiferol (**140**), a novel analogue of the thyrsiferol family of compounds, prompted us to investigate its potential anticancer properties. To examine the pharmacological profile of **140**, we selected sea urchin embryos as a biological system for the *in vitro* evaluation of antimitotic activity [76].

A preliminary screen of the antimitotic activity of 7,11-*epi*-thyrsiferol (**140**) using the sea urchin assay established that this compound inhibits the first cleavage of *S. purpuratus* embryos in a concentration dependent manner with 50% inhibition occurring at approximately 7 μg/mL [11 μM, Fig. (**9**)]. At a concentration of 30 μg/mL (48 μM), this compound produced 100% inhibition of the first mitosis without lysis or morphological abnormalities at any of the concentrations used [77].

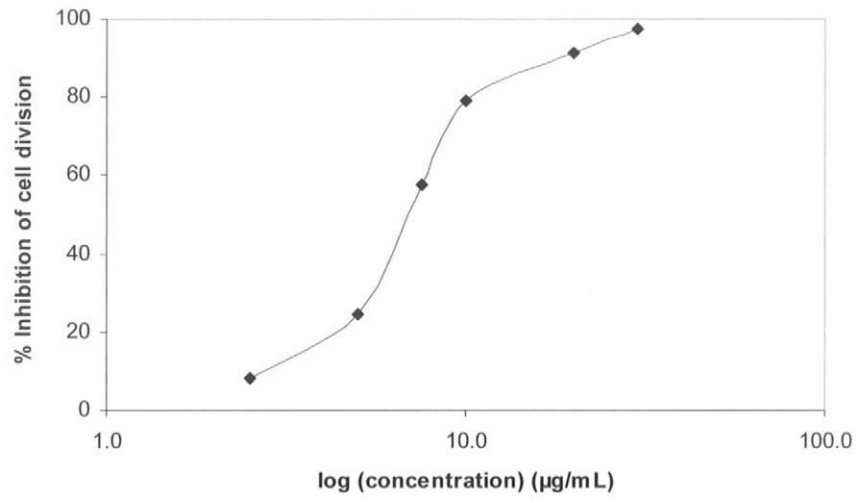


Fig. 9. Log concentration response curve for inhibition of *S. purpuratus* first embryonic cell division by 7,11-*epi*-thyrsiferol (**140**, $IC_{50} \approx 7$ μg / mL).

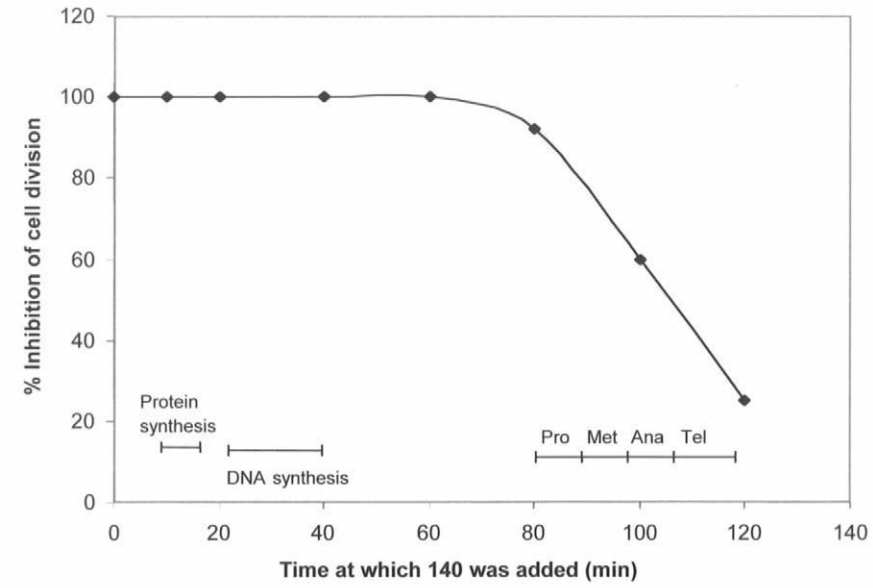


Fig. 10. The effect of 7,11-*epi*-thyrsiferol (**140**) at different stages of the cell cycle in *S. purpuratus* embryos. 7,11-*epi*-Thyrsiferol (48 μM) was added to embryo suspensions at the times indicated post fertilization. Incubation was continued until control embryos had completed the first cleavage. Pro, Met, Ana and Tel are abbreviations for prophase, metaphase, anaphase, and telophase of mitosis, respectively.

The effect of **140** on different stages of the cell cycle was determined by adding 30 μg of it (*i.e.*, the concentration that produces 100% inhibition of the first mitosis) at 10 min intervals after fertilization to aliquots of sea urchin embryos. Preliminary results showed that the maximal inhibition of embryonic cleavage continued to occur even when **140** was added as late as 70 min after fertilization; the inhibition declined steeply when **140** was added 80 min after fertilization, Fig. (**10**). Thus, identification of cell cycle phases that are sensitive to the drug suggests that **140** is effective prior to metaphase of the cell cycle.

The actions of the natural products **1** and **3** on sea urchin embryonic cell division were determined for comparison with the synthetic

analogue **140**. Thyrsiferol (**1**) and dehydrothyrsiferol (**3**) inhibited the first cleavage of *S. purpuratus* embryos in a concentration dependent manner with 50% inhibition occurring at 85 ng/mL (0.14 μM) and 280 ng/mL (0.48 μM) respectively, Fig. (**11**).

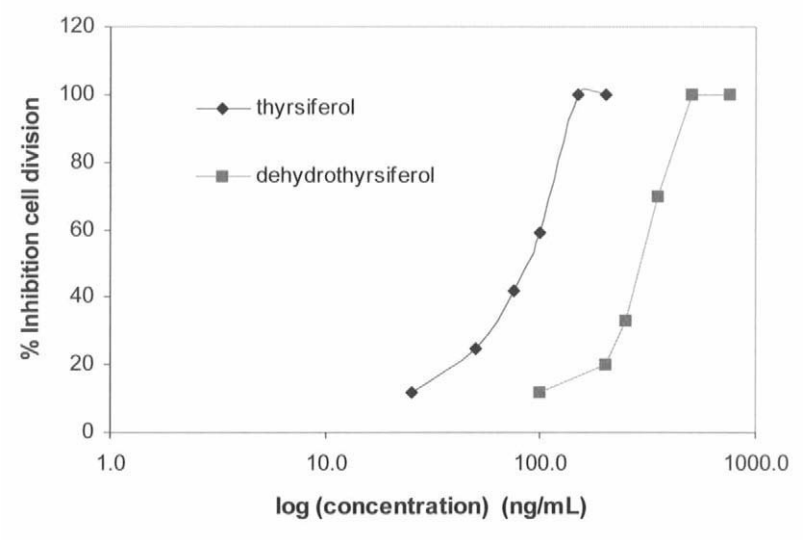


Figure 11. Log concentration response curve for inhibition of *S. purpuratus* embryos cleavage by thyrsiferol ($IC_{50} \approx$ 85 ng/mL) and dehydrothyrsiferol ($IC_{50} \approx$ 280 ng/mL).

The pharmacological results just presented have established the antimitotic activity of compound **140** to be 7 μg/mL [11 μM, Fig. (**9**)] in the sea urchin assay. A comparison between the antimitotic activitiy of synthetic analogue **140** with the naturally occurring products **1** and **3** determined thyrsiferol (**1**) to be the most potent substrate among the three compounds tested (Table 4).

Table 4. Antimitotic activities of 140, 1, and 3

Compound	IC_{50}
thyrsiferol (**1**)	0.14 μM
dehydrothyrsiferol (**3**)	0.48 μM
7,11-*epi*-thyrsiferol (**140**)	11 μM

Although the synthetic analogue 7,11-*epi*-thyrsiferol did not display comparable biological activities as the natural products thyrsiferol and

dehydrothyrsiferol, its total synthesis provided useful insights into the application of a titanium^{III}-mediated methodology in the synthesis of complex molecules.

Summary

As demonstrated by the synthetic routes outlined above, each strategy is elegant and innovative in its own way. A discussion of the difficulties associated with the construction of the A-ring of thyrsiferol is included in every publication. In addition, the stereoselective assembly of the tetrahydropyran central unit via hydroxyl group cyclizations onto epoxides has been shown to be an efficient tactic. An advantage is apparent in Forsyth's approach, with the construction of the A-ring independently and at the outset of the synthesis.

The titanium^{III}-mediated approach toward the ABC framework also proved to be an efficient methodology to access the *cis*-fused analogue (**140**) of thyrsiferol. The application of this method toward the *trans*-fused natural product remains to be investigated and if this execution proves to be successful, the application of this chemistry in the synthesis of cyclic ethers from *C*-glycosides will become even more valuable.

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BIOACTIVE MARINE METABOLITES FROM OKINAWAN WATERS

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ABSTRACT: Marine invertebrates such as ascidians, sponges and others are a prolific source of bioactive secondary metabolites. We have isolated a variety of marine natural products from the Okinawan marine invertebrates by using the sea urchin egg assay. Our recent work, the isolation, structure determination and activities of chlorinated macrolides, sesterterpenic acids, a bromotyrosine derivative, acetogenin derived endoperoxides, diterpene alkaloids, sesquiterpene quinones and spiro-sesquiterpenes, is presented in this article. The syntheses of these metabolites are also described.

INTRODUCTION

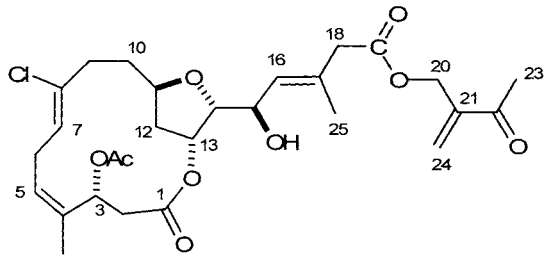
Okinawa is Japan's southernmost prefecture, and consists of hundreds of islands known as the Ryukyus, in an island chain over 1000 km long, which extends southwest from Kyusyu (the southwesternmost of Japan's main four islands) to Taiwan. The warm waters of the Kuroshio Current have developed and sustained the coral reefs of Okinawa, which are among biologically the most diverse and the richest coral reefs in the world.

Biotic stress factors due to predation are usually severe in coral reefs whose biota is characterized by rich and diverse fauna and flora. Many of sessile organisms such as sponges, ascidians, soft corals, etc. have had toxins and noxious compounds to protect themselves from the predators. These compounds may be important sources for natural products based drugs and medicines [1-3].

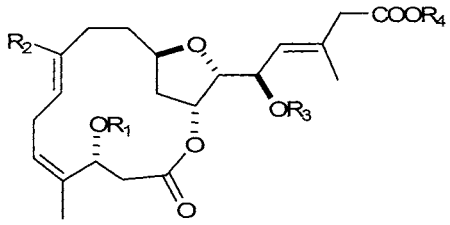
In our searching for inhibitors of the cell division of fertilized sea urchin eggs from Okinawan waters [4], we have isolated a number of chemically and biologically interesting marine natural products. In this article we will discuss the isolation, structure determination and activities of the marine metabolites which have been mainly isolated in our laboratory. Syntheses of these metabolites or their congeners are also described.

Chlorinated macrolides

In the early stage of our screening of the marine organisms of Okinawan waters, we found that the extracts of an ascidian and a sponge collected from the same coast in Hateruma Island had significant inhibitory activity against fertilized sea urchin eggs. The encrusting brown ascidian *Lissoclinum* sp. first was extracted with acetone and then the acetone extract was partitioned between EtOAc and H_2O. The toxic EtOAc-soluble material was subjected to bioassay-guided fractionation by a series of chromatographic processes to give haterumalide B (**1**, 0.000003% of wet weight) [5]. From the gold sponge of *Ircinia* sp., congeners of **1**, haterumalides NA (**2**, 0.065%), NB (**3**, 0.024%), NC (**4**, 0.027%) ND (**5**, 0.023%) and NE (**6**, 0.030%) were isolated in a similar manner described above for **1** [6].



1



2 : R_1 = Ac, R_2 = H, R_3 = H, R_4 = H
3 : R_1 = Ac, R_2 = H, R_3 = H, R_4 = ^{n}Bu
4 : R_1 = Ac, R_2 = OH, R_3 = H, R_4 = ^{n}Bu
5 : R_1 = Ac, R_2 = OH, R_3 = H, R_4 = H
6 : R_1 = H, R_2 = H, R_3 = H, R_4 = H
7 : R_1 = Ac, R_2 = H, R_3 = H, R_4 = Me

The molecular formula of **1**, $C_{28}H_{37}ClO_9$, was established by FDMS [*m/z* 554 $(M + 2)^+$ and 552 M^+], HRFABMS [*m/z* 575.2021 $(M + Na)^+$, Δ+0.3 mmu] and the ^{13}C NMR spectrum. The partial structures including four isolated spin systems could be assigned by 1D and 2D (COSY, HMQC and NOESY) NMR experiments, Fig. (**1**). HMBC correlations

were used to assemble the partial structures to give the planar structure of **1**, Fig. (**1**). The gross structures of **2**, **3**, **4**, **5** and **6** were determined in the same way as for **1**. The relative stereochemistry of **2** was clarified based on coupling constants and NOESY correlations shown in Fig. (**2**). The absolute stereochemistry at C15 in **2** was determined by the modified Mosher's method [7]. Methylation of **2** with CH_2N_2 gave methyl ester **7**, which was transformed into the (*R*)- and (*S*)-MTPA (methoxy-α-trifluoromethylphenylacetic acid) esters. The conventional treatment of $\Delta\delta_H$ (δ_S-δ_R, ppm) values established that the stereochemistry of C15 in **2** was *R*, Fig. (**3**). Trial for establishing the absolute stereochemistry of **2** on the basis of the absolute stereochemistry at C15 led to a wrong conclusion due to the abnormal $\Delta\delta_H$ values [6, 8]. The absolute stereochemistry of **7** was revised by the enantioselective synthesis of the enantiomer **8** of **7** by Kigoshi, Uemura and co-workers, Schemes (**1, 2** and **3**) [8].

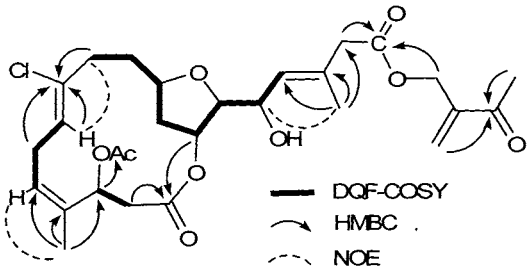


Fig. (1). Partial structures of haterumalide B (1) based on 2D NMR correlations.

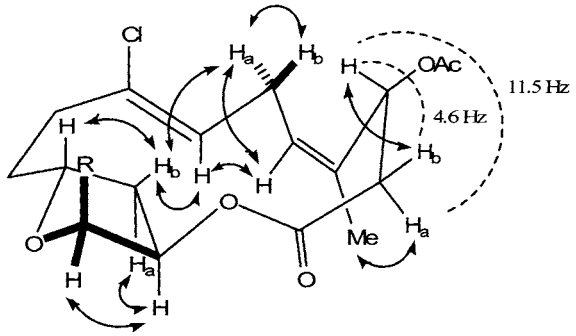


Fig. (2). A plausible conformation of macro ring based on coupling constants and NOESY correlations.

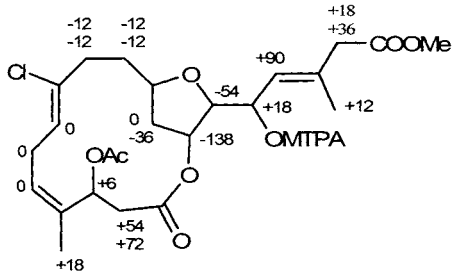


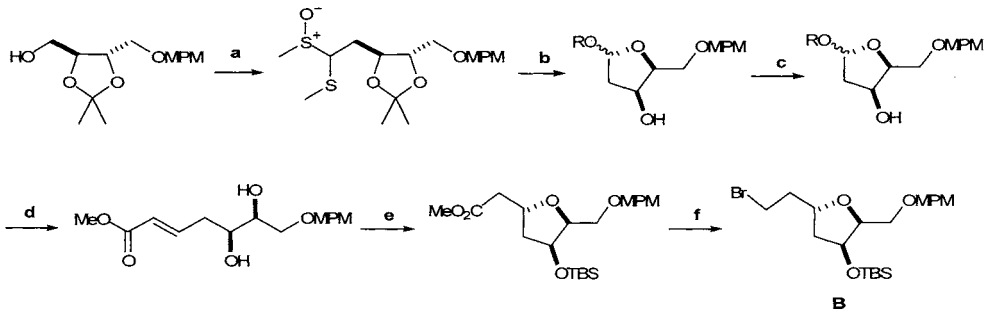
Fig. (3). $\Delta\delta_H$ (δ_S-δ_R, ppm) values for the MTPA esters in Hz.

The synthesis was achieved from commercially available (+)-2, 3-*O*-isopropylidene-L-threitol in 26 steps. The key steps are the stereoselective construction of a chloroolefin unit [step f in Scheme (**2**)] and the intramolecular Reformatsky-type reaction [step g in Scheme (**3**)]. Because the stereochemistry of C3, C11, C13 and C14 in synthetic **8** was undoubtedly constructed to be all *S* by the organic synthetic method, the absolute stereochemistries of C3, C11, C13 and C14 in natural **2** were determined to be *R*, *R*, *R* and *R*. On the other hand, since the absolute stereochemistry of C15 in natural **2** was determined to be *R* by the modified Mosher's method, the total absolute stereochemistry of haterumalide NA (**2**) was revealed, which revised the previously reported structure [8]. To confirm these results, **8** was converted into the (*R*)-MTPA ester, which was the enantiomer of the (*S*)-MTPA ester of the natural haterumalide NA methylester on comparison of their ^{1}H NMR spectra.

Haterumalide B (**1**), a chlorinated 14-membered ring macrolide, completely inhibited the first cleavage of fertilized sea urchin eggs at a concentration of 0.01 μg/mL. Haterumalides NA (**2**) exhibited cytotoxicity against P388 mouse leukemia cells [9] with an IC_{50} of 0.32 μg/mL, and moderate acute toxicity against mice with an LD_{50} of 0.24 g/kg. This showed **2** was a compound with potential as a lead for anticancer agent. Cytotoxicity of **2**, **3**, **4**, **5** and **6** against P388 mouse leukemia cells are shown in Table (**1**). Haterumalides NA (**2**) was the most cytotoxic against P388 cells of them.

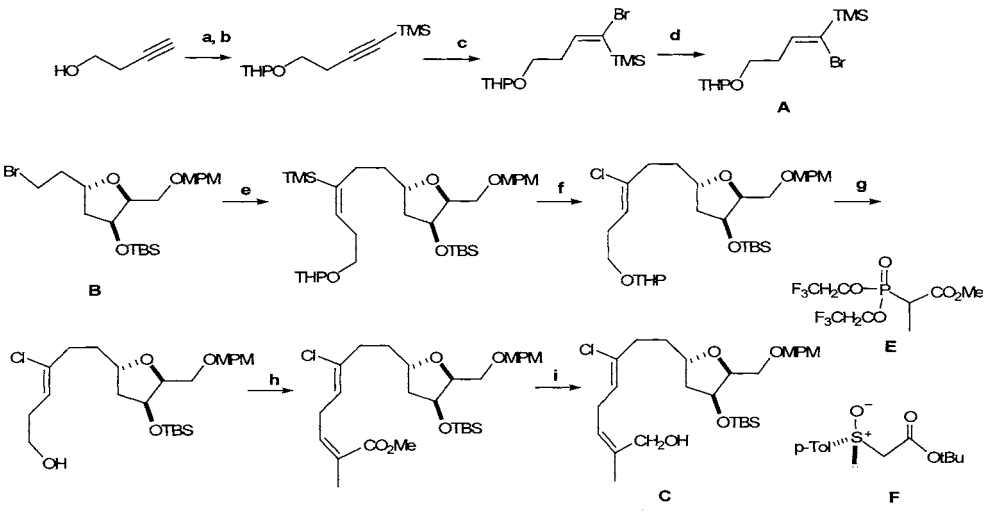
Strobel and co-workers isolated haterumalide NA/oocydin A (**2**) from bacteria *Serratia marcescens* [10]. Haterumalide NA was also obtained from a soil bacterium [11]. The isolation of similar compounds from two unrelated invertebrates such as the ascidian *Lissoclinum* sp. and the sponge of *Ircinia* sp., and the microorganisms, supports the potential microbial origin of haterumalides [12-15]. Although more than 200 marine macrolides have been recorded since the first isolation of the aplysiatoxins, halogenated marine macrolides are rarely found [16, 17].

Total syntheses of *ent*-haterumalide NA and haterumalide NA have been reported by Snider's and Hoye's groups, respectively [18, 19].



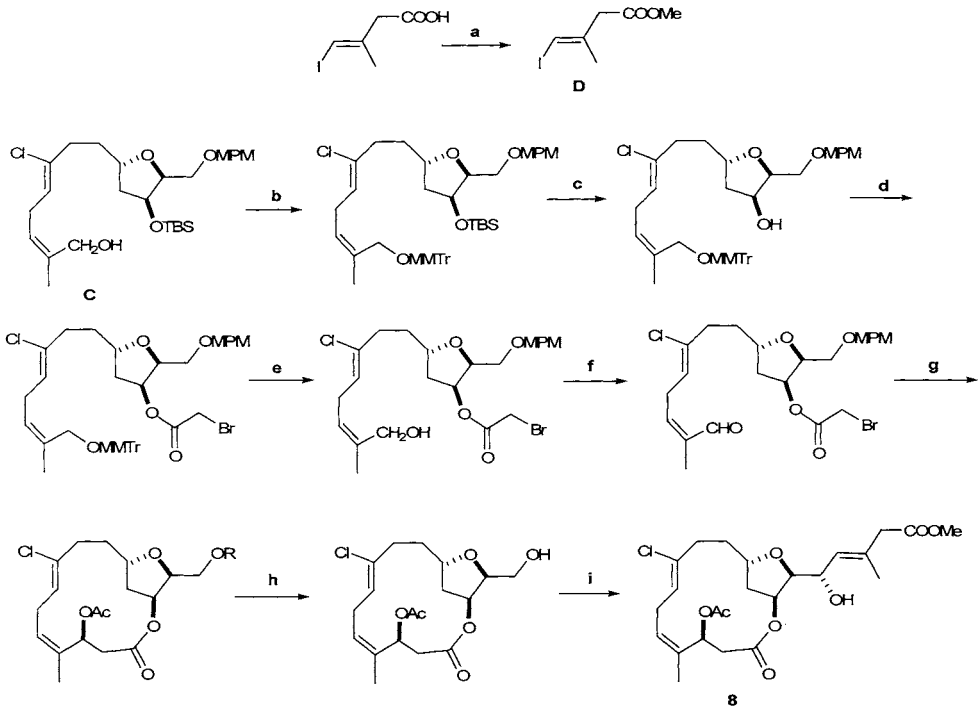
Reagents and conditions: (a) *p*-TsCl, pyridine. (b) NaI, $CaCO_3$, acetone (93%, 2 steps). (c) FAMSO, *n*-BuLi, THF-hexanes (87%). (d) concentrated HCl, MeOH (8:92) (58%). (e) 1 M HCl aq, THF (1:1). (f) Ph_3=$CHCO_2Me$, MeCN. (g) NaOMe, MeOH. (h) TBSCl, imidazole (70%, 3 steps). (i) $LiALH_4$, THF (100%). (j)) *p*-TsCl, pyridine. (k) LiBr, DMF (100%, 2 steps).

Scheme (1). Synthesis of a segment C9-C15.



Reagents and conditions: (a) DHP, *p*-TsOH (93%). (b) TMSCl, *n*-BuLi, ether-hexanes (74%). (c) (i) DIBAL, ether-hexanes; (ii) pyridine, ether; (iii) Br_2, CH_2Cl_2 (91%). (d) *hν*, Br_2, pyridine, CH_2Cl_2 (99%). (e) (i) **A**, *s*-BuLi, THF-hexanes; (ii) **B** in HMPA, THF (68%). (f) NCS, H_2O, DMF (45%). (g) AcOH, THF- H_2O (80%). (h) Dess-Martin periodinane, CH_2Cl_2. (i) **E**, KHMDS, 18-crown-6, THF-toluene (75%, 2 steps). (j) DIBAL, toluene (100%).

Scheme (2). Synthesis of a segment C3-C15.



Reagents and conditions: (a) $TMSCHN_2$, hexane-benzene-MeOH (74%). (b) MMTrCl, pyridine (100%). (c) TBAF, THF (99%). (d) $BrCH_2COBr$, pyridine, CH_2Cl_2. (e) AcOH, THF- H_2O (82% in 2 steps). (f) Dess-Martin periodinane, CH_2Cl_2 (93%). (g) (i) Et_2Zn, $RhCl(PPh_3)_3$, THF-hexanes; (ii) Ac_2O (9%). (h) DDQ, CH_2Cl_2-phosphate buffer (pH 5.9) (88%). (i) Dess-Martin periodinane, CH_2Cl_2. (j) **D**, $CrCl_2$, $NiCl_2$, DMSO (57%, 15*S*:15*R* = 11:1, 2 steps).

Scheme (3) . Synthesis of the enantiomer of the haterumalide NA methyl ester.

Table (1). Cytotoxicity of haterumalides against P388 cells.

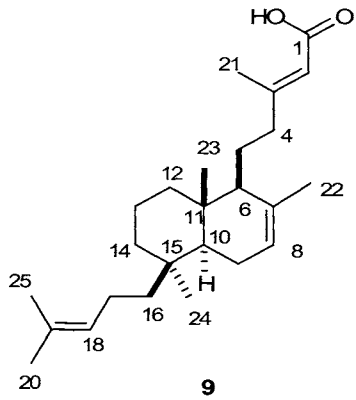
compound	IC_{50} (mg/mL)	compound	IC_{50} (mg/mL)
haterumalide NA (**2**)	0.33	haterumalide ND (**5**)	3.2
haterumalide NB (**3**)	10	haterumalide NE (**6**)	33
haterumalide NC (**4**)	0.46		

Sesterterpenic acids

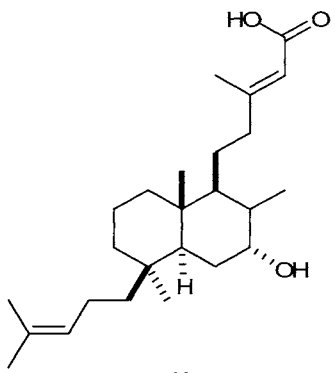
The EtOAc extract of the black sponge *Ircinia* sp. collected in Kohama Island showed inhibition activity against fertilized sea urchin eggs. Separation of the extract by chromatographic techniques gave two new sesterterpenic acids, kohamaic acids A (**9**, 0.0012% of wet weight) and B (**10**, 0.00001%) [20]. These molecular formulae were determined to be $C_{25}H_{40}O_2$ and $C_{25}H_{40}O_3$ by HRFABMS, respectively. Detailed analysis of 1D and 2D (DQF-COSY, HMQC, HMBC and NOESY) data for these compounds led to the structures of **9** and **10**.

The following derivatization was performed to determine the absolute stereochemistry of **9**, Scheme (**4**). Methylation of **9** gave ester **11**. Selective oxidation of the C18-double bond with OsO4, and the cleavage of a C18-C19 bond in the resulting diol by periodic oxidation followed by reduction with $NaBH_4$ gave alcohol **12**. Oxidation of **12** with OsO_4 yielded triol **13**. The relative stereochemistry of **13** was determined based on the NOESY correlations, Fig. (**4**). The absolute stereochemistry C8 in **13** was established by the modified Mosher's method [7]. The triol **13** was transformed into the (*R*)- and (*S*)-MTPA esters, **14** and **15**. The conventional treatment of these $\Delta\delta_H$ (δ_S-δ_R, ppm) values established that the stereochemistry of C8 in **13** was *R*. Thus, the absolute stereochemistry of kohamaic acids A was determined to be 6*R*, 10*S*, 11*S*, and 15 *R*, as depicted in formula **9**.

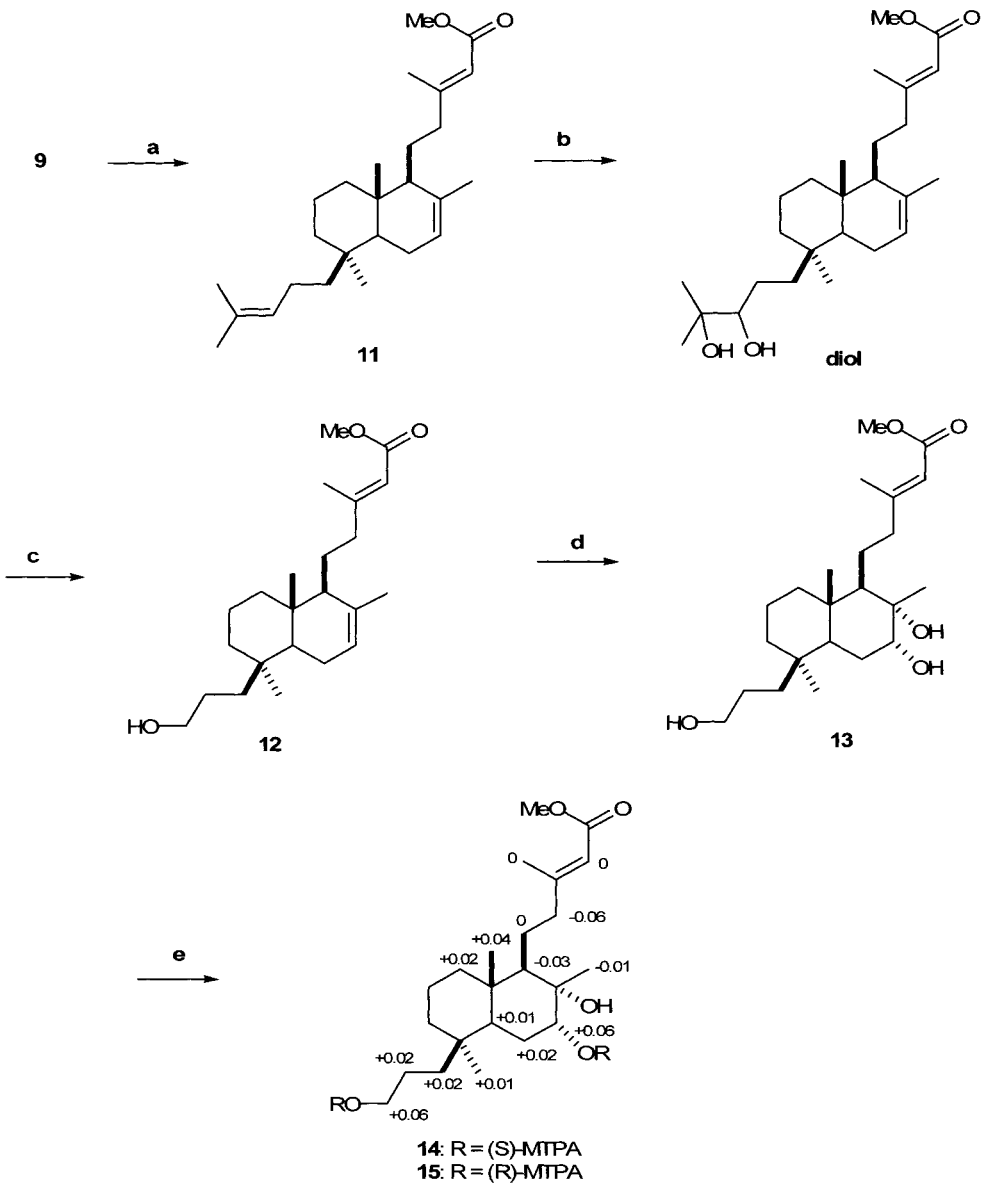
Kohamaic acids A (**9**) and B (**10**) exhibited cytotoxicity against P388 mouse leukemia cells, with an IC_{50} of 17 and 2.8 μg/mL, respectively. Kohamaic acids also completely inhibited the cleavage of fertilized sea urchin eggs at a concentration of 2 μg/mL.



9



10



Reagents and conditions: (a) CH_2N_2, Et_2O, 0 °C (87%). (b) OsO_4, THF, 0 °C; $NaHSO_3$, THF, H_2O, pyridine, rt (53%). (c) $NaIO_4$, dioxane-H_2O, rt; $NaBH_4$, MeOH, rt (45%). (d) OsO_4, THF, rt; $NaHSO_3$, THF, H_2O, pyridine, rt (54%). (e) (-)- or (+)-MTPACl, pyridine, rt (79%).

Scheme (4). Transformation of 9 into MTPA esters 14 and 15.

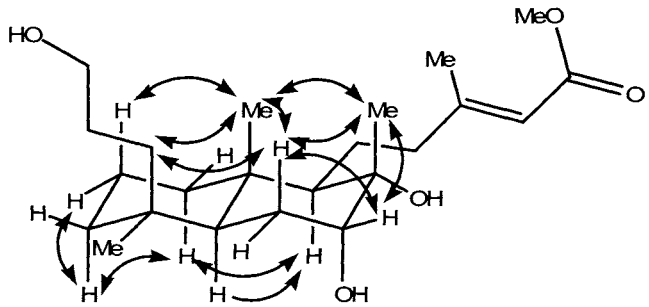


Fig. (4). Selected NOESY correlations of triol 13.

Further investigation on the activity of **9** revealed that **9** could selectively inhibit the activities of DNA polymerases (pol. α, β, γ, δ and ε) only from species in deuterostome branch in the animal kingdom, like sea urchin, fish and mammals, but not from protostomes including insects (fruit fly, *Drosophila melanogaster*) and mollusks (octopus and oyster), Fig. (**5**) [21].

Dysideapalaunic acid (**16**), which is closely related to kohamaic acids and inhibits an aldose reductase, was isolated from the marine sponge *Dysidea* sp. from the Palauan Sea by Nakagawa and co-workers in 1986 [22]. The Okinawan kohamaic acids have a different stereochemistry at C15 from **16** and previously isolated sesterterpenes, which are biosynthesized from (14*E*)-geranylfarnesyl pyrophosphate via the transition state of a chair-chair conformation, Fig. (**6**). It was presumed that kohamaic acids are biosynthesized from (14*Z*)-geranylfarnesyl pyrophosphate via the transition state of a chair-chair conformation, Fig. (**7**). Although (2*Z*)-and (6*Z*)-geranylfarnesyl pyrophosphates have been isolated, the existence of (14*Z*)-geranylfarnesyl pyrophosphate is yet unknown in nature [23]. Therefore, we could not rule out the possibility that kohamaic acids are biosynthesized from (14*E*)-geranylfarnesyl pyrophosphate via the transition state of boat-chair conformation, Fig. (**8**).

Total synthesis of (+)-dysideapalaunic acid (**16**) was achieved by Hagiwara and Uda, Scheme (**5**) [24]. Starting from an optically active Wieland-Miescher ketone analogue (8a*S*)-(+)-ene-dione, octahydro-dimethyl-(methylpent-3-enyl) naphthalene ethylene acetal has been synthesized in eight steps involving reductive alkylation, deoxygenation, and a Wittig condensation. Transformation of the ethylene acetal *via* methylaton at C2, Grignard addition, and a Horner-Emmons reaction furnished the (+) acid **16** in eight steps. Thus, the absolute stereochemistry of (+)-dysideapalaunic acid (**16**) has been determined to be 10*S*, 11*S* and 15*S*. (+)-Dysideapalaunic acid (**16**) and kohamaic acid (**9**) has the same absolute stereochemistries at the bridgehead carbons.

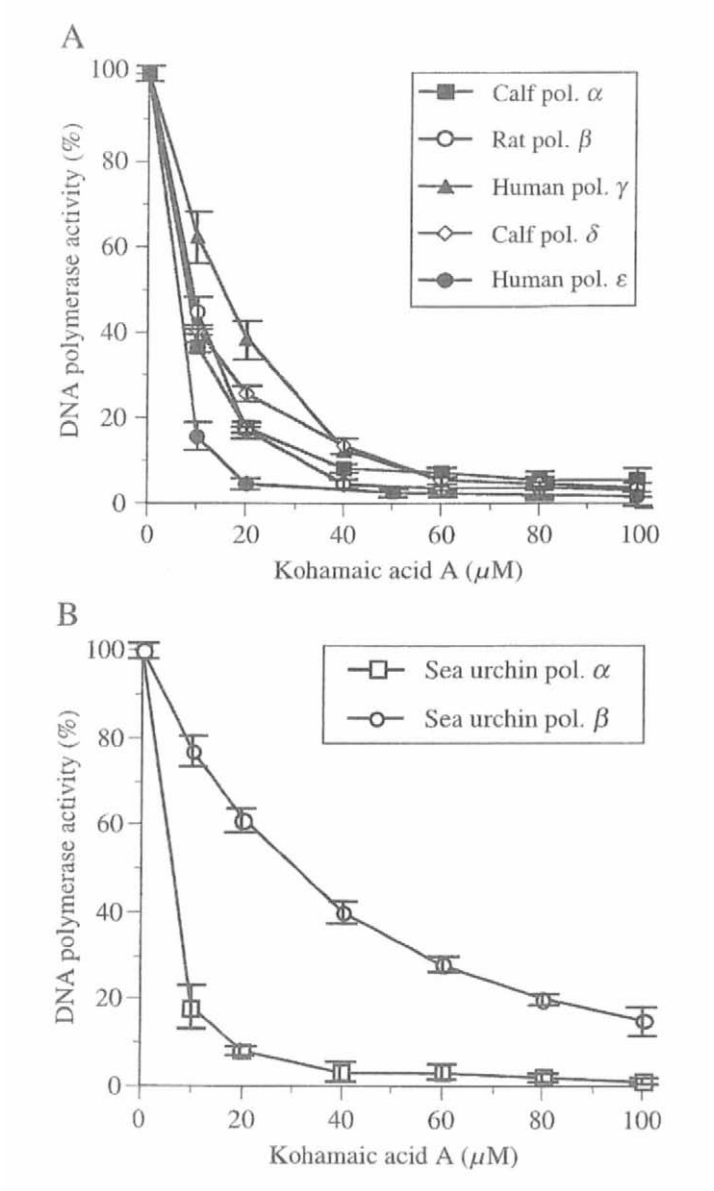


Fig. (5). DNA polymerase inhibition dose response curves of kohamaic acid. (A) Mammalian DNA polymerases. The enzymes used (0.05 units of each) were calf DNA polymerase α (■), rat DNA polymerase β (○), human DNA polymerase γ (▲), calf DNA polymerase δ (◊) and human DNA polymerase ε (●). (B) Sea urchin DNA polymerases. The enzymes used (0.05 units of each) were DNA polymerase α (□) and DNA polymerase β (○). The DNA polymerase activities were measured as described in the Material and methods. DNA polymerase activity in the absence of these compounds was taken as 100%. Data are shown as mean S.E. for three independent experiments.

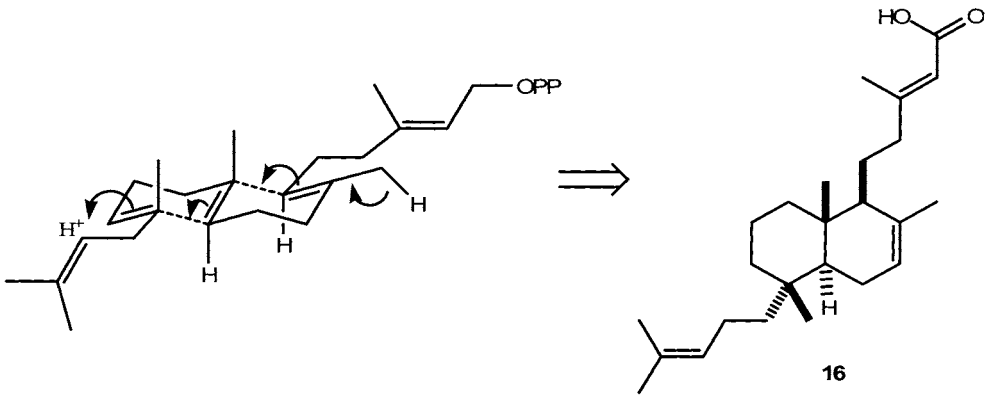


Fig. (6). Biosynthetic pathway of dysideapalaunic acid (16).

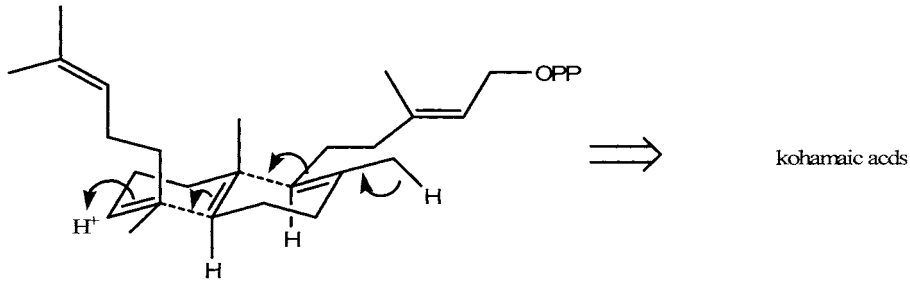


Fig. (7). Plausible biosynthetic pathway 1.

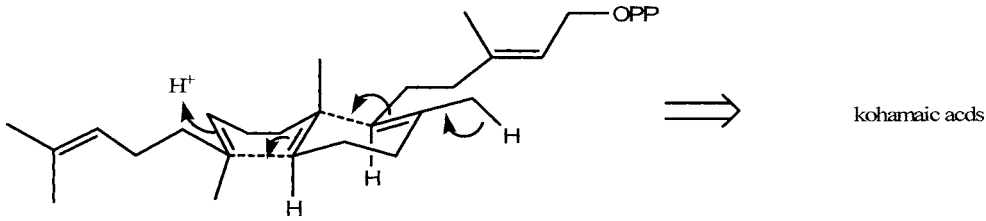
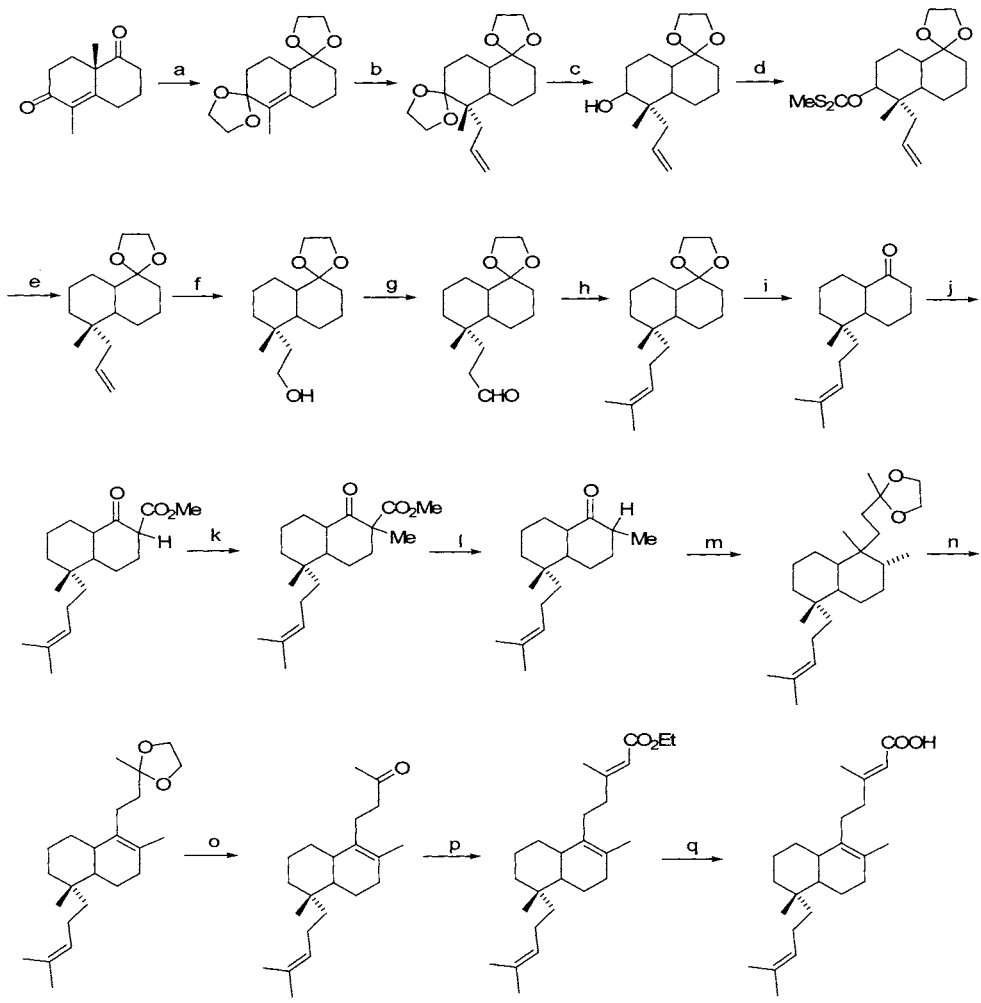


Fig. (8). Plausible biosynthetic pathway 2.



Reagents and conditions: (a) butan-2-one ethylene acetal, $(CH_2OH)_2$, D-CSA, 45°C. (b) Li, liq. NH_3, CH_2=$CHCH_2Br$, water (1 mol equiv.). (c) Li AlH_4, Et_2O, -50°C. (d) BuLi, THF, CS_2, MI, rt. (e) Bu_3SnH, AIBN, xylene, reflux. (f) BH_3-THF, THF, 0°C, then NaOH, H_2O_2, 0°C then rt (g) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -60 to -20°C. (h)Ph_3P=$C(Me)_2$, Et_2O, 0°C then rt. (I)PPTS, aq. acetone, reflux. (j) NaH, $(MeO)_2CO$, THF, rt. (k) NaH, MeI, THF, rt. (l) LiCl, HMPA, 130°C. (m) 4-bromobuta-2-one ethylene acetal, Mg, THF, ultrasonic irradiation then reflux. (n) $SOCl_2$, pyridine, 0°C. (o) PTSA-H_2O, aq. acetone, reflux. (p) NaH, $(EtO)_2P(=O)CH_2CO_2Et$, THF, reflux. (q) 15% aq. NaOH, EtOH, reflux.

Scheme (5). Synthesis of (+)-dysideapalaunic acid (16).

Bromotyrosine Derivative

Environmental pollution caused by metallic antifouling agents, such as copper (I) oxide and bis(tributyltin)oxide (TBTO), have become a globally serious problem [25]. In our continuing search for environmentally acceptable antifouling compounds [26, 27], we investigated the yellow sponge *Psuedoceratina purpurea* collected in Zamami Island.

Fractionation of the aqueous MeOH-soluble extract guided by antibacterial activity against *Rhodospirillum salexigens*, which has adhering properties and forms a microbial biofilm [28], led to the isolation of a novel bromotyrosine derivative, zamamistatin (**17**) [29]. In its ESIMS spectrum, **17** showed 1:4:6:4:1 quintet ion peaks at m/z 697, 699, 701, 703 and 705, indicative of the presence of four bromine atoms. The molecular formula was determined to be $C_{18}H_{18}Br_4N_2O_4$ by HRESIMS. The observation of only 9 carbons by ^{13}C NMR and the specific rotation of +248° suggested that **17** was an optically active dimmer with a symmetrical structure. Not many resonances in the ^{1}H and ^{13}C NMR spectra of **17** in contrast to its molecular formula made the structure determination of **17** difficult.

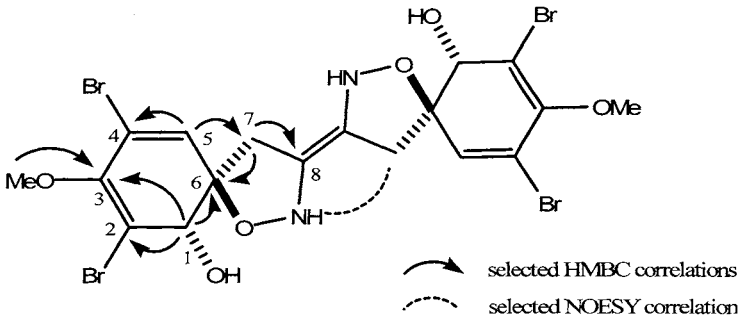
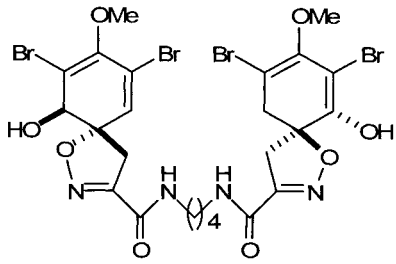


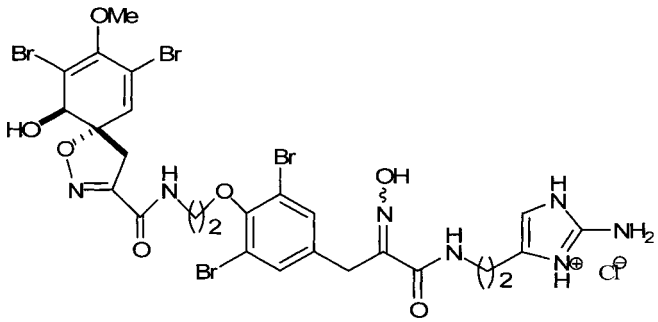
Fig. (9). Initially proposed structure of zamamistatin (17) and the HMBC correlations.

The planar structure, except the geometry of a C8 double bond, was initially proposed shown in Fig. (**9**) by the detailed analysis of 1D and 2D NMR (COSY, HMQC and HNBC) data and by comparison of its ^{1}H and ^{13}C NMR spectra with those of structurally related compounds such as aerothionin (**18**), purealin (**19**) and others [30-41]. Although the DQF-COSY spectrum of **17** showed few ^{1}H-^{1}H correlations, the HMBC spectrum was very useful for elucidating the planar structure of **17**. The geometry of C8 double bond was elucidated by a NOESY H7'/NH correlation to be *trnas*. The relative stereochemistry of **17** was clarified by

the observation of W coupling constant (1.1 Hz) between H1 and H5 and of NOESY correlations H1/H7b and H5/H7a, Fig. (**10**). This stereochemistry in **17** was supported by comparison of the chemical shift of H1 (δ_{1H} = 4.11) with those of related compounds: the ^{1}H chemical shift of the *trans* spiroisooxazoline moiety, which has a *trans* vicinal relationship between a hydroxyl group and an oxygen atom, is δ_{1H} = 4.08 and that of a *cis* spiroisooxazoline moiety is δ_{1H} = 4.40, which was reported by Yamamura and co-workers [34].



aerothionin (**18**)



purealin (**19**)

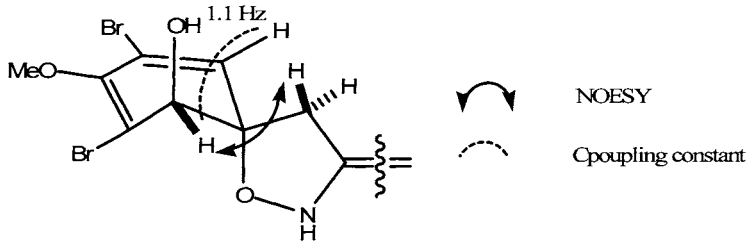


Fig. (10). Relative stereochemistry of zamamistatin (17).

Since zamamistatin (**17**) was an optically active alcohol with a *C2* symmetrical structure, the absolute stereochemistry was determined using the modified Mosher's method [7]. The diol **17** was transformed into the (*R*)- and (*S*)-MTPA esters. The calculated $\Delta\delta_H$ values (δ_S -δ_R, ppm) showed that the absolute stereochemistry at C1 was *S*, Fig. (**11**).

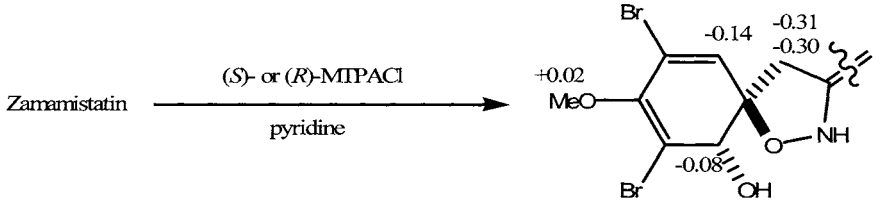
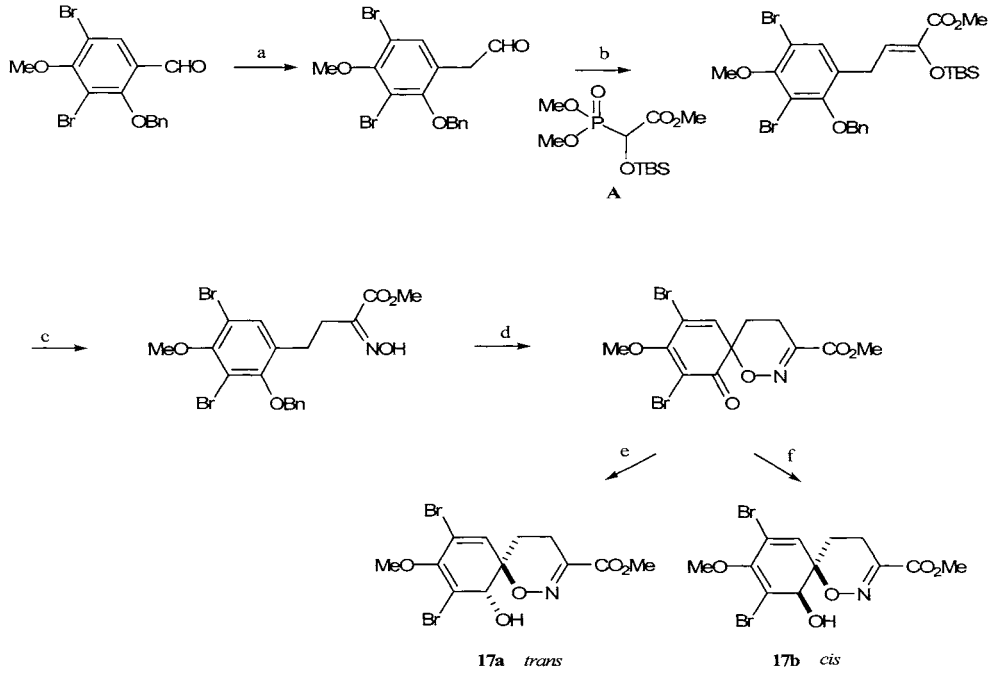


Fig. (11). $\Delta\delta_H$ values (δ_S-δ_R, ppm) for the MTPA esters of zamamistatin (17).

Kigoshi and co-workers recently isolated zamamistatin (**17**) from a sponge *Pseudoceratina* sp. [42]. In the spectral analysis of **17**, they found that the chemical shift of C6 (δ_{C6} 74.3) and C7 (δ_{C7} 26.7) in **17** were different from those (δ_{C6} 91.5 and δ_{C7} 40.2) of aerothionin (**18**), which let them reconsider its structure, Fig. (**12**). Considering the molecular formula of **17**, they proposed a structure possessing a dihydro-1,2-oxazine ring for **17**. To confirm this, they prepared dihydro-1,2-oxazine derivatives, **17a** and **17b**, Scheme (**6**) [42-45] and compared the ^{13}C NMR data for **17** with those of **17a, 17b** and the known **17c** [45]. The ^{13}C NMR data of synthetic **17a** and **17b** were similar to those of zamamistatin (**17**) rather than aerothionin-related compounds, Fig (**12**). Base on these observations it was concluded that the structure of zamamistatin should be revised to an endo-type dimmer.

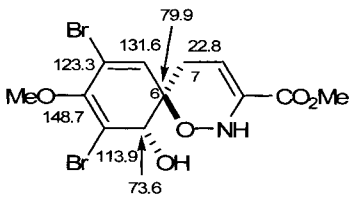


Reagents and conditions: (a) (i) (methoxymethyl)triphenylphosphonium chrolide, t-BuOK, THF, rt. (ii) 2M HCl, THF, reflux, (97%). (b) **A**, LHMDS, THF, -78°C, (98%). (c) (i) HF/pyridine, MeOH, rt, then NH_2OH/HCl, rt. (ii) H_2, Pd/C, 1,4-dioxane-AcOH, rt, (98%). (d) 2,4,4,6-tetrabromo-2,5-cyclohexadienone, MeCN, rt, (92%). (e) $Zn(BH_4)_2$, CH_2Cl_2, rt, (9%). (f) $NaBH_4$, MeOH, rt, (19%).

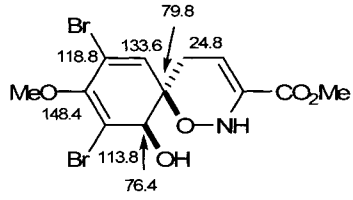
Scheme (6). Synthesis of dihydro-1,2-oxazine derivatives, 17a and 17b.

Biosynthesis of spiroisoozoline was proposed by K. T. Okamoto, J. Clardy et al. [36]. The dimmer structure of zamamistatin (**17**) may be biologically synthesized by reductive dimerization of the spiroisooxazolin moiety in a bromotyrosine precursor followed by decarboxylation, hydrolysis and recyclization, Fig. (**13**). Isomerization from an exo-type dimmer (previously proposed structure for zamamistatin) to an endo-type dimmer (revised structure) was supported by computational calculation, in which the latter is more stable than the former [42].

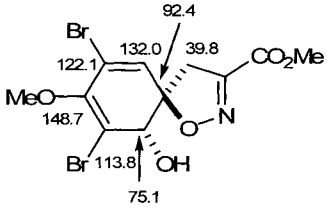
Zamamistatin (**17**) exhibited significant antibacterial activity against *Rhodospirillum salexigens* (21 mm, 1.6 μg/disk).



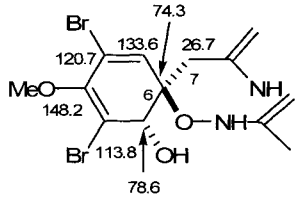
17a



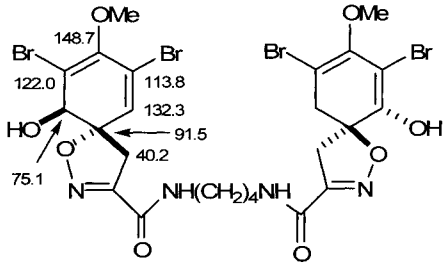
17b



17c

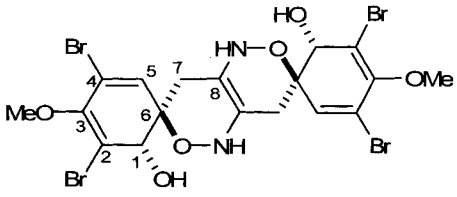


17



aerothionin (18)

Fig. (12). ^{13}C NMR data for compounds 17a, 17b and 17c, .zamamistatin (17), and aerothionin (18).



17 (revised)

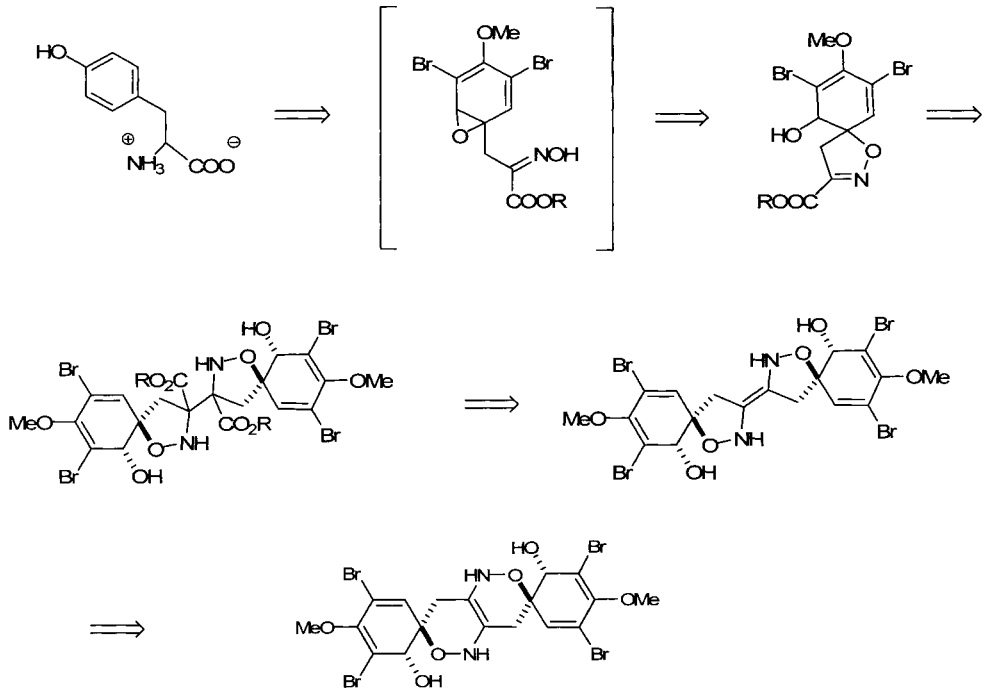
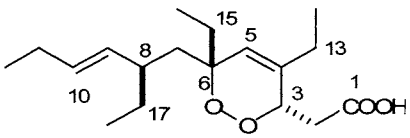


Fig. (13). Proposed biosynthesis of zamamistatin (17).

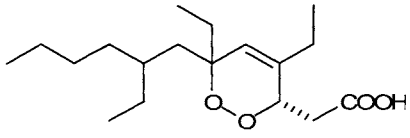
Acetogenin derived endoperoxides

In our continuing search for compounds that inhibit the cell division of fertilized sea urchin eggs, we found that the acetone extract of the sponge *Plakortis lita* De Lubenfels (order Homosclerophorida, family Plakinidae) completely inhibited the first cleavage of fertilized sea urchin eggs at 10 ppm. Marine sponges of the genus *Plakortis* have yielded a wide variety of endoperoxide-containing metabolites, such as plakortin, plakinic acid, placonin, plakortolide, plakinidine, peroxyplakoric acids, monadic acids, plakortones and plakortides [46-61]. These compounds have exhibited cytotoxicity, antibacterial and antifungal activity and Ca^{2+}-ATPase activation. The CH_2Cl_2-slouble fraction of the acetone extract of the white sponge *P. lita* collected in Hateruma Island was subjected to fractionation guided by cytotoxicity against P388 mouse leukemia cells using column chromatography (SiO_2 and ODS) and reversed-phase HPLC (ODS) to give haterumadioxins A (**20**, 0.038% yield based on wet weight) and B (**21**, 0.008%) as oils [62]. The molecular formulae of **20** and **21** were determined to be $C_{18}H_{30}O_4$ and

$C_{18}H_{32}O_4$ by HRFABMS, respectively. Extensive analysis of their 1D and 2D data led to the gross structures of **20** and **21,** which were belong to the same class of endoperoxides derived from fatty acids, Fig. (**14**). Compound **21** was a dihydo derivative of **20**.



Haterumadioxin A (**20**)



Haterumadioxin B (**21**)

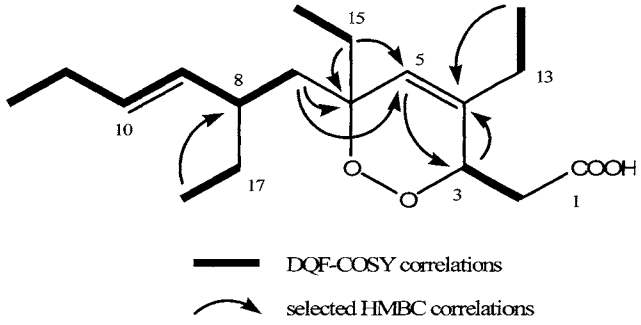


Fig. (14). Partial structures of haterumadioxin A (20) based on 2D NMR correlations.

The relative stereochemistry of **21** was established as follows. The magnitude of $J_{2a,3}$= 3.0 Hz and $J_{2b,3}$= 9.5 Hz suggested that H2a and H3 were located in a *gauche* arrangement and that H2b and H3 were located in an *anti* arrangement, Fig. (**15**). Similarly, the magnitude of $J_{7a,8}$= 8.1 Hz and $J_{7b,8}$= 3.7 Hz suggested that H7a and H8 were located in a *anti* arrangement and that H7b and H8 were located in an *gauche* arrangement, and H8 and H9 were located in a *anti* arrangement. These results and the NOESY correlations H2a/H13, H2b/H8, H2b/H10, H5/H7a, H5/H9, H5/H10, and H8/H10 suggested that one of the stable conformation of **20** was as shown in Fig. (**15**). The stability of this conformation may be due to π–π stacking between C4 olefin and C9 olefin and steric repulsion between the C6-ethyl group and the C8-ethyl group. Therefore, the relative stereochemistry in **20** was determined to be 3*S**, 6*R** and 8*R**. The conformational calculation using Macromodel 6.0 and the MM2*

force field suggested this result. To be more precise, the conformational calculation gave 10 conformations having 2 kJ/mol energy greater than that of the most stable conformation. All these conformations had alkyl side chains turned at C7, as shown in Fig. (**15**).

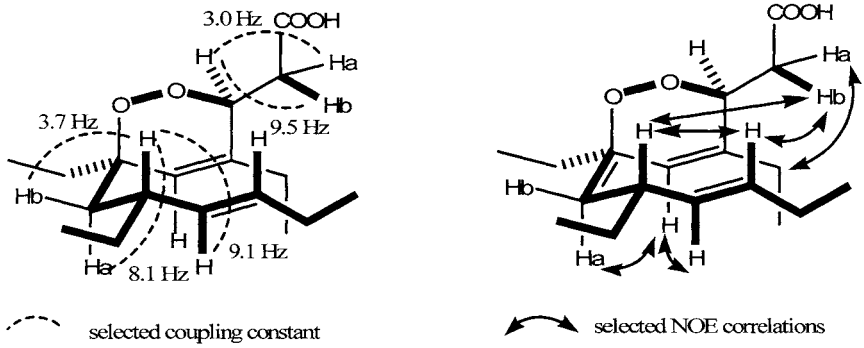


Fig. (15). Relative stereochemistry of haterumadioxin A (20).

The absolute stereochemistry of **20** was determined using the modified Mosher's method [7]. Reduction of **20** with $LiAlH_4$ followed by TBDPSCl (*tert*-butyldiphenylsilyl chloride)/imidazole gave TBDPS ether **20A**, Fig. (**16**). Treatment of **20A** with (*R*)- and (*S*)-MTPACl gave (*R*)- and (*S*)-MTPA esters, **20B** and **20C**, respectively. The results, as shown in Fig. (**16**), established that the configuration at C3 was 3*S*. Therefore, the absolute stereochemistry of haterumadioxin A (**20**) was determined to be 3*S*, 6*R* and 8*R*.

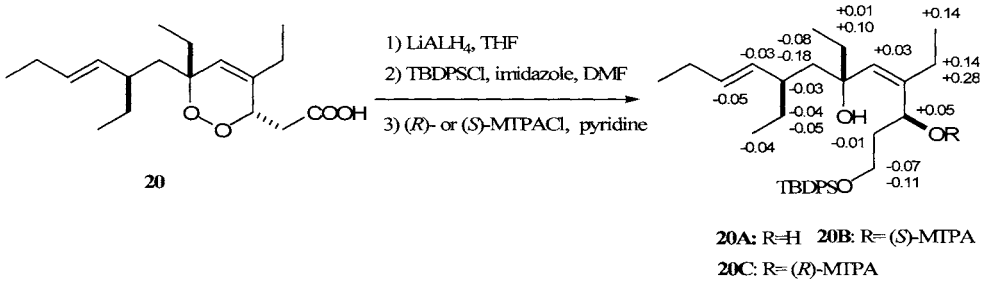


Fig. (16). Degradation reactions of haterumadioxin A (20) and $\Delta\delta_H$ values (δ_S-δ_R, ppm) for the MTPA esters.

Haterumadioxins A (**20**) and B (**21**) showed significant cytotoxicity against P388 mouse leukemia cells, with IC_{50}s of 11 and 5.5 ng/mL, respectively. Haterumadioxin A (**20**) was evaluated against a human

cancer cell line panel [63]. Fig. (**17**) shows the mean graph of **20** based on the growth inhibition, which seems to be more effective against a melanoma cancer cell. The COMPARE analysis of the mean graph revealed that **20** was correlated with doxifluridine, an antimetabolite (γ = 0.767).

Diterpene alkaloids

It has been amply demonstrated that ascidians are prolific producers of novel bioactive secondary metabolites [64-66]. A significant number of ascidian-derived compounds have entered into preclinical trials as antitumor agents [67]. Examples include didemnin B (went through phase II clinical trials but was withdrawn by NCI because it proved to be too toxic to use as a drug) [68], aplidine (currently under phase II clinical trials in Europe and Canada) [69], and ecteinacidine 743 (passed through phase II clinical trials for treatment of sarcoma, and is enlisted for phase III trials in Europe) [70]. The biomedical potential of the ascidian metabolites has resulted in these primitive chordates. As part of our ongoing chemical and biological studies on Okinawan marine organisms, we investigated an ascidian *Lissoclinum* sp. collected off the coast of Hateruma Island. A lipophilic extract of the ascidian showed that it could inhibit the division of fertilized sea urchin eggs.

The encrusting grey ascidian *Lissoclinum* sp. collected off the coast of Hateruma Island was extracted with acetone. The acetone extract was first partitioned between H_2O and EtOAc. The EtOAc extract was suspended in aqueous MeOH (50%) and the suspension was successively partitioned with hexanes, $CHCl_3$ and 1-BuOH. Only $CHCl_3$ extract inhibited the division of fertilized sea urchin eggs. Bioassay-directed fractionation of $CHCl_3$ extract by a series of chromatographic processes, including silica gel and ODS column chromatography, and silica gel and ODS HPLC, led to the isolation of haterumaimides A-K (**22**, 2.0 x 10^{-3}% of wet ascidian; **23**, 3.0 x 10^{-3}%; **24**, 2.0 x 10^{-3}%; **25**, 3.0 x 10^{-3}%; **26**, 2.0 x 10^{-4}%; **27**, 4.9 x 10^{-4}%; **28**, 1.1 x 10^{-3}%; **29**, 1.1 x 10^{-3}%; **30**, 2.4 x 10^{-3}%; **31**, 7.5 x 10^{-4}%; **32**, 3.2 x 10^{-4}%) and N-Q (**33**,1.43 x 10^{-3}%; **34**, 1.1 x 10^{-4}%; **35**, 1.0 x 10^{-4}%; **36**, 2.5 x 10^{-4}%) [71-74] together with dichlorolissoclimide (**37**, 1.0 x 10^{-2}%) [75] and chlorolissoclimide (**38**, 1.0 x 10^{-2}%) [76]. Haterumaimides L (**39**) and M (**40**), and 3β-hydroxychlorolissoclimide (**41**) were recently reported from the mollusks *Pleurobranchus albiguttatus* and *Pleurobranchus forskalii* by Schmitz and co-workers [77]. Labdane alkaloids of this type have attracted considerable interest because of their potential use as protein synthesis inhibitor [78], as antitumor drugs [79], and due to their unusual

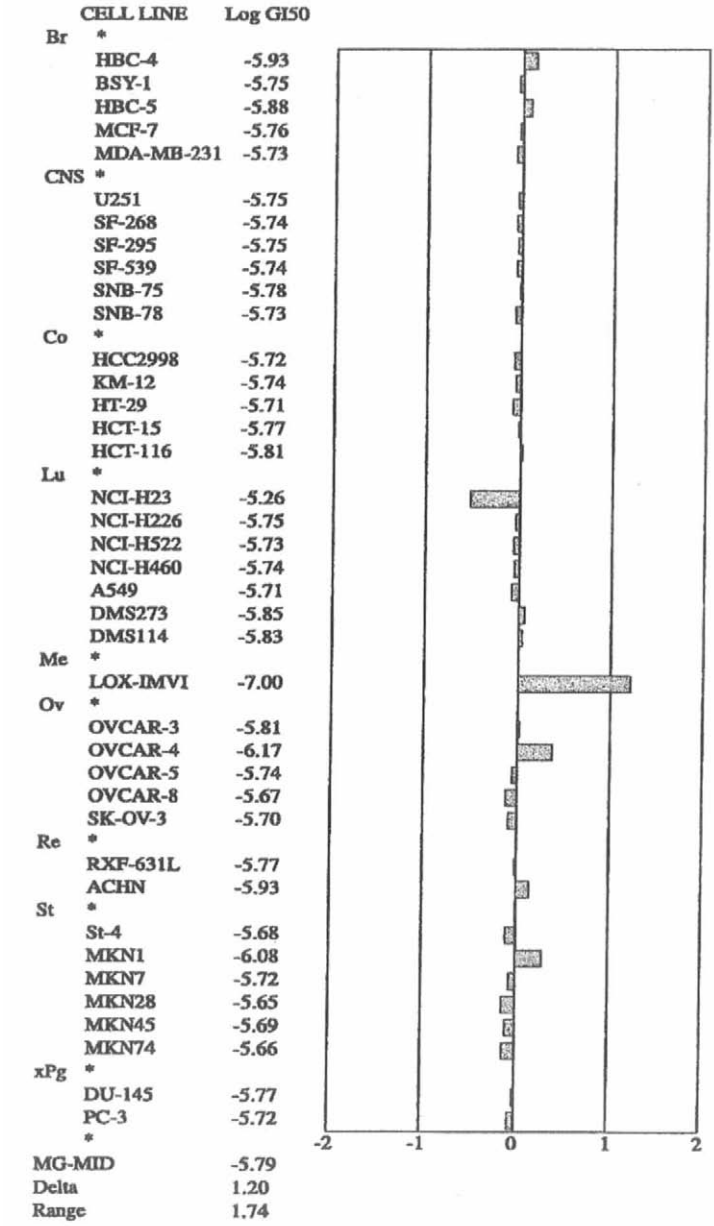
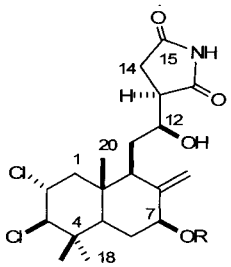


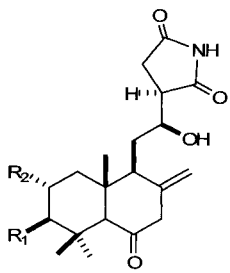
Fig. (17). Growth inhibition against a panel of 38 human cancer cell lines. The log GI_{50} for each cell lines is indicated; columns extending to the right, sensitivity to haterumadioxin A; columns extending to the left, resistance to haterumadioxin A. One scale represents one logarithm difference. MG-MID, the mean of log GI_{50} values for 38 cell lines. Delta, the logarithm of difference between the MG-MID and the log GI_{50} of the most sensitive cell line. Range, the logarithm of difference between the log GI_{50} of the most resistance cell line and the log GI_{50} of the most sensitive cell line.

structural features [80-82].

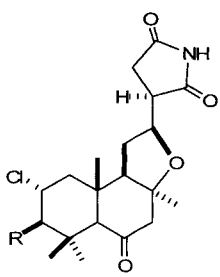
Haterumaimide A (**22**) had a molecular formula of $C_{22}H_{32}Cl_2NO_5$, as deduced from HRFABMS [*m/z* 460.1657 $(M + H)^+$, Δ-0.1 mmu]. The IR spectrum of **22** contained characteristic absorption bands at ν_{max} 3505 (OH), 3400 (NH), 1720 (C=O), 1705 (C=O), 1700 (C=O) and 1605 (C=C), and the UV spectrum showed one absorption maxima at λ_{max} (log ε) 215 nm (3.6). The detailed analysis of ^1H and ^{13}C NMR data of **22** indicated the presence of three methyl groups, an exomethylene, two oxymethines, a chloromethine, ester and imide (or amide) carbonyls.



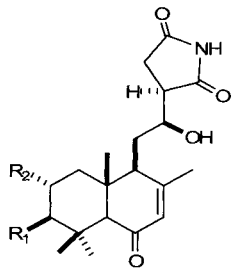
22 R=Ac
37 R=H



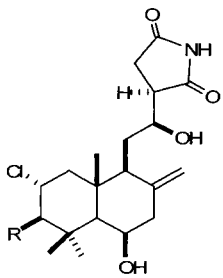
23 R1=Cl R2=Cl
28 R1=H R2=Cl
40 R1=Cl R2=H



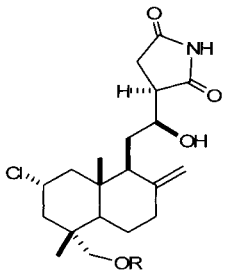
24 R=Cl
30 R=H



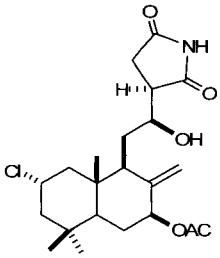
25 R1=Cl R2=Cl
29 R1=H R2=Cl
39 R1=Cl R2=H



26 R=Cl
27 R=H



31 R=H
32 R=Ac



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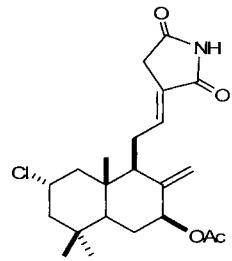

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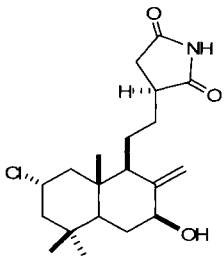

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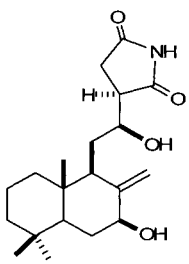

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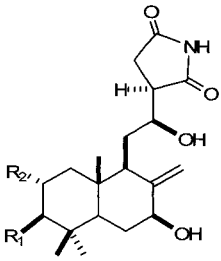
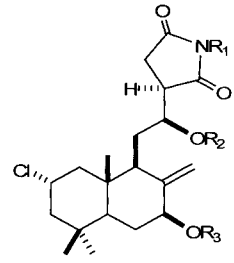


38 R1=H R2=Cl
41 R1=OH R2=Cl



42 R_1=Me R_2=H R_3=H
43 R_1=H R_2=Ac R_3=Ac
44 R_1=Me R_2=Ac R_3=Ac

Further detailed analysis of DOF-COSY and HOHAHA spectral data allowed us to elucidate three partial structures, C1-C3, C5-C7 and C9-C14, Fig. (**18**). The connectivity of these partial structures was established from the HMBC correlations to give the gross structure of **22**, Fig. (**18**). The gross structures of **23-36** were determined in the same manner as described above for **22**. The relative stereochemistry of **24** was determined to be 2*R**, 3*R**, 5*R**, 8*S**, 9*R**, 10*R** and 12*S** from the NOESY correlations and the magnitude of vicinal coupling constants, Fig. (**19**). It was more challenging to assign the relative configuration at C-13 because of the rotational freedom enjoyed by the succinimide moiety in contrast to the bulk of the molecule. However, a careful examination of the NOESY correlations of H11α/H13, H11β/H13, H11β/H14α, H11β/H14β and H12/H13, the weak NOESY correlation of the H12/H14β and the magnitude of $J_{12,13}$ = 4.3 Hz suggested the restricted rotation of the bond between C12 and C13. Because **24** adopted the plausible conformation shown in the Fig. (**19**), the relative stereochemistry of C13 was determined to be 13*R**. The relative stereostructures of **22, 23** and **25-36** were determined in the same manner as described above for **24**.

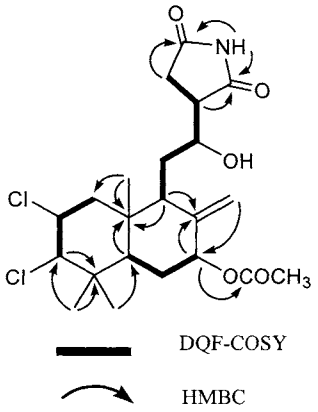


Fig. (18). Planar structures of 22.

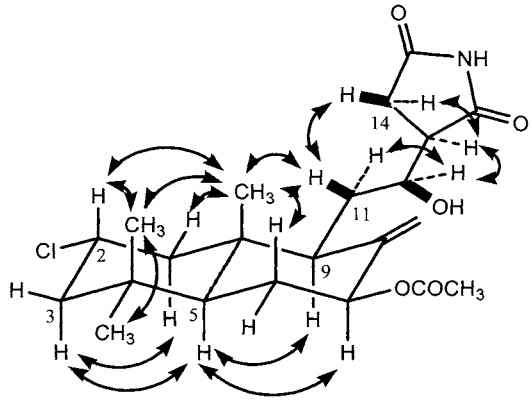


Fig. (19). Selected NOEs of 24.

The absolute stereochemistry of C12 in **25** was determined using the modified Mosher's method [7]. The results, as shown in Fig. (**20**), established that the configuration at C12 was 12*S*. Therefore, the absolute stereochemistry of haterumaimide D (**25**) was determined to be 2*R*, 3*R*, 5*R*, 8*S*, 9*R*, 10*R*, 12*S* and 13*R*. The absolute stereochemistry of C12 in haterumaimide H (**29**) was also established to be 12*S* by the modified Mosher's method [7].

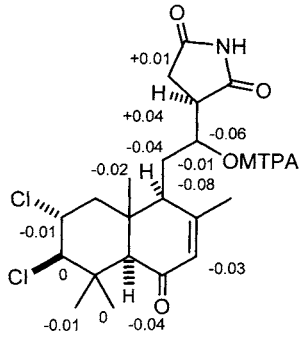
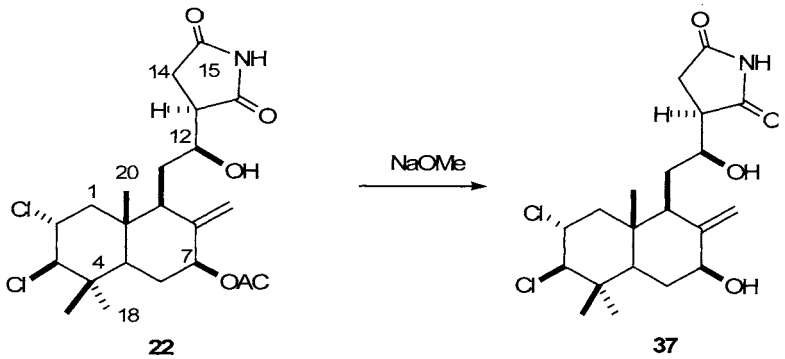
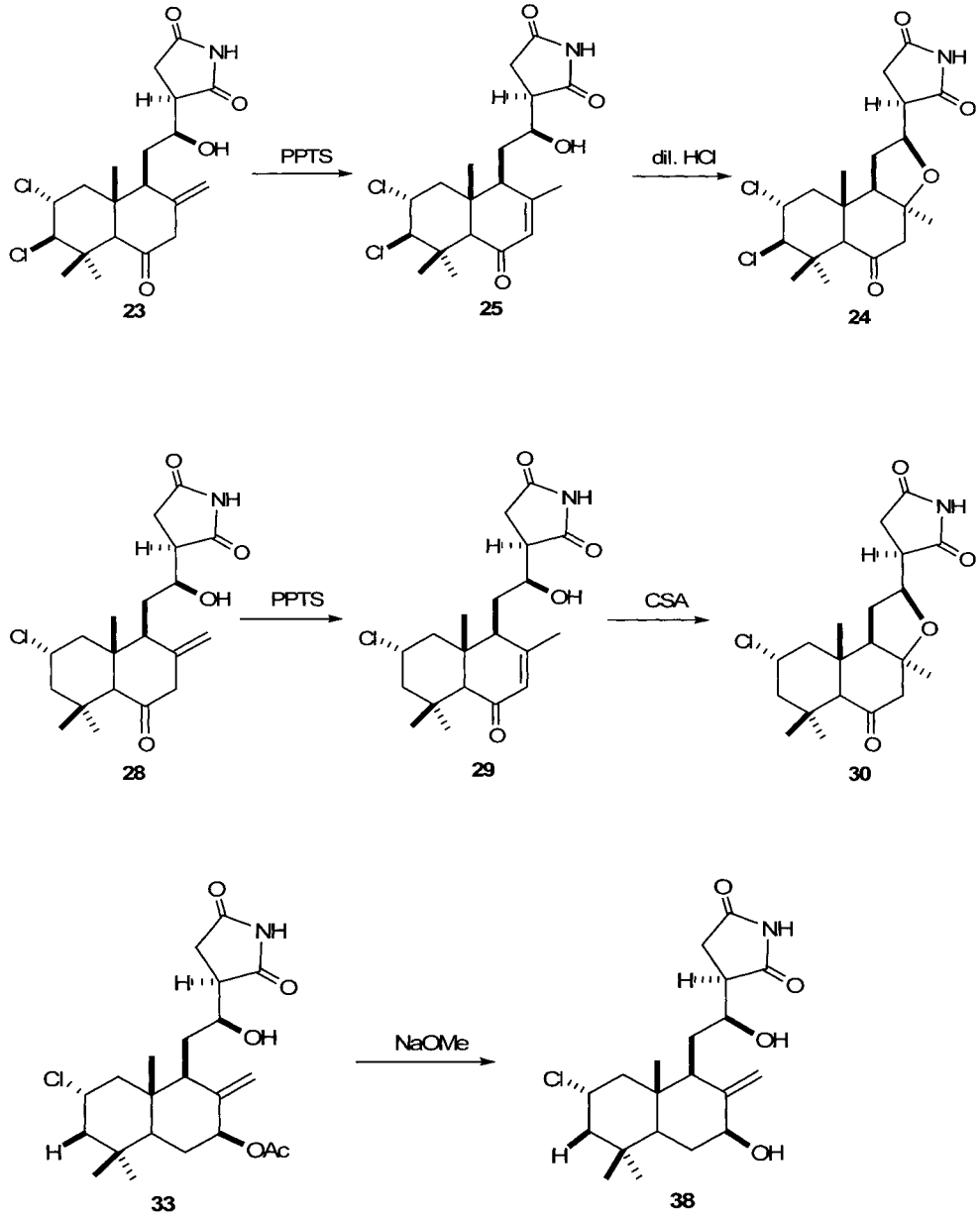


Fig. (20). $\Delta\delta_H$ values (δ_S-δ_R, ppm) for the MTPA esters of 25.

The absolute stereochemistries of haterumaimides A-C (**22-24**), F –I (**27-29**) and N (**33**) were determined by their chemical transformation into the absolute stereochemistry-known congeners or *vice versa* as follows, Schemes (**7-1** and **7-2**). Methanolysis of **22** gave the known dichlorolissoclimide (**37**). Acid treatment of **25** afforded **24**. Isomerization of the double bond of **23** by PPTS in methanol gave the conjugated ketone **25**. Acid-catalyzed rearrangement of **28** furnished **29** and the acid treatment of **29** afforded **30**. Base-catalyzed hydrolysis of **33** furnished the known chlorolissoclimide (**38**).



Scheme (7-1). Chemical transformation of haterumaimide A.



Scheme (7-2). Chemical transformation of haterumaimides.

So far we have isolated a total of 17 labdane alkaloids (**22-38**) with a unique succinimide moiety, including two known compounds [75, 76]. Six of these are dichlorinated (**22-26, 37**), 10 are monochlorinated (**27-35, 38**), and one is a dechloro analogue (**36**). Beyond the basic skeletal structure and the chlorine substituent(s), this class of alkaloids possesses a wide variety of functionalities. Due to their intrinsic structural variety and impressive biological activities, we were interested in establishing the structure-activity relationships (SAR) of natural haterumaimides A-K (**22-32**) and N-Q (**33-36**). We also prepared three synthetic derivatives of **38** (**42, 43** and **44**) to examine the effects of the hydroxyl group at C-12 and the imide NH in ring **C** on toxicity. N-methyl compound **42** was obtained by the reaction between **38** and CH_2N_2. Compounds **38** and **42** were treated with Ac_2O/pyridine to give **43** and **44**, respectively.

The cytotoxicity of compounds **22-38** and **42-44** was evaluated against mouse lymphocytic leukemia (P388) cells. The toxicity of these compounds against fertilized sea urchin eggs was also evaluated. The results are shown in Table (**2**).

Compound **31** was the most cytotoxic against P388 cells, with IC_{50} value of 0.23 ng/mL, followed by **32** at 0.45 ng/mL. Thus, acetylation of the hydroxyl group at C18 of **31** has no significant effect on toxicity. In a comparison of **31** and **32** with **38** and its acetyl congener **33**, the cytotoxicity increases three-fold when the hydroxyl group at C7 in **38** and the acetoxyl group at C7 in **33** are replaced with those at C18 in **31** and **32**, respectively. Compounds **26** and **27** showed pronounced toxicity with IC_{50} values of 4.1 ng/mL and 5.5 ng/mL. Oxidation of a C6 hydroxyl group in **26** or **27** to a keto group in **23** or **28** significantly reduced the toxicity, which suggested that the relative hydrophilicity of this part of the molecule contributes to the activity. The conversion of an exomethylene double bond in **23** or **28** to a trisubstituted double bond in **25** or **29** does not markedly change the cytotoxic activity. The effect of the hydroxyl group at C12 and the imide NH in ring **C** on the cytotoxicity is more pronounced, since compounds **34, 35, 24, 30, 42, 43** and **44** show remarkably low cytotoxicity compared with **38**. There is a 2-fold decrease in cytotoxicity with the acetyl group at C7 in **22** or **33**, compared to the free alcohol **37** or **38**. Comparison of the cytotoxicity of dichloro and monochloro analogues clearly showed that the chlorine atom at C3 does not significantly contribute to the cytotoxicity. The chlorine atom at C2 has a pronounced effect on the cytotoxicity, since the dechloro congener **36** shows about a 30-fold decrease in toxicity compared with the monochloro analogue **38**. A similar trend in the structure-activity relationship for **22-38** and **42-44** was observed in the assay with fertilized sea urchin eggs, Table (**2**).

Table 2. Bioactivities of haterumaimides A-K and N-Q, and compounds 37, 38 and 42-44.

Hateruma-imides	IC_{50} (mg/mL) against P388	Inhibition (%) of the division of fertilized sea urchin eggs	Hateruma-imides	IC_{50} (mg/mL) against P388	Inhibition (%) of the division of fertilized sea urchin eggs
A (**22**)	3.5×10^{-3}	100	K (**32**)	4.5×10^{-4}	100
B (**23**)	2.1	100	N (**33**)	3.4×10^{-3}	100
C (**24**)	>10	10	O (**34**)	1.3	80
D (**25**)	0.9	100	P (**35**)	1.2	100
E (**26**)	4.1×10^{-3}	100	Q (**36**)	5.0×10^{-2}	100
F (**27**)	5.5×10^{-3}	100	**37**	1.0×10^{-3} †	100
G (**28**)	>10	100	**38**	1.7×10^{-3} ‡	100
H (**29**)	2.7	100	**42**	1.4	10
I (**30**)	>10	50	**43**	2.4	0
J (**31**)	2.3×10^{-4}	100	**44**	0.14	50

† data taken from ref. 74

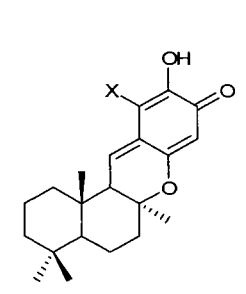
‡ data taken from ref. 76

Metabolites **22-38** with chlorine and a succinimide moiety are rare in nature. Based on the SAR results, it appears that several structural features of haterumaimides, such as the presence of hydroxyl groups at C6, C7, C12 and C18, a chlorine atom at C2 and an imido NH in ring **C**, are very important for the cytotoxicity. The hydrophilic OH and $(CO)_2NH$ groups in haterumaimides might increase cell membrane permeability to the molecules and/or enhance the stereo-electronic interaction between the molecules and a target molecule.

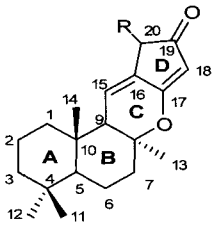
To the best of our knowledge there is no report on the total synthesis of lissoclimides and haterumaimides [80].

Sesquiterpene quinones

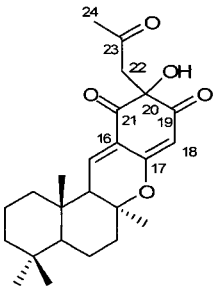
Since the EtOAc extract of the yellowish sponge *Dysidea* sp. collected in Hateruma Island showed potent activity against fertilized sea urchin eggs, we examined constituents of the sponge and isolated puupehenone and its congeners [83]. Puupehenone (**45**) and its derivatives are an important group of marine metabolites because they display a wide range of bioactivities such as cytotoxic, antiviral, antifungal, antitumor and antimalarial activities [84-90]. Moreover, some of them inhibit reproduction of HIV, activity of cholesteryl ester transfer protein (CETP) and activity of topoisomerase II, which have heightened the interest in this class of compounds [91-93]. Puupehenone (**45**), which inhibits DNA and protein synthesis, and the growth of *M. tuberculosis* (H37Rv), is commercially available.



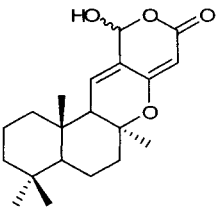
45 X=H 51 X=Cl



46 R=H 49 R=OH



47, 48



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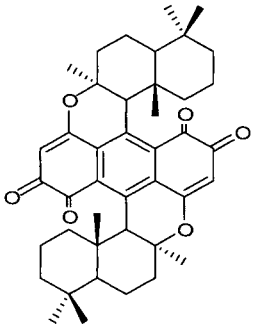

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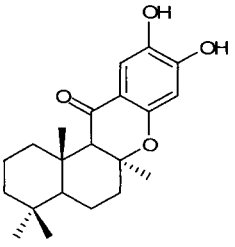

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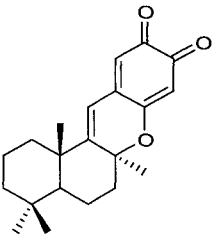

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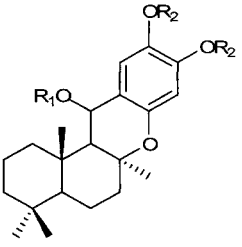


54 $R_1 = CH_3$ $R_2 = H$
55 R_1 = Ac R_2 = Ac

The yellowish sponge was extracted with acetone. The acetone extract was initially partitioned between EtOAc and water. Fractionation of the cytotoxic EtOAc-soluble material by a series of chromatographic processes, including silica gel and ODS column chromatography, and silica gel and ODS HPLC, led to the isolation of haterumadienone (**46**, 0.0017% of wet weight), acetone adducts **47** and **48** as a mixture [**47**/**48** (10:9), 0.0005%] [83] and compounds **49** and **50**, together with the known puupehenone (**45**, 0.05%), chrolopuupehenone (**51**). dipuupehenone (**52**) and 15-oxopuupehenol (**53**) and puupehedione (**54**) [84-89]. This mixture of **47** and **48** was then purified by repeated ODS HPLC with 30% H_2O/MeOH to give pure **47** and **48**.

Analysis of **46** by ^{13}C NMR and HRESIMS [*m/z* $(M + Na)^+$ 323.2017, Δ+2.9 mmu]. provided a molecular formula, $C_{20}H_{28}O_2$. Although the ^1H NMR signals in the aliphatic region were almost identical to those of puupehenone (**45**), **46** showed one less carbon than **45**. The partial structures (**a**, **b** and **c**) and other fragments (C11-C4-C12, C10-C14, C13-C8 and C16 -C17) were connected using HMBC correlations to give the planar structure of the **A**-, **B**- and **C**-ring moiety, Fig. (**21**). The presence of a cyclopentenone ring with an isolated methylene (C20, *J* =

20 Hz) [94] was also confirmed by an HMBC experiment. The relative stereochemistry of **46** was established based on NOEDS data. The NOEs observed between Me14/H2b, Me14/Me11, M14/H15, H1a/ H5 and Me13/H9 revealed a *trans-anti-cis* fusion of **A**/**B**/**C** rings, which was the same as in puupehenone (**45**), Fig. (**21**).

The molecular formula of **47**, $C_{24}H_{32}O_5$, was established by HRESIMS [*m/z* (M + Na)$^+$ 423.2154, Δ+0.5 mmu] and the ^{13}C NMR spectrum. Interpretation of the 1D and 2D NMR spectra indicated that **47** had the same **A**-, **B**- and **C**-ring moiety as **45** or **46**. In the remaining part of **47**, the presence of an acetonyl group, two ketones, an oxygenated quarternary carbon (C20) and a hydroxyl group were revealed based on ^1H, ^{13}C and HMBC NMR data. The HMBC correlations between these partial structures established the structure of ring **D**, Fig. (**22**). Stereochemistry for the **A**, **B** and **C**-ring moiety in **47** identical with that of **45** or **46** based on the NOE data.

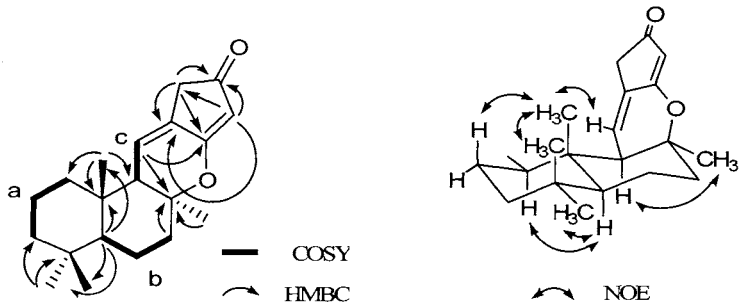


Fig. (21). Planar structure and stereostructure of haterumadienone (46) based on 2D NMR data.

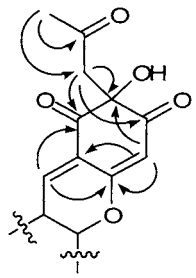


Fig. (22). Partial structure and HMBC correlations of acetone adduct 47.

The NMR spectra of compound **48** are almost identical to those of **47**. The structure of **48** was spectroscopically determined by 2D NMR

experiments and by comparison of its NMR data with those of **47**. The structure of compounds **49** and **50** were determined in the same manner as described above for **46**.

The absolute stereochemistry of C20 in **49** was determined using the modified Mosher's method [7]. The alcohol **49** was transformed into the (*R*)- and (*S*)-MTPA esters. The most stable conformation of each MTPA moiety and the relative stereochemistry of C20 were confirmed by the results of NOE experiments, Fig. (**23**). The calculated $\Delta\delta_H$ values (δ_S-δ_R, ppm), as shown in Fig. (**24**), established that the configuration at C20 was 20*R*. Therefore, the absolute stereochemistry of **49** was determined to be 2*R*, 3*R*, 5*R*, 8*S*, 9*R*, 10*R*, 12*S* and 13*R*.

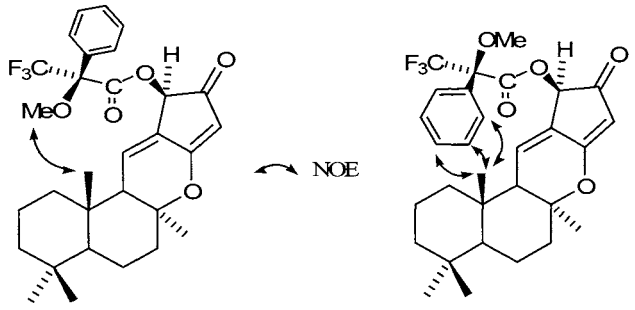


Fig. (23). NOE correlations in *S*-MTPA and *R*-MTPA esters of compound 49.

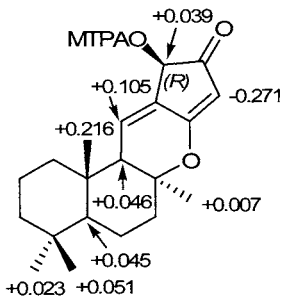


Fig. (24). $\Delta\delta_H$ values (δ_S-δ_R, ppm) for the MTPA esters of 49.

An interesting ring-contraction biogenetic pathway from puupuhenone (**45**) to haterumadienone (**46**) might proceed *via* benzylic acid rearrangement of the diketone form of **45**, followed by oxidative decarbonylation. Haterumadienone (**46**) is the first ring-contracted

congener which possesses ring **C** of **45** [87]. Compounds **47** and **48,** which might be formed by aldol condensation between the trione hydrate **55** and acetone in the extraction process, were epimers at C20 of each other, Scheme (**8**). These acetone adducts are the most reasonable adducts because they have less electrostatic interactions between the ketonic groups than other adduct structures, 1, 2-diketones, Scheme (**8**). The trione hydrate **55** could be detected in the sponge extract with ethyl acetate instead of acetone [83]..

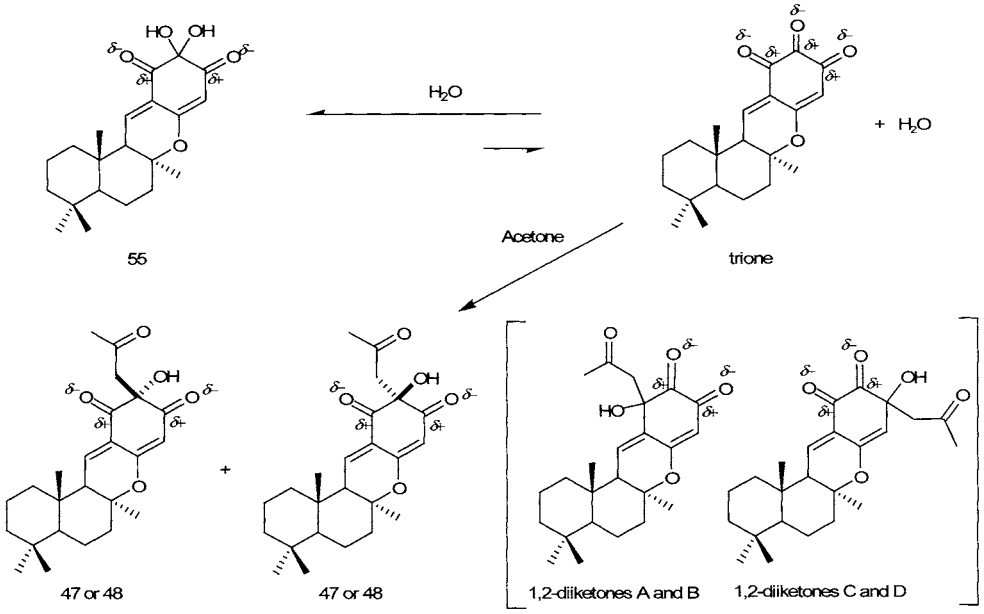
The toxicity of the isolated compounds and two synthetic derivatives **56** and **57**, which were prepared by the reactions of **45** and CH_2N_2, and of **45** and Ac_2O, respectively, against fertilized sea urchin eggs was evaluated. The results are shown in Table (**3**). Acetone adducts **47** and **48** were the most toxic against fertilized sea urchin eggs followed by puupehedione (**54**), chloropuupehenone (**51**) and puupehenone (**45**). These compounds bear a quinone-type structure in the **D**-ring moiety as a common structural unit. Compounds which have an aromatic or a five-membered ring in the **D**-ring moiety showed weak toxicity against fertilized sea urchin eggs.

Table (3). Inhibition of the first cleavage of fertilized sea urchin eggs.

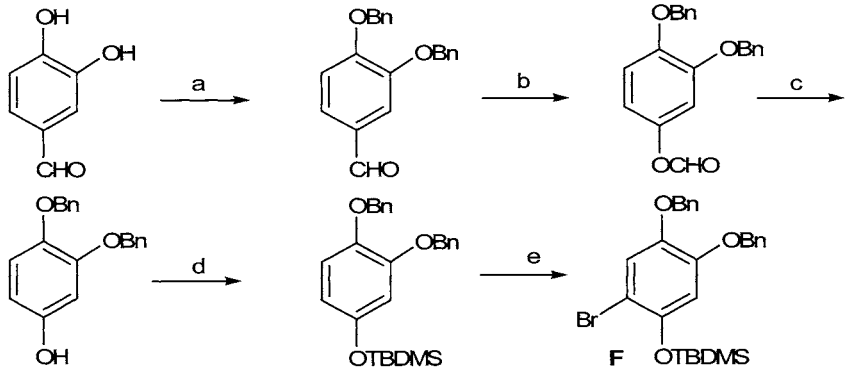
Compound	% (5 ppm)	% (3 ppm)	% (1 ppm)
45	100	100	32
46	40	14	9
47	100	100	100
48	100	100	100
49	9	4	2
50	47	18	13
51	100	100	47
52	21	7	4
53	100	100	49
54	11	7	5
55	100	34	16

The first synthesis of (±) puupehenone was reported in 1978 by Trammel [95]. Enantiospecific synthesis of (+)-puupehenone (**45**) has been achieved by Barrero and co-workers, Schemes (**9** and **10**) [96, 97]. Starting from protocatechualdehyde and the drimanic acetoxyaldehyde, whose efficient preparation from (–)-sclareol has been reported, **45** has been synthesized in 13 steps. The key steps of the synthesis are the organoselenium-induced cyclization of the mixture of two regioisomers (*endo*- and *exo*-isomers) to give two selenyl derivatives with complete diastereoselectivity, and the simultaneous removal of benzyl and phenyl selenyl groups of the selenyl derivatives by treating with Raney Ni [steps

k and l in Scheme (**10**)]. Syntheses of puupehenone congers have been reported by Maiti and co-workers [98].

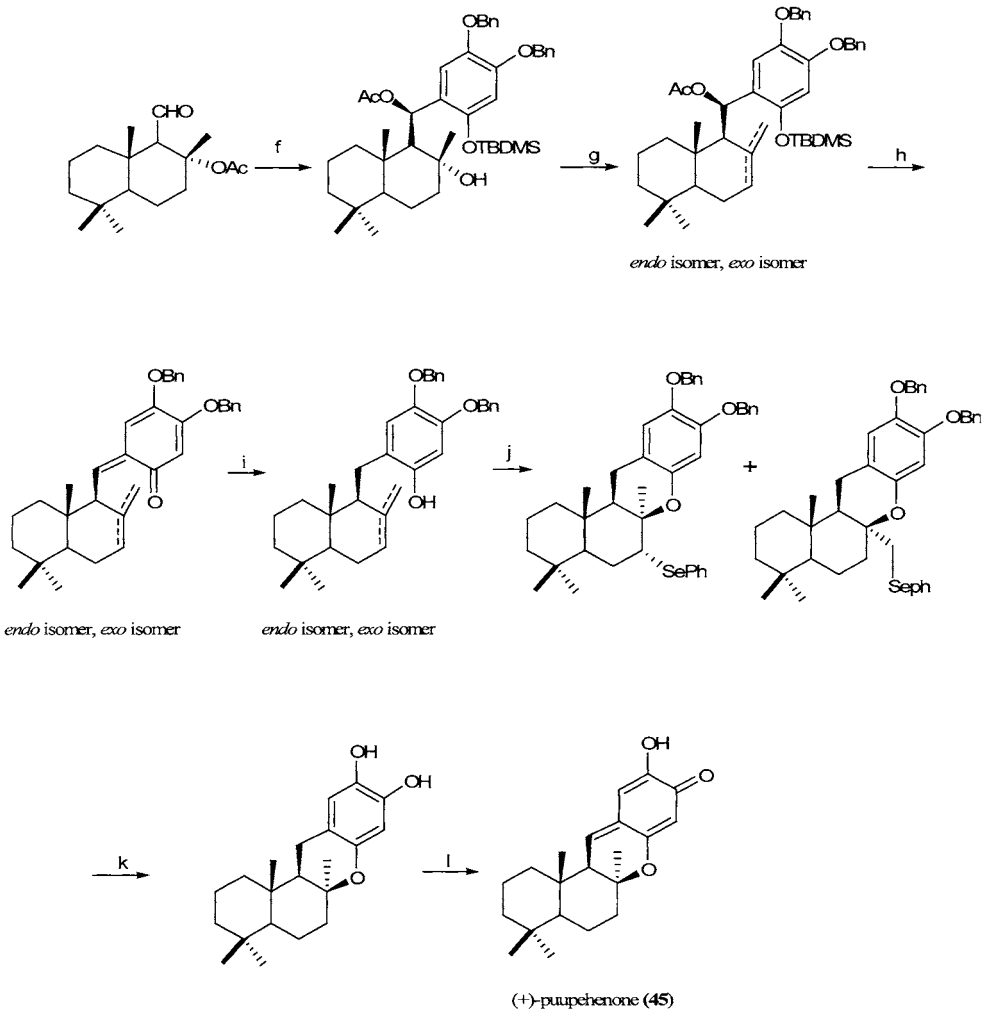


Scheme (8). Formation of acetone adducts.



Reagents and conditions: (a) BnBr, K_2CO_3, acetone, reflux, 15h, (92%). (b) MCPBA, CH_2Cl_2, rt, 12 h, (95%). (c) 6N NaOH-MeOH, rt, 5 min, (96%). (d) TBDMSCl, imidazole, DMF, rt, 15 h, (92%). (e) NBS, CCl_4, Silicagel (97%).

Scheme (9). Synthesis of the aromatic synthon F.



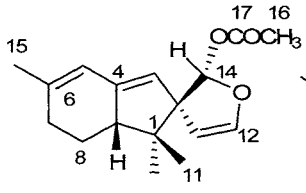
Reagents and conditions: (f) compound **F** in Scheme (**8**), *t*-BuLi, Et_2O, -78°C (88%). (g) $SOCl_2$, pyridine, rt, 1 h (94%). (h) TBAF, THF, rt, 15 min (81%). (i) $NaBH_4$, EtOH, rt, 20 min (91%). (j) NPSP, $SnCl_4$, CH_2Cl_2, -78°C, 2h (91%). (k) Raney Ni, THF, rt, 20 h (75%). (l) PDC, CH_2Cl_2, rt, 3h (70%).

Scheme (10). Synthesis of (+)-puupehenone.

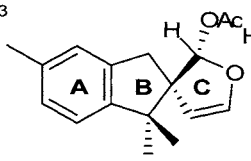
Sesquiterpenes

Marine sponges of the genus *Dysidea* are a rich source of structurally unique and biologically active compounds, including spiro-sesquiterpenes such as spirodysin and dehydroherbadysinolide, furanosesquiterpenes based on the furodysinin and furodysin skeletons, brominated diphenyl ethers, polychlorinated alkaloids and other compounds [99-103].

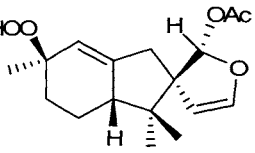
As part of our continuing chemical studies of marine organisms from Hateruma Island, we examined the constituents of the sponge *Dysidea chlorea* de Laubenfels whose crude extract strongly inhibited cell division of fertilized sea urchin eggs. Bioassay-guided fractionation of the extract coupled with ^{1}H NMR measurement led to the isolation of four new tricyclic spiro-sesquiterpenes, haterumadysins A, B, C and D (**58**–**61**), together with two known compounds, spirodysin (**62**) and dehydroherbadysinolide (**63**). Compounds **62** and **63** were first isolated from the Australian sponges *Dysidea herbacea* and *Dysidea* sp., respectively, and have a rare spirolactol or spirolacton framework [99, 102].



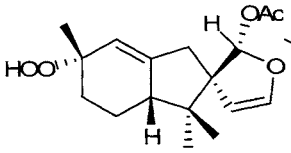
58



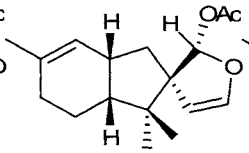
59



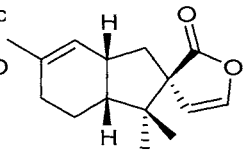
60



61



62



63

The light blue sponge was collected by hand from the coast of Hateruma Island. The acetone extract was initially partitioned between EtOAc and water. The cytotoxic EtOAc extract was fractionated by silica-gel column chromatography and ODS HPLC to furnish haterumadysin A (**58**, 0.0010% of wet weight), haterumadysin B (**59**, 0.00047%), haterumadysin C (**60**, 0.00022%), haterumadysin D (**61**, 0.00042%), (–)-spirodysin (**62**, 0.0030%), and (–)-dehydroherbadysinolide (**63**, 0.00042%).

Analysis of ^{13}C NMR and HRESIMS data [*m/z* 297.1475 $(M + Na)^+$, Δ +0.8 mmu] for **58** provided a molecular formula of $C_{17}H_{22}O_3$, which accounted for seven degrees of unsaturation. The MS and NMR data suggested that **58** was the dehydro analogue of spirodysin (**62**). The planar structure of **58** was determined by the interpretation of the 2D NMR data (COSY, HMQC and HMBC), Fig. (**25**). The NOEs observed between H9/H$_3$10, H9/H14, and H$_3$10/H14 implied that these protons were on the same face of the molecule. Haterumadysin B (**59**) had a molecular formula of $C_{17}H_{20}O_3$, as determined by HR ESIMS [*m/z* 295.1298 $(M + Na)^+$, Δ −1.2mmu], which accounted for an additional degree of unsaturation compared to **58**. The planar structure of **59** was established by analysis of the COSY, HMQC and HMBC data. The relative stereochemistry of **59** was established based on NOE data. The NOE observed between H$_3$10 and H14 confirmed that these protons were on the same face of the molecule.

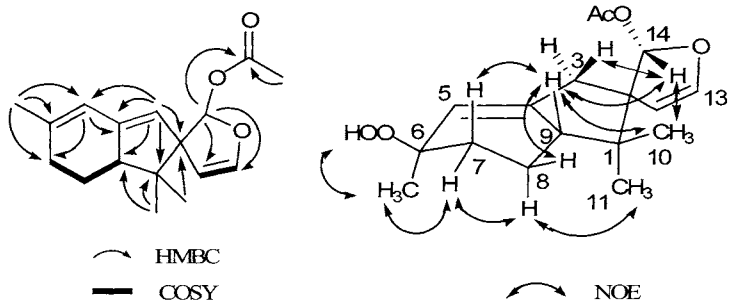
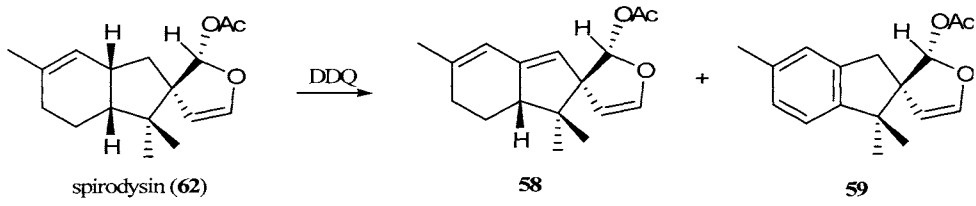


Fig. (25). Planar structure of haterumadysin A (58) and stereostructure of haterumadysin C (60) based on 2D NMR data.

Treatment of **62** with 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone (DDQ) at 80 °C for 2 h afforded **58** (20%) and **59** (25%), Scheme (**10**) [104]. Thus, haterumadysin A (**58**) and haterumadysin B (**59**) were



Scheme (10). Transformation of spirodysin (62) into 58 and 59.

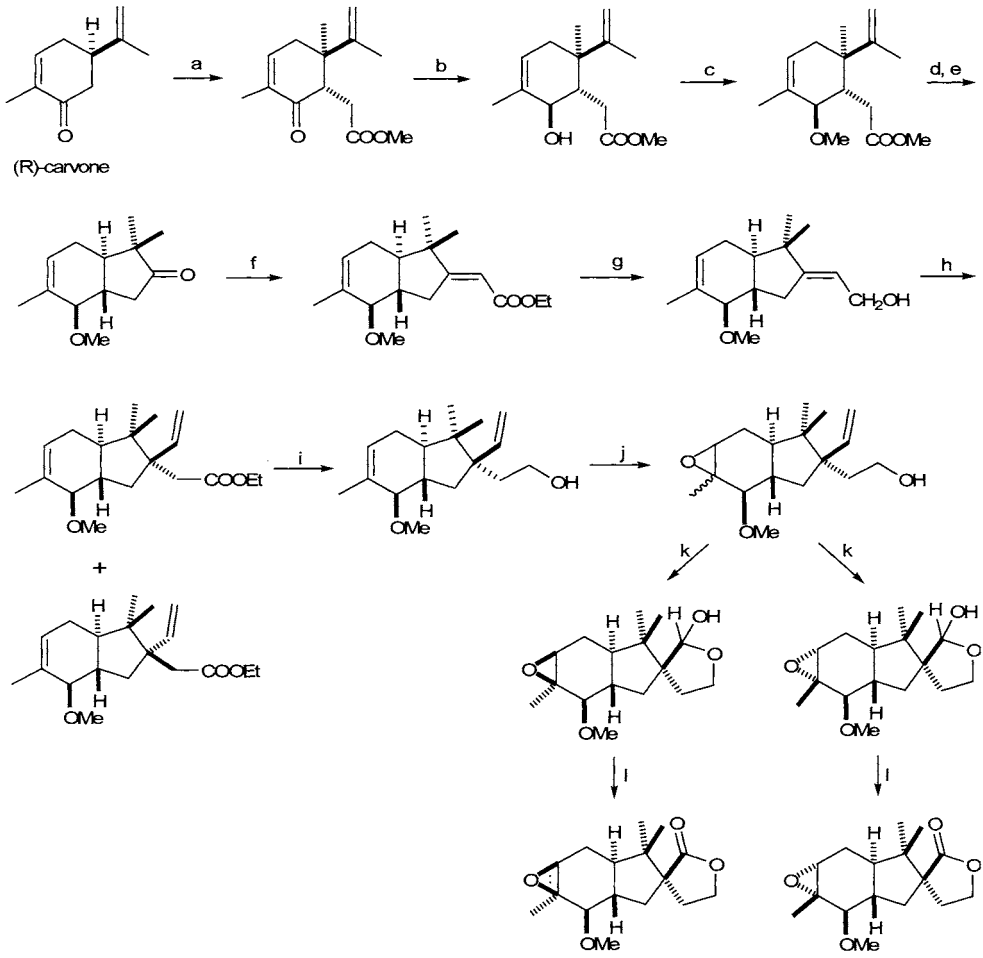
confirmed to be dehydro analogues of spirodysin (**62**).

Analysis of haterumadysin C (**60**) by HRESIMS [*m/z* 331.1505 $(M + Na)^+$, $\Delta -1.6$ mmu] provided a molecular formula of $C_{17}H_{24}O_5$. The NMR data and the molecular formula revealed the presence of a trisubstituted double bond at C4 and a hydroperoxy group in compound **60**. A positive iodine-starch test further supported the presence of the hydroperoxy group in **60**. The relative stereochemistry of **60** was determined by NOEDS experiments, Fig. (**25**). Haterumadysin D (**61**) had the same molecular formula as that of **3**, $C_{17}H_{24}O_5$, as deduced from HRESIMS [*m/z* 331.1510 $(M + Na)^+$, $\Delta -1.1$ mmu]. The ^1H and ^{13}C NMR spectra were almost identical to those of haterumadysin C (**60**). Extensive analysis of 1D and 2D NMR data and comparison of the NMR data with those of **60** led to the same planar structure as that of **60**. Since the NOEs observed for the portions in the **B**- and **C**-rings in **61** resembled those described above for **60**, both compounds had to possess the identical stereochemistry at C9, C12 and C14. The methyl group at C6 in **61** was assigned to be β based on NOEs observed between $H_315/H7\beta$ and $H_315/H9$, therefore compounds **61** was determined to be the C6 epimer of **60**.

Haterumadysins A, B, C and D (**58–61**) completely inhibited the first cleavage of fertilized sea urchin eggs at 1 ppm and spirodysin (**62**) showed 90% inhibition of the first cleavage of fertilized sea urchin eggs at 1 ppm. However dehydroherbadysinolide (**63**) showed no activity against fertilized sea urchin eggs even at 3 ppm. Accordingly the lactol group in the **C**-ring seems to be very important for this activity.

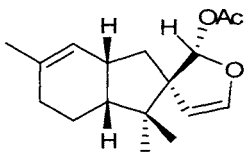
Compounds **58–61** belong to a small group of sesquiterpenes which contain a furan spirofused to bicyclo[4.3.0]nonane and have a rare spirolactol moiety. We, however, could not conclude that compounds **60** and **61** are natural products, because it is possible that compounds **60** and **61** derive from **62** by oxidation with molecular oxygen in the isolation process.

The spirodysins are a small group of sesquiterpenes isolated from *Dysidea herbacia* and are probably biogenetic precursors of furodysins and furodysinins. Although several congeners of spirodysin containing a furan spirofused to bicyclo[4.3.0]nonane were isolated so far since the first isolation of spirodysin (**62**) in 1978, Fig. (**26**) [99-102, 105,106], there is a report on the synthesis of spirodysins. Srikrishna and co-workers recently succeeded in enntiospecific construction of the tricyclic framework of spirodysins, Scheme (**12**) [107]. The key step is the construction of the bicyclo[4.3.0]nonane-8-one employing a lithium and liquid ammonia mediated carbanion cyclization of the δ-methyl-δ, ε-unsaturated ester [steps d and e in Scheme (**12**)].

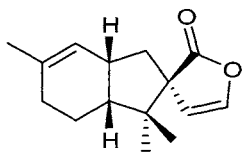


Reagents and conditions: (a) LDA, THF, -70°C; $BrCH_2COOMe$, 70°C, rt, 76%. (b) $NaBH_4$, MeOH, -30°C, 81%. (c) NaH, THF, Bu_4NI, MeI, reflux, 8h, 70%. (d) Li, liquid NH3, THF, -30°C, 1h, 88%. (e) PCC, NaOAc, CH_2Cl_2, rt, 2h, 88%. (f) NaH, $(EtO)_2P(O)CH_2COOEt$, sealed tube, 120°C, 85%. (g) LAH, Et_2O, -40°C, 86%. (h) $MeC(OEt)_3$, EtCOOH, sealed tube, 180°C, 72h. (i) LAH, Et_2O, 0°C, 86%. (j) *m*-CPBA, $NaHCO_3$, CH_2Cl_2, rt, 2h, 81% (1:1). (k) O_3/O_2, CH_2Cl_2/MeOH (5:1), -70°C; Me_2S, rt, 8h, 90-93%. (l) PCC, NaOAc, CH_2Cl_2, rt, 2h, 82-84%.

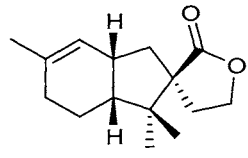
Scheme (12). Synthesis of furans spirofused to bicyclo[4.3.0]nonane.



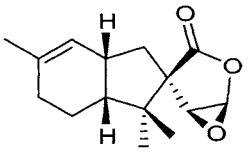
spirodysin (**62**)



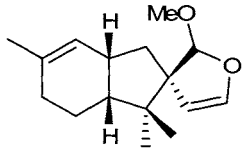
dehydroherbadysinolide (**63**)



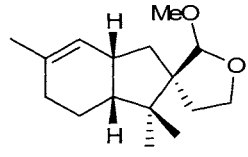
herbadysinolide (**64**)



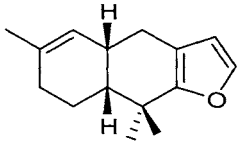
dysehterin (**65**)



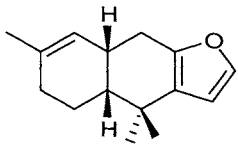
14-deacetoxy14-methoxy-spirodysin (**66**)



12, 13-dihydro-14-deacetoxy-14-methoxyspirodysin (**67**)



furofysin (**68**)



furodysinin (**69**)

Fig. (26). Spirodysins, furodysin and furodysinin isolated from *Desidea herbacia*.

ABREVIATIONS

DQF-COSY	=	Double-quantum filtered correlation spectroscopy
FDMS	=	Field desorption mass spectrometry
HMBC	=	Heteronuclear multiple-quantum correlation
HMQC	=	Heteronuclear multiple-quantum correlation
HOHAHA	=	Homonuclear Hartmann-Hash spectroscopy
HPLC	=	High performance liquid chromatography
HRESIMS	=	High resolution electrospray ionization
HRFABMS	=	High resolution first atom bombardment massspectrometry
NOEDS	=	Nuclear Overhauser effect difference spectrum
NOESY	=	Nuclear Overhauser effect spectroscopy
AIBN	=	Azobisisobutyronitrile
BnBr	=	Benzyl bromide
CSA	=	10-Camphorsulfonic acid
DDQ	=	2, 3-Dichloro-5, 6-dicyano-1, 4-benzoquinone

DHP	=	3, 4-Dihydro[2*H*]pyran
DIBAL	=	Diisobutylaluminium hydride
DMSO	=	Dimethyl sulfoxide
FAMSO	=	Formaldehyde dimethyldithioacetal S-oxide
HMPA	=	Hexamethylphosphoric triamide
KHMDS	=	Potassium hexamethyldisilazide
LAH	=	Lithium aluminium hydride
LDA	=	Lithium diisopropylamide
MCPB, *m*-CPBA	=	*m*-Chloroperbenzoic acid
MMTrCl	=	4-(Methoxyphenyl)diphenylmethyl chloride
MPM	=	Methoxy pheny methyl
NBS	=	*N*-Bromosuccinimide
NCS	=	*N*-Chlorosuccinimide
NPSP	=	*N*-Phenylselenophthalimide
ODS	=	Octadecylsilyl
PCC	=	Pyridinium chlorochromate
PDC	=	Pyridinium dichromate
PPTS	=	Pyridinium *p*-toluenesulfonate
TBAF	=	Tetra-*n*-butylammonium fluolide
TBDPSCl	=	*tert*-Butyldiphenyllsilyl chloride
TBSCl, TBDMSCl	=	*tert*-Butyldimethylsilyl chloride
THF	=	Tetrahydrofuran
TMSCl	=	Tetramethylsilyl chloride
p-TsCl	=	*p*-Toluenesulfonyl chloride
Br	=	Breast (cancer cells)
CNS	=	Central nervous system (brain cancer cells)
Co	=	Colon (cancer cells)
CETP	=	Cholesteryl ester transfer protein
HIV	=	Human immunodeficiency virus
IC_{50}	=	Inhibitory concentration fifty
LD_{50}	=	Lethal dose fifty
Lu	=	Lung (cancer cells)
Me	=	Melanoma (cancer cells)
Ov	=	Ovarian (cancer cells)
Re	=	Renal (cancer cells)
SAR	=	Structure-activity relationship
St	=	Stomach (cancer cells)
xPg	=	Plostate (cancer cells)

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NATURAL MARINE ANTIVIRAL PRODUCTS

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ABSTRACT: The oceans are unique resources that provide a diverse array of natural products, primarily from invertebrates such as sponges, tunicates, bryozoans and molluscs and from marine bacteria and cyanobacteria. Pharmacologic research with marine chemicals continue to contribute potentially novel chemical leads in the ongoing global search for therapeutic agents in the treatment of multiple diverse categories. As infectious diseases evolve and develop resistance to existing pharmaceuticals, the marine environment provides novel leads against fungal, parasitic, bacterial and viral diseases. Limitations in our current antiviral treatment options and the continuing emergence of new pathogenic viruses have contributed to a growing need for new and effective chemotherapeutic agents to treat viral diseases. The search for potent antiviral agents is urgent in view of the dramatic situation of the global human immunodeficiency virus (HIV) epidemic, a possible spread of avian influenza and of other viral diseases. Effective antiviral therapeutics are not available, and the presently approved therapy for HIV has been recognized to be toxic, unable to eradicate the causative virus, and to induce severe drug resistance. The marine environment provides a rich source of chemical diversity for the screening and identification of new compounds with desirable antiviral properties. Antiviral testing has revealed numerous compounds from structural classes including polysaccharides, terpenoids, steroids, alkaloids and peptides that potentially inhibit both RNA and DNA viruses. This review presents an account of some research directed toward the discovery of new antiviral agents from marine sources.

INTRODUCTION

The marine environment is a prolific resource for the isolation of less exploited organisms and microorganisms. There are in fact untapped habitats in the sea with unique characteristics. In addition, the potential contribution of marine sources to the discovery of new bioactive molecules has recently been recognized. These activities probably

represent a mixture of novel metabolites and products previously undiscovered from terrestrial isolates.

Since current antiviral therapies are limited by their toxicity and the tendency of transfected viruses to assume drug-resistant forms, there is a need to find new substances with antiviral activity. All possible approaches towards the development of new antiviral drugs should therefore be pursued. One potential source of these inhibitors is the marine environment [1-5]. Natural marine products have been found to be an important source of drugs and drug leads. These natural products are secondary metabolites which enhance survival fitness and may serve as chemical weapons against bacteria, fungi, viruses and small or large animals [6-8]. Most of the natural products of interest to the pharmaceutical industry are secondary metabolites, and several such compounds derived from marine organisms and microorganisms have been used in clinical trials as experimental antiviral drugs.

The inhibitory effects of marine extracts on the replication of the herpes simplex virus (HSV) and other viruses were reported almost four decades ago. However, these observations did not generate much interest, because the antiviral action of the extracts was considered to be largely non-specific. Shortly after the identification of the human immunodeficiency virus (HIV) as the causative agent of acquired immunodeficiency syndrome (AIDS) in 1984, several natural marine products and extracts were found to be potent and selective inhibitors of HIV replication in cell culture [9,10]. Their activity spectrum has been shown to extend to various enveloped and non-enveloped viruses, including those that emerge as opportunistic pathogens, e.g., HSV and cytomegalovirus (CMV) in immunosuppressed (e.g., AIDS) patients. However, the marine environment provides a never-ending set of fascinating problems for the chemists. Many of the most intriguing problems concern compounds available in only minute quantities. One solution is to focus on bioassay-guided separations.

This work reviews the recent literature (1990-early 2005) on antiviral secondary metabolites from marine organisms and microorganisms and reports a selection of results from more than one hundred bioactive compounds. Under the heading of each respective virus, this article reviews the research on the antiviral activity of extracts and compounds present in the marine environment. This review demonstrated how far the search for new bioactive metabolites from

marine organisms and microorganisms is an active sector of the chemistry of natural products.

HUMAN IMMUNODEFICIENCY VIRUS

HIV is the retrovirus that causes AIDS. The development of anti-HIV microbicides for either topical or *ex vivo* use is of considerable interest, mainly due to the difficulties in creating a vaccine that would be active against multiple clades of HIV. In this chapter we discuss the anti-HIV activity of extracts and compounds isolated from marine sources. Most reports recorded in the literature on anti-HIV activity in the marine environment include marine macro and microalgae and sponges.

The results suggest that the extracts from algae are a promising source of antiviral agents which may act on different stages of the virus replication cycle [11,12]. For example, Nakamura *et al.* [13] investigated boiling water extracts of 25 species of marine algae for their inhibitory activities on the growth of HIV in the MT-4 cell line by the preliminary microplate screening assay *in vitro*. Of the 75 samples assayed, 38 algae were found to be active. Additionally, 47 species of marine macroalgae from the coast of Korea have been screened for the presence of inhibitory compounds against human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) and HIV-integrase [14,15]. One Chlorophyta, 8 out 17 Phaeophyta and 6 out 26 Rhodophyta showed inhibitory activity against HIV-1 RT. Five species (*Ecklonia cava*, *Ishige okamurae*, *Sargassum confusum*, *Sargassum hemiphyllum* and *Sargassum ringgoldianum*) belonging to Phaeophyta were shown to inhibit the 3'-processing activity of HIV-1 integrase.

The blue-green filamentous algae *Spirulina platensis* also inhibited HIV-1 replication in human T-cell lines, peripheral mononuclear blood cells and Langerhans cells [16]. The extract inactivated HIV-1 infectivity directly when preincubated with the virus before addition to human T-cell lines. These data indicate that aqueous *Spirulina platensis* extract contains antiretroviral activity that may be of potential clinical interest. Antiviral activities were also reported from the water-soluble fraction of the marine diatom *Haslea ostrearia*, which delayed HIV-1-induced sincytia formation on MT-4 cells [17].

Nicoletti *et al.* [18] investigated the antiviral activity of the green algae *Caulerpa taxifolia*. The chloroform-methanol residue showed an interesting inhibitory effect *in vitro* toward the feline immunodeficiency

virus, a valid model for studying AIDS. This extract reduced virus-induced sincytia in cultured cells, viral RT activity and viral capsin protein p24 expression.

Most of the research on marine compounds with anti-HIV activity has focused on sulphated polysaccharides (PS) and proteins. Sulphated polymannuroguluronate is a marine sulphated PS which has entered phase II clinical trials in China as the first anti-AIDS drug candidate obtained from marine brown algae. Miao *et al.* [19] investigated the binding site(s) receptors of this compound in lymphocytes mediating its anti-AIDS activities. These results indicate that the interaction of this PS and CD4 may provide a mechanistic explanation of its immunopotentiating and anti-AIDS activities in HIV-infected individuals.

The presence of sulphate groups in PS is necessary for anti-HIV activity, and potency increases with the degree of sulphation [20,21]. One of these compounds, calcium spirulan (Ca-SP) has been isolated from the sea alga *Spirulina platensis* as an antiviral component [22,23]. Anti-HIV-1 activity of this compound was measured by three different assays: viability of acutely infected CD4-positive cells, determination of HIV-1 p24 antigen released into culture supernatants, and inhibition of HIV-induced syncytium formation. Ca-SP may be a candidate agent for anti-HIV therapeutics that might overcome the disadvantages observed in many sulphated PS. When the role of chelation of calcium ion with sulphate groups was examined by removing the calcium, or replacing it with sodium the presence of the calcium ion in the molecule was also shown to be essential for the dose-dependent inhibition of the cytopathic effect (CPE) and syncytium formation induced by HIV-1.

Examples of sulphated PS with anti-HIV activity also included sulphated α-D(1→3)-linked mannans from *Nothogenia fastigiata* [24], from *Schizymenia dubyi* [25], anti-HIV PS from the brown seaweed *Fucus vesiculosus* [26] and from the marine microalga *Cochlodinium polykrikoides* [27]. Sulphated PS displaying antiviral activities against HIV-1 and human immunodeficiency virus type 2 (HIV-2) were also isolated from marine *Pseudomonas* and *Dinoflagellata* [28].

Reports on anti-HIV compounds isolated from marine algae also included proteins. Cyanovirin N, an 11-kDa protein, was identified in the search for antiviral agents [29]. Boyd *et al.* [30] isolated and sequenced this protein from cultures of the cyanobacterium *Nostoc ellipsosporum*. Cyanovirin N irreversibly inactivated diverse laboratory strains and

primary isolates of HIV-1, as well as strains of HIV-2 and simian immunodeficiency virus [31-34]. In addition, cyanovirin N aborts cell-to-cell fusion and transmission of HIV-1 infection. The antiviral activity of cyanovirin N is due, at least in part, to unique high-affinity interactions of this protein with the viral surface envelope glycoprotein gp120. Cyanovirin N contains four cysteines which form two intrachain disulfide bonds [35]. The positions of the disulfide linkages were established by fast atom bombardment mass spectral studies of peptide fragments generated by a tryptic digestion of the native protein. Reductive cleavage of these crosslinks resulted in loss of anti-HIV activity. More recently, Barrientos and Gronenborn [36] confirmed that cyanovirin N's antiviral activity appears to involve unique recognition of N-linked high-mannose oligosaccharides on the viral surface glycoproteins.

Another anti-HIV protein of marine origin was griffthisin. Griffthisin was isolated from the red alga *Griffithsia* spp. [37]. This protein displayed potent antiviral activity against laboratory strains and primary isolates of HIV-1. Griffthisin also aborted cell-to-cell fusion and transmission of HIV infection, blocking CD4-dependent gp120 binding receptor-expressing cells and binding to viral coat glycoproteins (gp120, gp41 and gp160) in a glycosylation-dependent manner. Taken together, these data suggest that griffthisin is a new type of protein that binds to various viral glycoproteins in a monosaccharide-dependent manner. This compound could be a potential candidate microbicide to prevent the sexual transmission of HIV and AIDS.

Besides algae, marine sponges had a prolific source of anti-HIV proteins. For example, anti-HIV bioassay-guided fractionation of aqueous extracts of the Caribbean sponge *Niphates erecta*, led to the isolation of a novel anti-HIV protein named niphatevirin [38]. Niphatevirin potently inhibited the CPE of HIV-infection in cultured human lymphoblastoid cells. Niphatevirin bound to CD4 in a manner that prevented the binding of gp120, but did not directly bind gp120. Other anti-HIV proteins were isolated from the purple fluid of the sea hare *Bursatella leachii* [39], and from aqueous extracts of the cultured cyanobacterium *Scytonema varium*, which yielded scytovirin, a protein with potent anticytophatic activity against laboratory strains and primary isolates of HIV-1 [40]. Scytovirin binds to viral coat proteins gp120, gp160 and gp41, but not to cellular receptor CD4 or other tested proteins.

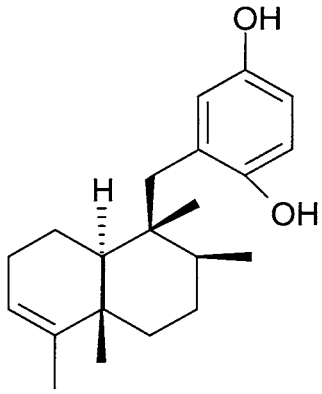

Fig. (1). Structure of avarol

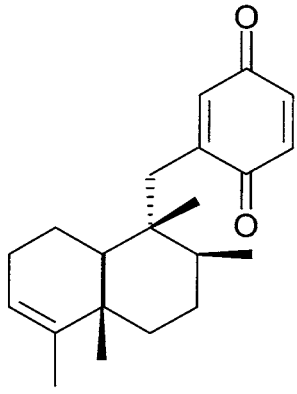

Fig. (2). Structure of avarone

Anti-HIV compounds from marine sources also included terpenoids, steroids, peptides and alkaloids. Avarol, Fig. **(1)** and avarone, Fig. **(2)**, sesquiterpenoid hydroquinones from the marine sponge *Dysidea cinerea*, are promising anti-HIV compounds [41,42]. Three new

sesquiterpene hydroquinones, rietone, 8'-acetoxyrietone and 8'-desoxyrietone, were also isolated from the soft coral *Alcyonium fauri* [43]. Rietone exhibited moderate activity in the National Cancer Institute's *in vitro* anti-HIV bioassays.

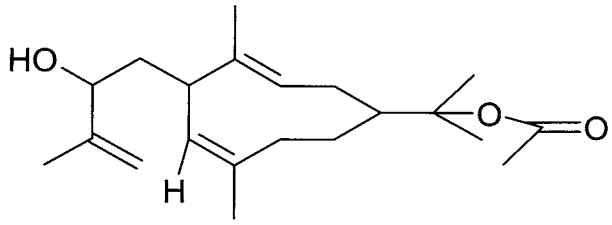

Fig. (3). Structure of cembranoid diterpenes

Bioassay-guided fractionation of an aqueous extract from a Philippine Islands collection of the soft coral *Lobophytum* spp. yielded cembranoid diterpenes, Fig. **(3),** which exhibited moderate HIV-inhibitory activity in a cell-based *in vitro* anti-HIV assay [44], while new isomalabaricane triterpenes, Fig. **(4),** have been isolated from the sponge *Stelletta* spp. [45]. Other anti-HIV diterpenes also included the dolabellane diterpenes isolated from the Brazilian brown algae *Dictyota pfaffi* [46] and *Dictyota menstrualis* [47]. To investigate the effect of these diterpenes in the reverse transcription of the viral genomic RNA, the recombinant HIV-1 RT was assayed *in vitro* in the presence of each compound. All compounds inhibited the RNA-dependent DNA-polymerase activity of HIV-1 RT and consequently virus replication.

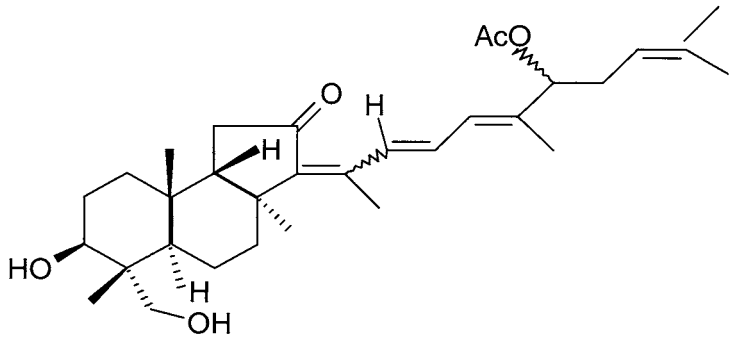

Fig. (4). Structure of isomalabaricane triterpenes

McKee *et al.* [48] evaluated a total of 22 sulphated sterols isolated from marine sponges for their antiviral activity against HIV-1 and HIV-2. In general, sterols with sulphate groups at positions 2, 3 or 6 were the most active. However, those compounds which were sulphated on the sterol D ring were completely inactive against both HIV-1 and HIV-2. New steroid sulphates, which have proved to be cytoprotective against HIV, were also isolated from the marine sponge *Pseudoaxinissa digitata* [49].

Among anti-HIV marine peptides, microspinosamide is a new cyclic depsipeptide incorporating 13 aminoacid residues which was isolated from extracts of an Indonesian collection of the marine sponge *Sidonops microspinosa* [50]. Microspinosamide inhibited the CPE of HIV-1 infection in an *in vitro* assay. Another HIV-inhibitory depsipeptide, neamphamide A, was isolated from the Papua New Guinea marine sponge *Neamphius huxlegi* [51]. Kahalalides are bioactive peptides isolated from the marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* spp., which possess activity against AIDS-opportunistic infections [52].

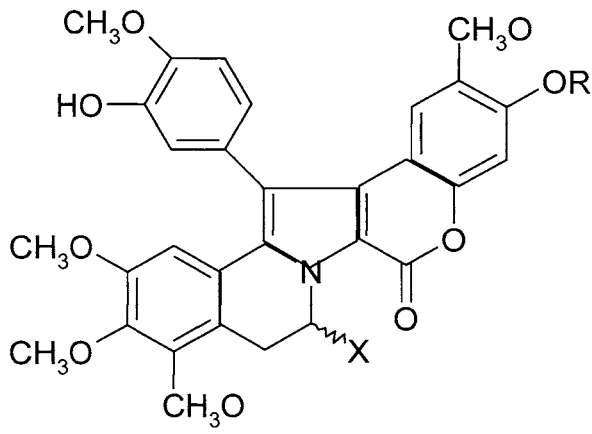

Fig. (5). Structure of lamellarins

Marine alkaloids were also reported to possess activity *in vitro* against AIDS-opportunistic infectious diseases such as tuberculosis and toxoplasmosis [53,54]. Examples of these alkaloids included the ascidian alkaloids lamellarins, Fig. **(5)** [55], and dragmacidin F, Fig. **(6)**, a new

antiviral bromoindole alkaloid from the Mediterranean sponge *Halicortex* spp. [56]. The lamellarins form a group of more than 30 polyaromatic pyrrole alkaloids isolated from diverse marine organisms, mainly but not exclusively, ascidians and sponges. Inhibition of HIV-1 integrase by lamellarin-α-20-sulphate, along with other effects on nuclear proteins, provide an experimental basis indicating that DNA manipulating enzymes are important targets for lamellarins [57].

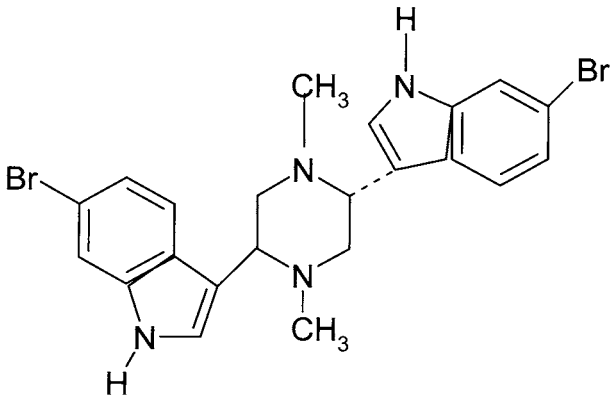

Fig. (6). Structure of dragmacidin F

Two novel alkaloids named manadomanzamines A and B were isolated from the Indonesian sponge *Acanthostrongylophora* spp. [58]. The compounds exhibited activities against HIV-1 and AIDS-opportunistic fungal infections. Oral and intravenous pharmacokinetic studies indicated that the compounds have low metabolic clearance, a reasonably long pharmacokinetic half-life, which supports the value of these compounds as potential leads for further preclinical assessment and possible development [59]. Another marine sponge, *Petrosia similis*, afforded two compounds belonging to bis-quinolizidine alkaloids, namely petrosin and petrosin A [60]. Cell assays indicated that these compounds inhibited the early steps of HIV replication. In the extracellular HIV-1 RT inhibition assay, the compounds inhibited HIV-1 RT.

Two new polycyclic guanidine alkaloids, cambrescidins 826 and dehydroaranobine A, were isolated from the marine sponge *Monanchora* spp. [61]. The pentacyclic guanidine alkaloids inhibit HIV-1 envelope-

mediated fusion *in vitro*. Sorbicillactone A is another alkaloid isolated from a strain of *Penicillium chrysogenum* cultured from a sample of the Mediterranean sponge *Ircinia fasciculata* [62]. It possesses a unique bicyclic lactone structure. The compound exhibits promising activities in several viral test systems, in particular the ability to protect human T cells against the CPE of HIV-1.

Loya *et al.* [63] investigated the activity against HIV-1 RT in the organic extract of the red sea sponge *Toxiclona toxius*. Five novel natural compounds, namely toxiusol, Fig. **(7)**, shaagrockol B and C, toxicol A, all of which are sulphated hexaprenoid hydroquinones, and toxicol E, the *p*-hydroquinone derivative of toxicol A, were isolated. All compounds exhibited inhibitory activity of both DNA polymerizing functions of HIV-1 RT, but failed to inhibit RT-associated ribonuclease H activity. Prenylhydroquinone sulphates, which inhibit HIV-integrase enzymes, have been isolated from the marine sponge *Ircinia* spp. collected from New Caledonia [64].

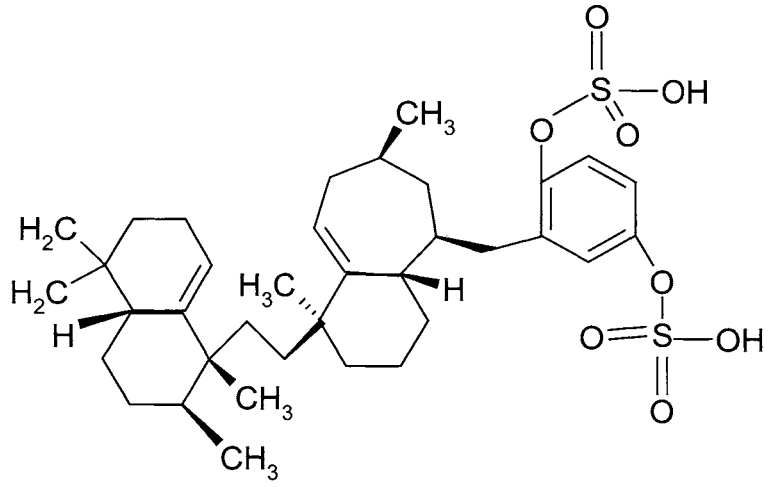

Fig. (7). Structure of toxiusol

Examples of other anti-HIV compounds also included four phlorotannin derivatives, eckol, 8,8'-dieckol, 8,4'''-dieckol and phlorofucofuro-eckol A, isolated from the brown alga *Ecklonia cava* [65]. Among these compounds, 8,8'-dieckol and 8,4'''-dieckol exhibited an inhibitory effect on HIV-1 RT and protease. An enzyme kinetic assay

showed that these compounds non-competitively inhibited RNA-dependent DNA synthesis activity of HIV- RT.

HERPES SIMPLEX VIRUS

Infections with HSV range from simple cold sores and fever blisters to severe central nervous system diseases. Approximately 16-35%, 40-80% and >90% of the population is sero-positive for or infected by herpes simplex virus type I (HSV-1), herpes simplex virus type II (HSV-2) and varicella zoster virus (VZV), respectively. More alarmingly, over the past decade, the incidence and severity of infections caused by HSV have increased due to the growth in the number of immunocompromised patients produced by aggressive chemotherapy regimes, expanded organ transplantation and a greater occurrence of HIV infections. Unfortunately, prolonged therapies with acyclovir, the most successful antiherpetic drug, have resulted in some undesirable complications and also induced the emergence of drug-resistant viruses. There is thus an urgent need for novel anti-HSV agents, especially those with a different mode of action from acyclovir.

In order to find new sources of antiviral agents with different mechanisms of action, extracts of marine algae from all over the world were assayed for anti-HSV activity. The first screening of 89 types of seaweed collected from British Columbia, Canada and Korea for antiviral activity was reported by Kim *et al.* [66]. *Analipus japonicus* was the most potent anti-herpes algae. Extracts from 13 types of Korean seaweed previously shown to contain antiviral activity were investigated in more detail in order to learn their mechanism of action [14]. Four species, *Enteromorpha linza*, *Colpomenia bullosa*, *Scytosiphon lomentaria* and *Undaria pinnatifida* were active against HSV. In experiments to determine the site of action of these antiviral extracts, the predominant activity was virucidal (i.e., direct inactivation of virus particles) rather than inhibition of virus replication.

Ohta *et al.* [67] screened 106 microalgae for CPE on Vero cells of HSV-1. The green alga *Dunnliella primolecta* had the highest anti-HSV-1 activity, since 10 μg/ml of extract from this alga completely inhibited the CPE. The antiviral activity was apparently excited during HSV adsorption and invasion of the cells. Examples of anti-HSV marine algae also included *Laminaria abyssalis*, a Brazilian marine alga [68], and the red marine alga *Polysiphonia denudata* from the Bulgarian Black Sea coast,

which selectively inhibited the reproduction of HSV-1 and HSV-2 in cell cultures [69]. An extract from another red marine alga, *Ceramium rubrum* from the Bulgarian Black Sea coast also inhibited the reproduction of HSV-1 and HSV-2 in cell cultures [70].

Spirulina (Arthospira), a filamentous unicellular alga, is one of the most extensively studied from the chemical, pharmacological and toxicological points of view [71]. A sulphated PS, named Ca-SP, has been isolated from *Spirulina platensis* as an antiviral component [22,72]. The inhibitory effect of polyanionic substances, such as sulphated PS, on the replication of HSV and other viruses was reported almost four decades ago. Since 1988, the activity spectrum of sulphated PS has been shown to extend to various enveloped and non-enveloped viruses [21].

The anti-HSV-1 activity of Ca-SP was assessed by plaque yield reduction and compared with those of dextran sulphate as a representative sulphated PS. These data indicate that Ca-SP is a potent antiviral agent against HSV-1, as even at low concentrations of Ca-SP, no enhancement of virus-induced syncytium formation was observed, as occurred in dextran sulphate-treated cultures. Recently, Lee *et al.* [73] investigated the effects of structural modifications of Ca-SP on antiviral activity. Calcium ion binding with the anionic part of the molecule was replaced with various metal cations, and their inhibitory effects on the replication of HSV-1 were evaluated. Replacement of calcium ion with sodium and potassium ions maintained the antiviral activity, while divalent and trivalent metal cations reduced the activity. Depolymerization of sodium spirulan with hydrogen peroxide decreased the antiviral activity as its molecular weight decreased.

The cell-wall sulphated PS of the red microalga *Porphyridium* spp. also appears to be a good candidate for the development of an anti-HSV drug [74,75]. Treatment of cells with 1 μg/ml PS resulted in 50% inhibition of HSV infection as measured by the plaque assay. Inhibition of the production of new viral particles was also shown when pre-infected cell cultures were treated with the PS. It seems therefore that the PS is able to inhibit viral infection by preventing adsorption of the virus into the host cells and/or by inhibiting the production of new viral particles inside the host cells. The cell-wall sulphated PS of this red microalga *Porphyridium* spp. also had impressive antiviral activity against VZV [74].

Zhu *et al.* [76] isolated another PS from the brown alga *Sargassum patents* as an antiviral component against HSV-1 and HSV-2. The gas

chromatography assay showed that the PS consisted of fucose, galactose, mannose, xylose, glucose and galactosamine. Fucose is the major constituent sugar (35.3%) followed by galactose (18.4%). This PS inhibited the replication of HSV-2, and significantly inhibited the virus attachment to its host cells [77]. All the results from this study suggested that the antiviral mode of action of this compound could be ascribed to the inhibition of virus adsorption, which is different from that of the current drug of choice acyclovir. A sulphated PS was isolated from another *Sargassum* spp., the brown alga *Sargassum horneri* [78,79]. Fucose was also detected as the main component sugar of this PS. This compound showed potent antiviral activity against HSV-1. Time-of-addition experiments suggested that it inhibited not only the initial stages of viral infections, such as attachment to and penetration into host cells, but also later replication stages after virus penetration.

In order to evaluate the potency of novel antiviral drugs, 11 natural sulphated PS from 10 green algae (*Enteromorpa compressa, Monostroma nitidum, Caulerpa brachypus, Caulerpa okamurai, Caulerpa scapelliformis, Chaetomorpha crassa, Chaetomorpha spiralis, Codium adhaerens, Codium fragile* and *Codium latum*) were assayed for anti-HSV-1 activity [80]. Except for one from *Enteromorpha compressa*, all PS showed potent anti-HSV-1 activity while having low cytotoxicities. These experiments demonstrated that some sulphated PS not only inhibited the early stages of HSV-1 replication such as virus binding to and penetration into host cells, but also interfered with later steps of virus replication. A sulphated PS fraction was isolated from another green alga *Caulerpa racemosa* [81]. The polymer contained galactose, glucose, arabinose and xylose as the major component sugars, and contained 9% sulphate hemiester groups. This compound was a selective inhibitor of reference strains of HSV-1 and HSV-2 in Vero cells, and lacked any cytotoxic effects.

Other sulphated PS with known antiviral activity are carrageenans. Natural carrageenans of diverse structural types isolated from the red seaweed *Gigartina skottsbergii* were recently identified as potent and selective inhibitors of HSV-1 and HSV-2 [82,83]. Time-of-addition and attachment studies suggested that the main target for antiviral action of the carrageenans was virus adsorption, whereas no effect on virus internalization or early or later protein synthesis was detected [84]. However, the λ-carrageenan was still significantly inhibitory when added

any time after adsorption. Carrageenans were also extracted from Chilean samples of *Stenogramme interrupta*, with promising antiherpetic activity [85]. This carrageenan is composed predominantly of 0.5 M KCl-insoluble and 1 M KCl-soluble fractions. The insoluble fraction contained γ-carrageenan as the major component, with α-carrageenan and pyruvated carrageenan as minor components.

Other sulphated PS with known antiviral activity are galactans and agarans. Talarico *et al.* [86] presented the chemical composition and antiviral activity against HSV-1 and HSV-2 of sulphated galactans obtained from two red seaweeds collected in Brazil, *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. The galactans lacked cytotoxic effects and showed a broad spectrum of antiviral activity against HSV-1 and HSV-2. No direct virus inactivation was observed after virion treatment with the galactans. The mode of action of these compounds can be mainly ascribed to an inhibitory effect on virus adsorption. Most importantly, significant protection against murine vaginal infection with HSV-2 was afforded by topical treatment with the sulphated galactans. Another sulphated galactans was isolated as the major component of an aqueous extract of the seaweed *Undaria pinnatifida* [87]. This PS was evaluated for antiviral activity against 32 clinical strains of HSV: 14 strains of HSV-1 and 18 strains of HSV-2. The mode of action of the compound was shown to be the inhibition of viral binding and entry into the host cells. Additionally, some agarans sulphates isolated from the red seaweed *Acantophora spicifera* showed very selective and potent antiviral activity against both HSV-1 and HSV-2 [88]. These sulphated agarans are made up of A-units highly substituted with sulphate groups.

Other anti-HSV sulphated PS included sulphated galactans from the marine alga *Bostrychia montagnei* [89], the red seaweed *Pterocladia capillacea* [90], extracts of *Cryptopleura ramosa* and *Nothogenia fastigiata*, two red seaweeds from the South American coast [91,92], and fucoidans from the brown seaweed *Adenocystis utricularis* [93].

Besides sulphated PS, reports on anti-HSV compounds isolated from marine algae are recorded in the literature. Specimens of the brown alga *Dictyota pfaffii* from Atol das Rocas, northeast Brazil, afforded the rare dolabellane diterpene 10,18-diacetoxy-8-hydroxy-2,6-dolabella-diene and the new 10-acetoxy-8,18-dihydroxy-2,6-dolabella-diene [46]. These substances showed strong anti-HSV-1 activity *in vitro*. Several antiviral diterpenes were isolated from other brown algae of the *Dictyota* genus,

Dictyota dichotoma and *Dictyota linearis* [94]. The diterpenes isopachydictyolal from *Dictyota dichotoma*, and 4α-acetyldictyodial, Fig. **(8)** from *Dictyota linearis* are new natural products which showed potent antiviral activity against HSV-1 using Vero cells as hosts.

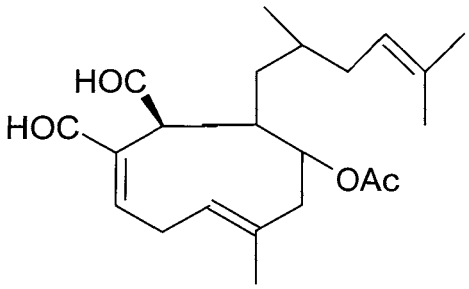

Fig. (8). Structure of 4α-acetyldictyodial

A 6-cyano-5-methoxy-12-methylindolo[2,3-α]carbazole and 6-cyano-5-methoxyindolo[3-α]carbazole were isolated from the blue-green alga *Nostoc sphaericum* [95]. These compounds are active against HSV-1. Larsen *et al.* [96] isolated three new chlorine-containing β-carbolines, bauerines A-C, from the blue-green alga *Dichothrix baueriana*. These alkaloids show activity against HSV-2.

Other marine organisms and microorganisms in addition to marine algae have been reported to produce anti-HSV compounds, including terpenoids, steroids, alkaloids, peptides and sulphated PS.

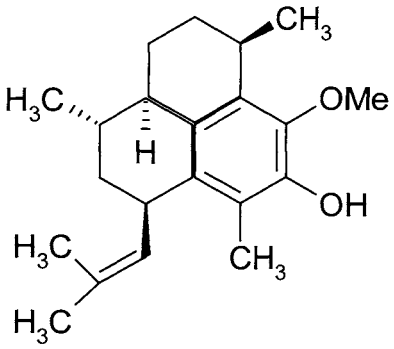

Fig. (9). Structure of pseudopterosins

Betancour *et al.* [97] reported for the first time the antiherpetic activity of a series of sponge-derived diterpenes. A family of 13 new diterpene glycosides, pseudopterosins P-Z, Fig. **(9)**, and seco-pseudopterosins H and I have been isolated from two collections of the marine sponge *Pseudopterogorgia elisabethae* from the Southwestern Caribbean sea in Colombia [98]. Pseudopterosin P displayed strong antiviral activity against HSV-1, HSV-2 and VZV. Seven new diterpenes, helioporins A-G, Fig. **(10)** have also been isolated from the blue coral *Heliopora coerulea* [99]. Helioporins A and B showed antiviral activity against HSV-1. Anti-HSV terpenoids of marine origin also included norsesterterpene peroxide acids from the red sea sponge *Diacarmus erythraeanus*, which showed *in vitro* antiviral activity against HSV-1 [100], and two new trisulphated triterpene glycosides, liouvillosides A and B, from the Antarctic sea cucumber *Staurocucumis liouvillei*, which were found to be virucidal against HSV-1 [101]. Comin *et al.* [102] isolated disulphated polyhydroxysteroids with inhibitory effect on the replication of HSV-2 from the Antarctic ophiuroid *Astrotoma agassizii*.

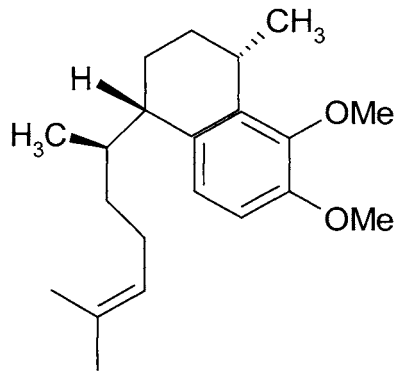

Fig. (10). Structure of helioporins

Marine sponges have been shown to be a prolific source of anti-HSV alkaloids. Dragmacidin F, Fig. **(6)** is a new antiviral bromoindole alkaloid isolated from the Mediterranean sponge *Halicortex* spp. collected from the south coast of Ustica Island, Italy [56]. This compound,

containing an unprecedented carbon skeleton that is very likely derived from cyclization of a partially oxidized form of dragmacidin D, showed *in vitro* antiviral activity against HSV-1, thus proving itself responsible for the antiviral property exhibited by *Halicortex* extracts. The crude extract from the marine sponge *Aaptos* spp. collected in Abrolhos, Bahia, Brazil, afforded a new alkaloid 8,9-dimethoxy-4-methyl-4H-benzo[*D*][1,6]naphthyridine and the known demethyloxyaaptamine [103]. Both compounds showed potent antiviral activity against HSV-1 and low toxicity to Vero cells, suggesting that they may be selectively targets for the inhibition of virus replication. Ichiba *et al.* [104] reported the isolation of a β-carboline alkaloid, 8-hydroxymanzamine A, Fig. **(11)** from the sponge *Pachypellina* spp., which exhibited moderate anti-HSV-2 activity.

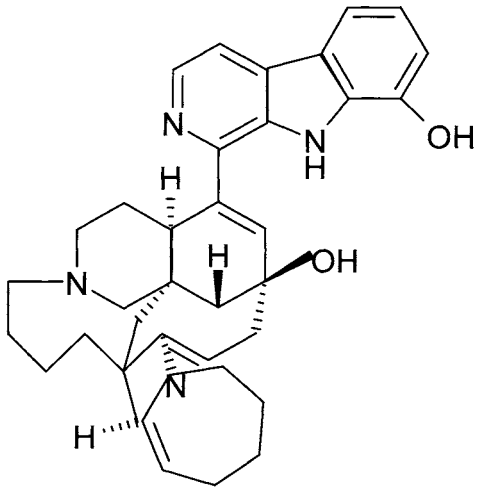

Fig. (11). Structure of 8-hydroxymanzamine A

A marine *Pseudomonas* species WAK-1 strain simultaneously produced extracellular glycosaminoglycan and sulphated PS which showed anti-HSV-1 activity in RPMI 8226 cells [105], while the water-soluble fraction of the marine diatom *Haslea ostrearia* delayed HSV-1-induced syncitia formation [17]. A novel acid PS, nostoflan, was isolated from the cyanobacterium *Nostoc flagelliforme*, which had a broad antiviral spectrum against enveloped viruses, including HSV-1 and HSV-2, whose cellular receptors are carbohydrates [106]. Two new

caprolactans with antiviral activity towards HSV-2 have been isolated from another marine bacterium [107].

As we can see above, marine microorganisms represent an under-explored resource for the discovery of novel antiviral agents. Rowley *et al.* [108] described a series of peptides designated halovirs A-E, Fig. **(12)** that are produced from a marine-derived fungus of the genus *Scytalidium*. These lipophilic and linear peptides are potent *in vitro* inhibitors of HSV-1 and HSV-2. Evidence is presented that the halovirs directly inactivate HSV, a mechanism of action that could be applicable in the prevention of HSV transmission. Recently, Rowley *et al.* [109] presented structure-activity relationships defining key structural elements for optimal viral inhibition. Results demonstrate that an N(α)-acyl chain of at least 14 carbons and a dipeptide are critical for maintaining the antiviral activity.

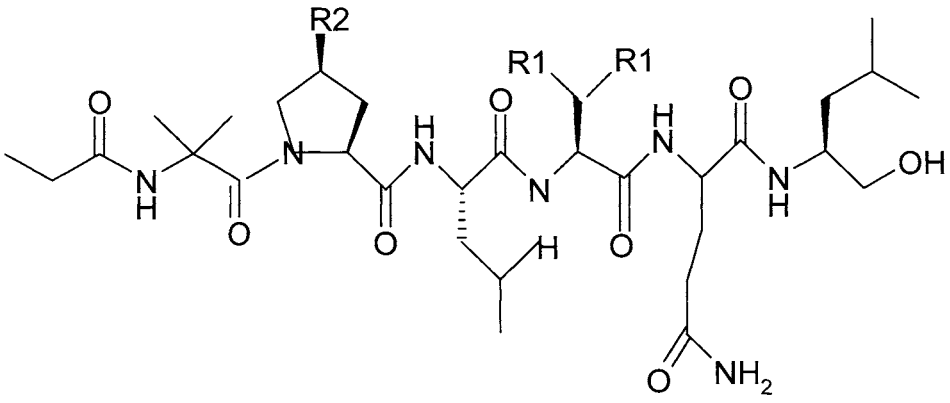

Fig. (12). Structure of halovirs

Examples of other antiviral compounds of marine origin also included two new saponins isolated from the holothurian *Thyone aurea* which showed interesting activity against HSV-1 [110], and fatty acids from the soft coral *Nephthea* spp. with antiviral activity against VZV [111].

CYTOMEGALOVIRUS

In terms of their biological and pathogenic properties, HSV fall naturally into several subfamily groupings, including CMV, although detailed classification is at present premature. Nevertheless CMV clearly constitute a group of their own with internal consistency. Human CMV is, together with HSV-1, one of the agents responsible for opportunistic infections in HIV-infected people.

As we can see in the present review, sulphated PS show antiviral activity against enveloped viruses, including human pathogens such as HIV, HSV and human CMV [112]. Examples included Ca-SP from the blue-green alga *Spirulina platensis* which inhibited, among others, human CMV [23]. This alga also yielded sulfolipids which have been found to be active against CMV [113].

A few reports on anti-CMV compounds of marine origin belonging to other structural types have also been found in the literature. Examples included two plastoquinones isolated from the brown alga *Sargassum micracanthum* which was found to have potent antiviral activity against human CMV [114], and pseudopterosin P, Fig. **(9)** from the sea whip *Pseudopterogorgia elisabethae* [98].

INFLUENZA VIRUS

Influenza continues to have a significant impact on public health. Airborne transmission, facile mutation, vaccine shortages and actual and perceived side effects and limitations of both vaccines and prophylactic drugs contribute to the search for new therapies and preventive medicines. For the past decades, besides a variety of synthetic antiviral drugs with different molecular targets, a number of natural marine products and extracts, mainly with a marine algae origin, have been recognized to control infections caused by the influenza virus.

Reports on the anti-influenza virus effects of extracts from marine algae from all over the world have been found in the literature [115]. These results show that the blue-green algae (cyanobacterium) are able to produce compounds with anti-influenza activity that may be of potential clinical interest. For example, aqueous and methanolic extracts of cultured cyanobacteria of several genera, *Microcystis*, *Nodularia*, *Oscillatoria*, *Scytonema*, *Lyngbya* and *Calothrix* were evaluated for their *in vitro* antiviral activity against the influenza A virus in Madin Darby kidney cells [116,117]. The further analysis of methanolic extracts of cultured strains of genus *Microcystis* revealed a remarkable antiviral

activity against the influenza A virus for *Microcystis aeruginosa*, *Microcystis ichthyoblabe* and *Microcystis wesenbergii*. The antiviral activity observed was associated with protease inhibitory activity of approximately 90%, which may be the agent responsible for reducing virus replication.

Nowotny *et al.* [118] investigated in more detail the antiviral activity of one of these species, *Microcystis aeruginosa*. Virus-specific protein synthesis decreased if the extract was present throughout the whole replication time. The antiviral effect was verified in the Allantois on shell systems using different subtypes of influenza virus A and B [119]. Virus replication was inhibited for 90% with only 10-20 μg/ml of extract. An extract of the red marine alga *Ceramium rubrum* from the Bulgarian Black Sea coast considerably inhibited the reproduction of influenza virus type A and B *in vitro* and *in ovo* [70]. The inhibition affected adsorption as well as the intracellular stages of viral replication. Extracts from other marine organisms besides algae have been shown to possess anti-influenza virus activity. Extracts prepared from economically important marine bivalves were found to possess high antiviral activity when tested against influenza virus type A and B [120].

As a result of these investigations, reports on the isolation of anti-influenza active compounds from marine sources have been found in the literature. Structures of pure compounds have been determined mainly as sulphated PS. A novel acid PS, nostoflan, was isolated from the cyanobacterium *Nostoc flagelliforme* [106]. Nostoflan showed potent antiviral activity against the influenza A virus. Sulphated PS with antiviral activity *in vitro* against a number of human and avian influenza viruses have been isolated from the green marine alga *Ulva lactuca* [121], the marine microalga *Cochlodinium polykrikoides* [27], marine *Pseudomonas* spp. [122] and the blue-green alga *Spirulina platensis* [23]. However, sulfolipids isolated from *Spirulina platensis* have also been found active against the influenza virus [113].

Another anti-influenza compound from marine origin included a new sesquiterpene hydroquinone, named strongylin A, which was isolated from the marine sponge *Strongylophora hartmani* [123].

OTHER VIRUSES

Reports on the antiviral activity found in the marine environment against other viruses have also been found in the literature. Screening of

lipophilic and hydrophilic extracts from marine algae for antiviral activity in different *in vitro* systems revealed some species with interesting effects. For example, Hudson *et al.* [14] and Kim *et al.* [66] screened 89 types of seaweed collected from British Columbia, Canada, and Korea for antiviral activity against the Sindbis virus in Vero cell monolayers. The antiviral activities were proportionately more frequent in the Korean extracts (56% compared with 27% of the Canadian extracts), but in general the more potent extracts were of Canadian origin.

Examples of antiviral activity of extracts from marine algae also included *Sargassum wightii*, a seaweed which showed highest activity against the vaccinia virus [124]. Nakano and Kamei [125] examined the antiviral efficacy of an extract from another *Sargassum* species, *Sargassum hemiphyllum*, that markedly promotes production of interferon-β in MG-63 cells in culture. Antiviral tests revealed the therapeutic efficacy of this alga in mice infected with the Aujeszky's disease virus. These results suggest that this extract manifests its antiviral activity by modulating the host's immunodefense systems.

Aqueous extracts from the marine microalgae *Porphyridium cruentum*, *Chlorella antotrophica* and *Ellipsoidon* spp. produced a significant inhibition of the *in vitro* replication of the haemorrhagic septicaemia virus and African swine fever virus [126], while extracts of the seaweeds *Cheilosporum spectabile* and *Rhizophora mucronata* were found to be effective in protecting mice from the lethal Semliki forest virus infection [127]. Extracts from four species of Brazilian marine algae collected from the coast of Rio of Janeiro State, were active against human T-cell lymphotropic virus type 1- (HTLV-1)- induced syncytium formation *in vitro* [128].

Other marine organisms and microorganisms besides algae have also been found to be active against several viruses, such as marine *Vibrio* spp. which was active against infections caused by the hematopoietic necrosis virus and Oncorhynchus masou virus [129], and the aqueous extracts of marine cephalopods, which inhibited the Moloney murine leukaemia virus RT activity [130].

Several studies were undertaken in order to investigate in more detail the nature of the antiviral compounds and their mechanisms of action. Structures of active pure compounds have been determined as PS, steroids, aminoacid derivatives, diterpenes, lipids and alkaloids.

As can be seen in the present review, most of the research on marine compounds with antiviral activity has focused on sulphated PS. Several sulphated seaweed PS show high antiviral activity against enveloped viruses, including important human pathogens such as HIV, HSV, human CMV, the dengue virus and respiratory syncytial virus [112]. Examples included the cell-wall sulphated PS of the red microalga *Porphyridium* spp. which was highly inhibitory for malignant cell transformation by the Moloney murine sarcoma virus [131] and the marine microalga *Cochlodinium polykrikoides* which produced extracellular sulphated PS that inhibited the CPE of the respiratory syncytial virus type A and B [27].

Another sulphated PS is produced by the marine microalga *Gyrodinium impudicum* strain KG03 from Korea exhibited impressive antiviral activity *in vitro* against the encephalomyocarditis virus [132]. This is the first reported marine source of antiviral sulphated PS against this virus. This PS may be useful in the development of marine bioactive PS for biotechnological and pharmaceutical products. Examples of other antiviral PS of marine origin also included a PS alginate isolated from the marine alga *Fucus gardneri* which showed activity against the potato virus [133], and κ/β-carrageenan obtained from the red marine alga *Tichocarpus crinitus*, which possesses antiviral effect against the tobacco mosaic virus in the early stages [134].

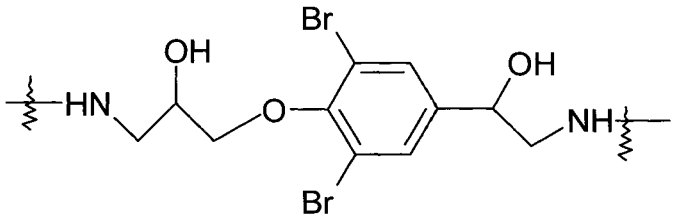

Fig. (13). Structure of fistularin 3

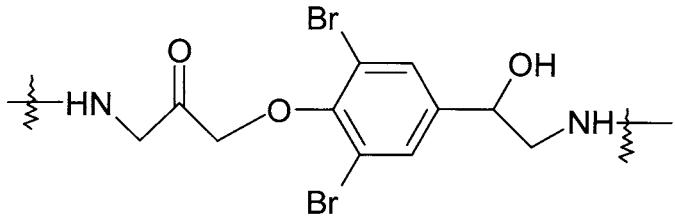

Fig. (14). Structure of 11-ketofistularin 3

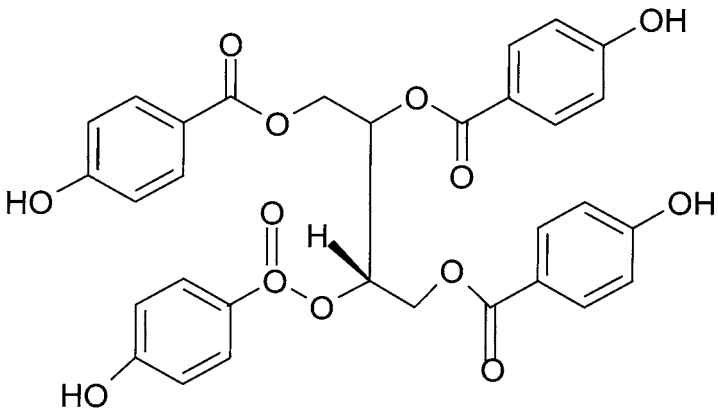

Fig. (15). Structure of kelletinin A

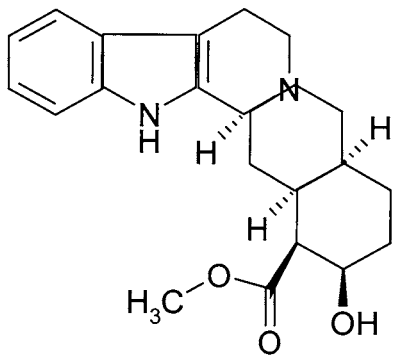

Fig. (16). Structure of halitunal

Gauvin *et al.* [135] isolated 5-α,8-α, epidioxy sterols from the marine sponge *Luffariella variabilis*, which showed inhibitory activity against the HTLV-1. Anti-HTLV-1 activity were also found in two brominated tyrosine metabolites, fistularin 3, Fig. **(13)** and 11-ketofistularin 3, Fig. **(14)** from the marine sponge *Aplysina archeri* [136], and kelletinin A, Fig. **(15)**, an ester of *p*-hydroxybenzoic acid extracted from the marine gastropod *Buccinulum corneum* [137]. From the marine alga *Halimeda tuna*, Koehn *et al.* [138] isolated an unusual diterpene aldehyde, halitunal, Fig. **(16)** which shows antiviral activity against murine coronavirus A59 *in vitro*. Examples of antiviral marine compounds also included neofolitispates, pentacyclic guanidine alkaloids isolated from the sponge *Neofolitispa dianchora* which show antiviral activity against the hepatitis B virus [139], and clavulone, Fig. **(17)**, a prostaglandin analog found in the soft coral *Clavularia viridis* which presented antiviral activity against the vesicular stomatitis virus [140].

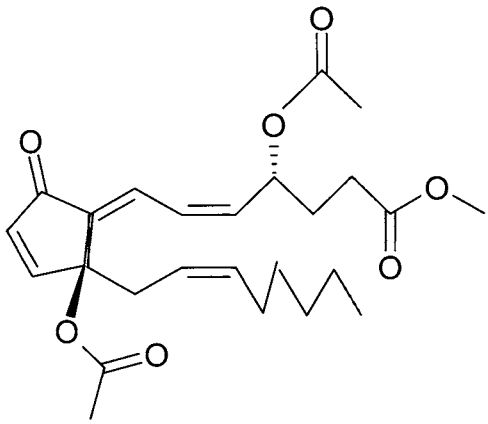

Fig. (17). Structure of clavulone

Chemical investigation of an extract of the soft coral *Nephthea* spp. showing antiviral activity against the Ranikhet disease virus *in vitro* afforded wax esters, cholesterol, 1-O-alkyl-glycerols and fatty acids [111]. Another glycolipid derived from a marine sponge, α-galactosylceramide, Fig. **(18)** is currently in human clinical trials as an

anticancer agent. However, it has also been shown to be effective in reducing the amount of hepatitis B virus DNA detected in mice [141]. It was assumed that the antiviral activities associated with α-galactosylceramide were mediated through the activation of natural killer Y cells.

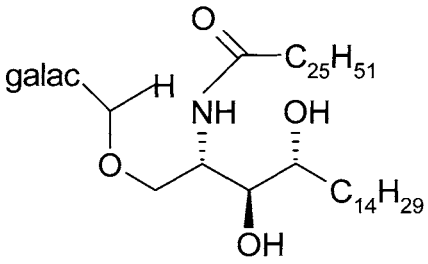

Fig. (18). Structure of α-galactosylceramide

A protein-bound pigment, allophycocyanin, purified from the blue-green alga *Spirulina platensis*, is the first compound reported to exhibit anti-enterovirus 71 activity [142]. Enterovirus 71 infection causes significant morbidity and mortality in children, yet there is no effective treatment. Allophycocyanin neutralized the enterovirus 71-induced CPE in both human rhabdomyosarcoma cells and African green monkey kidney cells. This compound was also able to delay viral RNA synthesis in the infected cells and to abate the apoptotic process in enterovirus 71-infected rhabdomyosarcoma cells, with evidence of characteristic DNA fragmentation decreasing membrane damage and declining cell sub-G1 phase.

ABBREVIATIONS

HSV = Herpes Simplex Virus
HIV = Human Immunodeficiency Virus
AIDS = Acquired Immunodeficiency Syndrome
CMV = Cytomegalovirus
HIV-1 = Human Immunodeficiency Virus type 1
RT = Reverse Transcriptase
PS = Polysaccharides

Ca-SP = Calcium Spirulan
CPE = Cytophatic Effect
HIV-2 = Human Immunodeficiency Virus type 2
HSV-1 = Herpes Simplex Virus type I
HSV-2 = Herpes Simplex Virus type II
VZV = Varicella-Zoster Virus
HTLV-1 = Human T-cell Lymphotropic Virus type 1

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TRITERPENE GLYCOSIDES FROM SEA CUCUCMBERS (HOLOTHURIOIDEA, ECHINODERMATA). BIOLOGICAL ACTIVITIES AND FUNCTIONS

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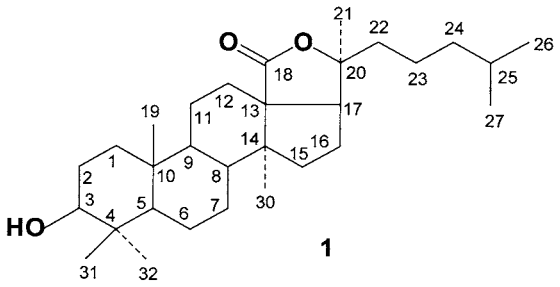
ABSTRACT: The holothuroid triterpene glycosides have strong membranolytic action against cellular and model membranes containing Δ^5-sterols as result of the formation of single-ion channels and more large pores that is the basis of hemolytic, antifungal, antitumor cytotoxic activities of these compounds. The binding presence of an 18(20)-lactone, and at any rate of one oxygen functional group near it for compounds with 9(11)-double bonds in lanostane aglycon moiety, is very important for the membranotropic action of these substances. A linear tetrasaccharide fragment in the carbohydrate chain is also essential for membranolytic action. A sulfate group at C-4 of the first xylose residue increases the effect against membranes. The absence of a sulfate group at C-4 of the xylose residue in biosides decreases the activities more than one order of magnitude. The presence of a sulfate at C-4 of the first xylose of branched pentaosides having 3-O-methyl group at a terminal monosaccharide increases activity but the same sulfate decreases the activity of branched pentaosides having terminal glucose residues. Sulfate groups attached to a C-6 position of terminal glucose and 3-O-methylglucose residues greatly decrease activity. Some glycosides may inhibit chemokine receptor subtype 5 (CCR 5). This activity is correlated with toxicity. Some glycosides possess immunostimulatory action in subtoxic doses. The most effective immunostimulants are monosulfated glycosides but di- and tri-sulfated are immunosuppressors. The network diagrams illustrating the relationships between glycoside structural elements and functional components (partial activities) as well as with general glycoside activity are shown to be useful for non-quantative predication of biological activity. The glycosides regulate of oocyte maturation in the sea cucumbers to synchronize maturation processes. This role is caused by the modifying action of the glycosides on the membranes of holothuroid eggs. The absence or very low concentration of Δ^5-sterols in oocyte membranes suggests another nature of the modifying action in comparison with that against membranes containing Δ^5-sterols. Holothuroid glycosides have defensive function against predators.

INTRODUCTION

For more than forty years triterpene glycosides from sea cucumbers (holothurians) have attracted the attention of chemists, biochemists, pharmacologists, and biologists-taxonomists. Until recently it was thought that triterpene oligoglycosides were found only in these marine invertebrates in the animal kingdom, but later similar substances have been found in sponges [1]. The majority of the known triterpene glycosides from different representatives of the class Holothurioidea have aglycons of a lanostane skeleton system possessing an 18(20)-lactone. They were classified as the so-called holostane series [2], being the derivatives of hypothetic holostan-3β-ol (**1**).



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Recently several new structural types of aglycons have been discovered in our laboratory [3-7]. Monosaccharide compositions of sea cucumber glycosides usually include D-xylose, D-quinovose, 3-O-methyl-D-glycose, D-3-O-methylxylose and D-glucose and rarely 6-O-acethyl-D-glucose [8,6].

These substances are specific for different taxonomic groups of sea cucumbers. This makes them a very convenient model for biochemical evolution studies and for application as chemical characters for improvement of holothurian taxonomy [9-11].

Triterpene glycosides of sea cucumbers demonstrate a wide spectrum of biological effects: antifungal, antitumor, hemolytic, cytostatic and immunomodulatory activities [12]. The application of many preparations from sea cucumbers in traditional oriental medicine has been reported [13]. The medicinal properties of these sea food preparations are attributed to triterpene glycosides [13].

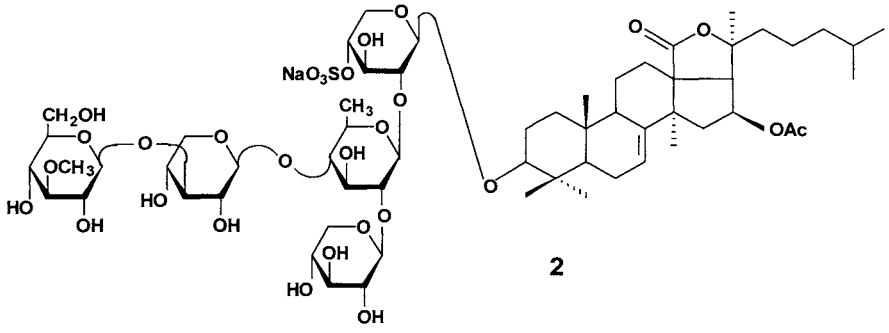
Although several reviews describe the biological activities and biological roles of these glycosides [14-19, 12, 20-22], they have nonetheless not covered all the recent work in this areas.

The present review covers the relevant publications up to 2005.

PARTIAL BIOLOGICAL ACTIVITIES OF HOLOTHURIAN GLYCOSIDES

Ichthyotoxicity and general toxicity

Many kinds of biological activities of holothurian triterpene glycosides are a result of their membranolytic action, i. e. their capability to induce disturbances in cellular membrane permeability up to lysis.



The ichthyotoxic action of aqueous extracts of sea cucumbers has been studied [23-25]. Aborigines of Guam and other regions of Indo-Pacific used holothurians such as *Bohdschia argus* and *Holothuria atra* to poison small lagoons of coral reefs at low tide for killing fish. Yamanouchi [25] and Nigrelli [24] showed the presence of ichthyotoxic substances in 30 species of sea cucumbers belonging to four different orders. The glycoside fractions, isolated from *Actinopyga agassizi* [26] and from *Holothuria leucospilota* (=*H. vagabunda*) [25], known as "holothurin", were studied in relation to their ichthyotoxicity. Histological analysis indicated the cause of fish death to be the destruction of gill capillaries [23]. Yamanouchi determined a lethal dose of "holothurin" against

earthworms, frogs and mice [25]. The LD_{50} for mice, under 24 hours of holothurin administration were of 0.75, 70 and 400 mg/kg at intravenous, hypodermic and peroral injection respectively. The LD_{50} of glycoside fraction "cucumarioside" isolated from *Cucumaria japonica* for mice was of 200 μg/mouse or 10 mg/kg at intraperitoneal injection [27].

The acute toxicity for frondoside A (**2**), the major saponin from *Cucumaria frondosa* as determined by the Behrens-Karber method was of 9.9 mg/kg (LD_{50}) at intraperitoneal injection [28].

Mutagenic activity

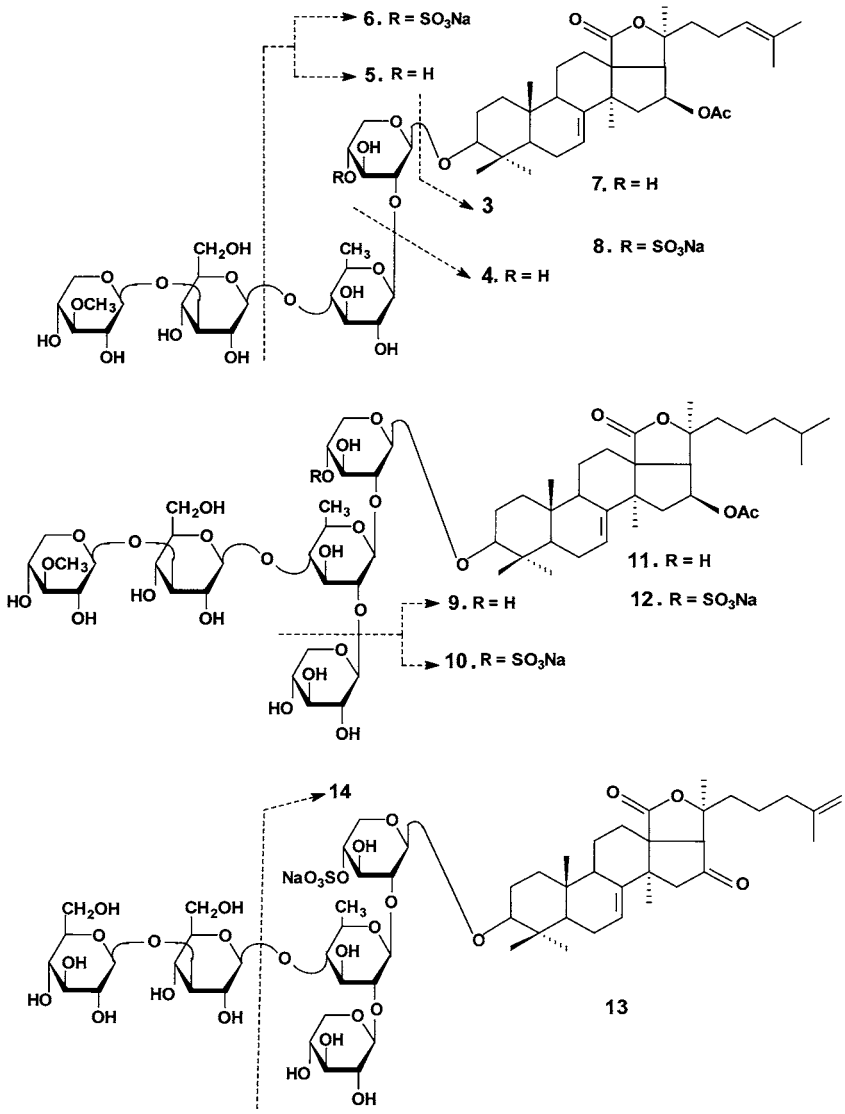
The crude holothurins from Philippine sea cucumbers belonging to Holothuriidae family have indirect mutagenic and clastogenic activity. Although no activity was noted *in vitro* (Rec- assay and Ames' test) after metabolic activation, these holothurins produced aberration scores in Swiss albino mice (micronuclear test) when administered intraperitoneally [29]. Nevertheless, at peroral injection, they exhibited a 35-fold reduction in activity, which indicates inactivation in the alimentary canal [29]. On the contrary, Polycarpova et al. showed the absence of mutagenic activity of cucumarioside - triterpene glycoside from the sea cucumber *Cucumaria japonica* [27].

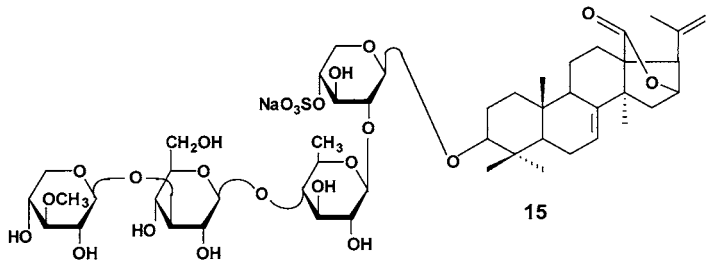
Hemolytic action

"Holothurin" shows a hemolytic action against rabbit erythrocytes [24, 25]. The hemolytic index of "holothurin" is 6 – 7 times more than that of the so-called "saponin" (the mixture of plant triterpene glycosides). Thron also found that "holothurin" has higher hemolytic activity in comparison with plant triterpene glycosides [30]. The hemolytic activity of "holothurin" has been observed *in vivo* by injection into the dorsal lymph spaces of the frog *Rana pipiens*. Hemolysis was followed by an intense hematopoetic activity [31]. The hemolytic activities of glycoside fractions from four species of Philippine sea cucumbers - *Holothuria fuscocinerea*, *H. pulla*, *Actinopyga lecanora* and *Opheodesoma*

grisea were studied by Poscidio [32]. The fraction from *O. grisea* had a minimal activity among the studied species.

We have studied hemolytic action of a series of triterpene glycosides and their derivatives (**3–15**) from sea cucumbers belonging to the order Dendrochirotida [33].





Cucumarioside G_1 (**8**) and cucumarioside G_2 (**15**) are native glycosides from the sea cucumber *Eupentacta fraudatrix*. Substances **3**, **4**, **5**, **6**, **7**, **9** and **10** are derivatives of cucumariosides G_1. Substances **11** and **12** are dihydroderivatives of cucumariosides C_2 and H from *E. fraudatrix*, correspondingly. Frondoside A (**2**) is a glycoside from the *Cucumaria frondosa*, cucumarioside A_4-2 (**13**) is a glycoside from *Cucumaria japonica*, and the substance **14** is a derivative of cucumarioside A_4-2. The data are shown in Table 1.

Table 1. The hemolytic activity, rate and delay time of hemolysis and the rate of K^+ loss induced by cucumariosides and their derivatives

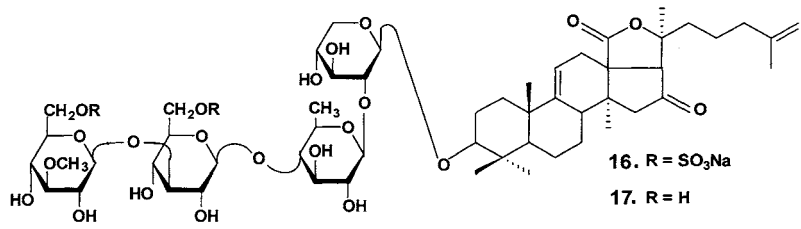
Substance	ED_{50} (M)	Delay time (sec)	Slope (1/sec)	V_K** (μM/min)
3	$>1.12x10^{-5}$	(not hemolytic up to 4 min)*		0.5
4	$0.64x10^{-5}$	105*	0.004	3
5	$0.25x10^{-5}$	11*	0.043	6
6	$0.78x10^{-6}$	8	0.041	150
7	$0.25x10^{-6}$	12	0.040	120
8	$0.25x10^{-6}$	9	0.31	140
9	$0.10x10^{-6}$	8	0.041	60
10	$0.10x10^{-6}$	3	0.63	100
11	$0.60x10^{-6}$	46	0.014	30
12	$0.40x10^{-6}$	27	0.025	35
2	$0.25x10^{-6}$	15	0.039	24
13	$0.20x10^{-5}$	3*	0.160	3
14	$0.10x10^{-5}$	1.5*	0.250	2
15	$0.35x10^{-5}$	24*	0.010	0.5

* - The concentration of substance is $1x10^{-5}$ M; all other cases - $1x10^{-6}$
**-The rate of K^+ loss at concentration of substance $1x10^{-6}$ M

These data show that hemolytic activity depends upon both aglycon and carbohydrate chain structures. The presence of a linear tetrasaccharide fragment in carbohydrate chains is significant for the activity. Moreover, a sulfate group, attached to the C-4 of the

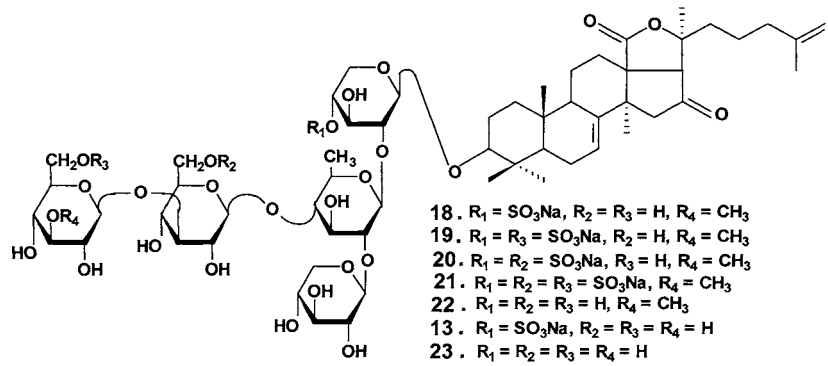
first xylose residue does not influence hemolytic properties significantly. Pentasaccharide derivatives, branched at the second monosaccharide unit, are a little less active in comparison with tetraosides. In contrast with pentaosides and tetraosides, the desulfation of the sulfated at first xylose unit bioside **5** leads to the significant decrease in activity. The presence of an 18(16)-lactone in the aglycon moiety of molecules is also an important requirement for hemolytic activity.

To investigate the influence of quantity and position of sulfate groups in carbohydrate moieties, we have studied kinetic parameters of hemolysis and K^+ loss from erythocytes of another series of triterpene glycosides and their derivatives from sea cucumbers in the order Dendrochirotida [34].



16. R = SO_3Na

17. R = H



18. $R_1 = SO_3Na$, $R_2 = R_3 = H$, $R_4 = CH_3$

19. $R_1 = R_3 = SO_3Na$, $R_2 = H$, $R_4 = CH_3$

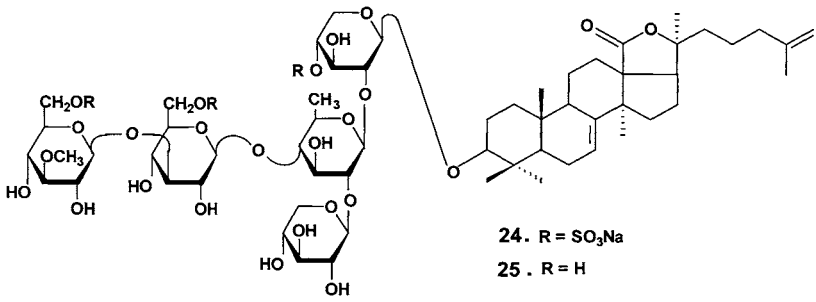
20. $R_1 = R_2 = SO_3Na$, $R_3 = H$, $R_4 = CH_3$

21. $R_1 = R_2 = R_3 = SO_3Na$, $R_4 = CH_3$

22. $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$

13. $R_1 = SO_3Na$, $R_2 = R_3 = R_4 = H$

23. $R_1 = R_2 = R_3 = R_4 = H$



24. R = SO_3Na

25. R = H

Psolusoside A (**16**) is a glycoside from *Psolus fabricii*. Substance **17** is a desulfated derivative of glycoside **16**. Cucumariosides A_2-2 (**18**),A_6-2 (**19**), A_3 (**20**), A_7-1 (**21**), A_4-2 (**13**), A_7-3 (**24**) are glycosides from *Cucumaria japonica*. Substances **22**, **23** and **25** are desulfated derivatives of glycosides **18**, **13** and **24**, respectively. The obtained data are shown in Table 2.

Table 2. The hemolytic activity, rate and the delay time of hemolysis induced by cucumariosides and their derivatives

Substance	ED_{50} (10^{-6} M)	Delay Time (sec)	Slope (1/sec)	V_K+ (μM/min)
16	**	not hemolytic up to 2 min*		16.6*
17	**	26*	0.006	12.0*
18	0.87	5	0.075	32.4*
19	1.9	105	0.010	6.6*
20	1.25	21	0.025	62.0*
21	2.5	120	0.005	21.6*
22	2.5	38	0.025	15.8*
13	2.5	not hemolytic up to 2 min		5.0*
23	1.9	13	0.015	20.0*
24	2.5	44	0.08	98.0*
25	0.87	1	0.14	73.0*

* - The concentration of substance is $2.5x10^{-6}$ M; all other cases - $5x10^{-6}$ M.
** - ED_{50} was no determined

These results showed that sulfate groups, attached to different positions of the carbohydrate chain differently influence on the hemolytic activity and K^+ loss. A sulfate group, attached to C-4 of the first xylose of branched pentaosides having 3-O-methyl group in terminal monosaccharide unit, increases the activity by kinetic parameters of hemolysis and by K^+ loss rate. Similar results were obtained by us for glycosides from *Eupentacta fraudatrix* [33].

Such sulfate groups of analogous glycosides having no 3-O-methyl group induce an opposite effect, i.e. suddenly decrease the activity.

Sulfate groups, attached to the C-6 position of monosaccharide units differently influence on the kinetic parameters of hemolysis and K^+ loss depending upon the position of a sulfated monosaccharide residue in carbohydrate chain. A sulfate group at C-6 of terminal residue of 3-O-methylglucose vastly decreases the activity both by hemolysis parameter and by K^+ loss rate. A sulfate at C-6 of the third monosaccharide unit also decreases the hemolytic activity. However this sulfate group increases the K^+ loss rate. It is possible that sulfate groups at C-6 of monosaccharide units prevent the generation of large water loaded pores in erythrocyte membranes. A sulfate group at C-6 of the third monosaccharide unit probably does not prevent the generation and maintenance of solitary ion channels and *vice versa* increased K^+ loss. The absence of 16-ketogroup in aglycon for glycosides with the 7(8)-double bond suddenly increases hemolytic activity and K^+ loss.

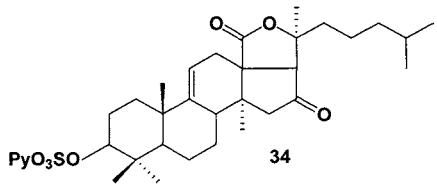
Antifungal action

Antifungal action of triterpene glycosides of sea cucumbers was first found by Shimada [35]. The glycoside fraction from *Apostichopus* (=*Stichopus*) *japonicus* ("holotoxin") inhibited a growth of different pathogenic fungi at the concentrations of 2.78 to 16.7 μg/ml, but did not influence on growth of bacteria and mycobacteria. Antifungal properties are characteristic of glycoside fractions from the genera *Bohadshia, Holothuria, Actinopyga, Stichopus, Thelenota* and *Eupentacta* (=*Cucumaria*) *fraudatrix* [36-38, 15, 39]. The triterpene glycoside fraction from *Cucumaria japonica* has some antifungal activity against *Candida albicans* and *Candida tropicalis* (30–50 μg/ml), but significantly less than those from other holothurians [40].

Table 3. The spectrum of antimicrobial action of triterpene glycosides from sea cucumbers of the family Stichopodidae and their derivatives

Substance	Minimal mycostatic concentration (μg/ml)						
	Saccharo-myces carlsbergensis	*Candida albicans*	*Torula utilis*	*Penicillum niger*	*Aspergillus niger*	*Hormodendron pedrosol*	*Trychophyton mentagra-phytes*
26	12.5	>100	12.5	>100	>100	>100	>100
27	1.55	6.25	1.55	12.5	6.25	12.5	1.55
28	12.5	25.0	12.5	6.25	12.5	50.0	6.25
29	6.25	12.5	6.25	12.5	25.0	25.0	25.0
30	1.55	6.25	1.55	1.55	0.75	12.5	0.75
31	6.25	25.0	6.25	25.0	6.25	25.0	6.25
32	12.5	25.0	12.5	>100	25.0	12.5	12.5
33	3.12	12.5	3.12	25.0	6.25	6.25	3.12
34	>100	>100	>100	>100	>100	>100	>100

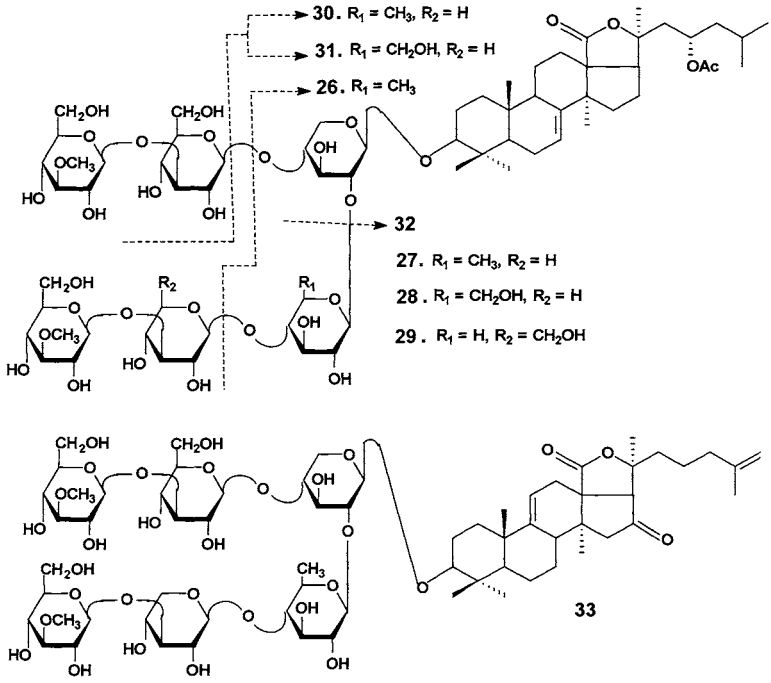
The antifungal activities of eight glycosides and their derivatives from sea cucumbers belonging to the family Stichopodidae have also been studied [41]. Stichoposides A (**26**), C (**27**), D (**28**), E (**29**) from the sea cucumber *Stichopus chloronotus*, thelenotosides A (**30**) and B (**31**) from *Thelenota ananas* and derivative **32**, obtained by Smith degradation of **27**, have the same aglycon. Holotoxin A_1 (**33**) from *Apostichopus* (=*Stichopus*) *japonicus* does has the same structure of carbohydrate chain as **27**, but differs in aglycon structure. The substance **34** is derivative of holotoxin A_1. The data are shown in Table 3.



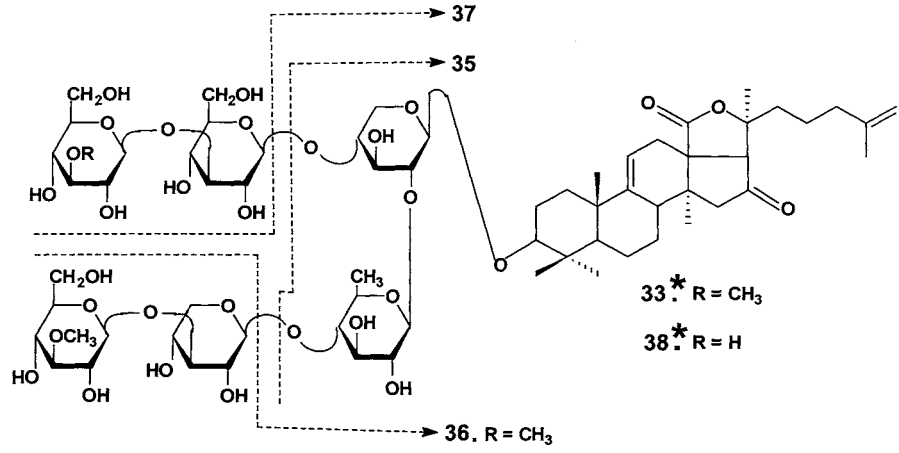
34

The sulfated aglycon **34** does not have antifungal activity while bioside **26** demonstrates a limited spectrum of antifungal activity. This confirms the importance of a carbohydrate chain to the activity. The optimal quantity of monosaccharide units is four. The activity of hexaosides does not exceed that of tetraosides. Moreover, quinovose-containing glycosides **27** and **30** are 2 - 8 times more active than substances **28** and **31**, containing glucose instead of quinovose. On the other hand, holotoxin A_1 (**33**), with the 9(11)-double bond and 16-keto group in aglycon moiety, is less

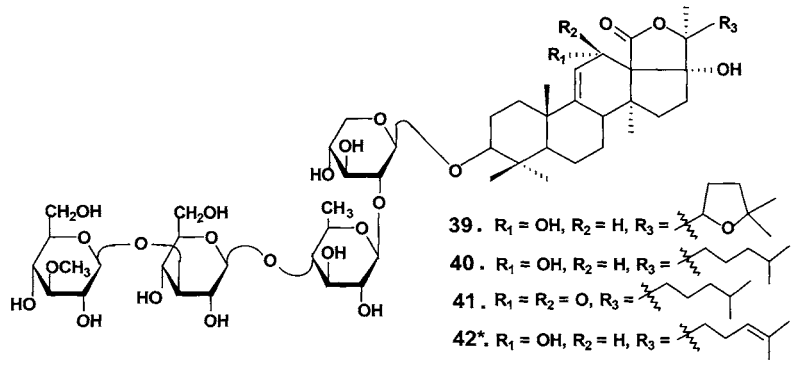
active than stichoposide C (**27**) with the 7(8)-double bond and C-23 acetate.



Kitagawa et al. studied antifungal activities of 27 triterpene glycosides and their derivatives (**35** - **58**) from sea cucumbers [42-45].



*- the structures corrected according with [46]



*- the structure corrected according with [47, 48]

The compounds **35**, **36** and **37** are derivatives of holotoxin A_1 from *Apostichopus* (=*Stichopus*) *japonicus*; **38** is holotoxin B_1; **39** is a desulfated derivative of holothurin A from *Holothuria leucospilota*; **40** is a desulfated derivative of echinoside A from *Actinopyga echinites*; **41** is a 12-oxoderivative of desufated echinoside A. Substance **42** is a desulfated derivative of 24-dehydroechinoside A from *Actinopyga agassizi*; **43** is stichloroside C_2 from *Stichopus chloronotus*; **44** is bivittoside B from *Bohadschia bivittata*; **45** is a desulfated pervicoside B from *Holothuria pervicax*; **46** is a desulfated pervicoside C from *Holothuria pervicax*; **47**, **48** and **49** are derivatives of holotoxin A_1 from *A.* (=*S.*) *japonicus*; **50**, **51**, **52** are bivittosides A, C, and D from *Bohadschia bivittata*; **53** is a 12-oxoderivative of bivittoside D; **54** and **57** are echinosides B and A from *Actinopyga echinites*; **55** is a desulfated derivative of echinoside B; **56** is a product of oxidation of echinoside B at C-12 of aglycon; **58** is a desulfated derivative of pervicoside A from *H. pervicax*. The obtained data are shown in Tables 4 and 5.

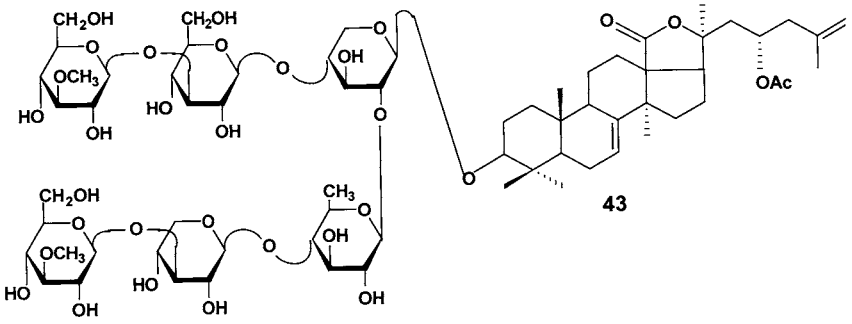
The biological activities depend on the structures of holothurian triterpene glycosides [42-44]. The structures of both an aglycon part and a carbohydrate chain are very important for the antifungal activity. The 18(20)-lactone presence is necessary for the activity, because the derivative **49** without the lactone does not show antifungal properties.

The presence of at least one oxygen-containing functional group, neighbouring an 18(20)-lactone (namely at 12, 16

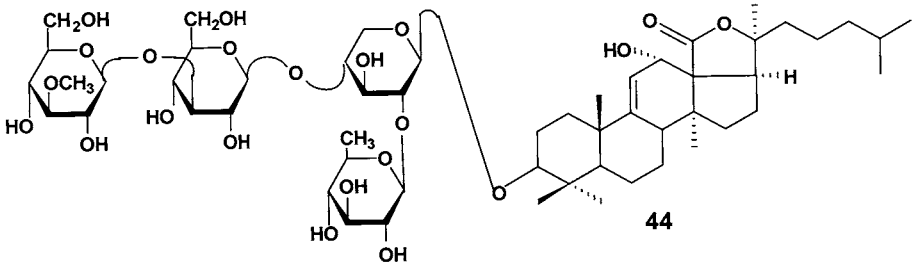
positions), for compounds with a 9(11)-double bound in aglycon moiety also plays an important role. For example, bivittoside C (**51**), lacking such a functional group, has almost no activity [44].

The linear tetrasaccharide fragment in carbohydrate chains is also very important. In fact, the branched tetraoside - bivittoside B (**44**) is less active than the linear tetraosides **30** and **31**. Progenin **36**, obtained from holotoxin A_1 (**33**), has no antifungal activity at concentrations up to 100 μg/ml.

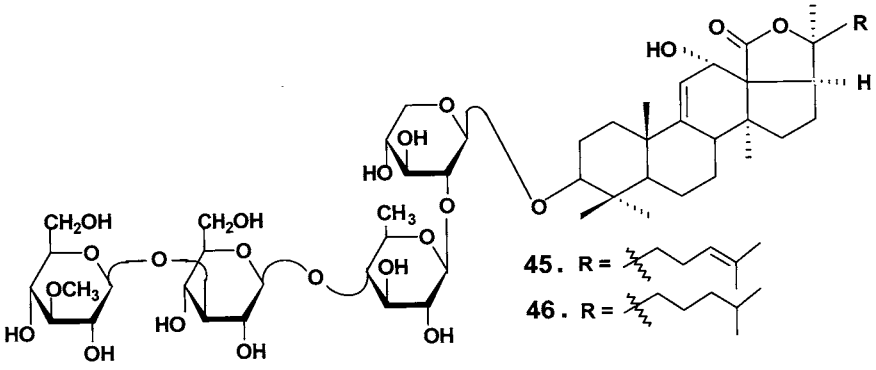
A sulfate group at C-4 of the first xylose residue in a carbohydrate chain does not have significant influence on the biological activities of tetrasaccharide derivatives, as shown by the comparison of activity data on echinoside A (**57**) and its desulfated derivative **40** [43]. However, the sulfate group significantly contributes to antifungal activities of biosides, as shown by comparison of antifungal activities of echinoside B (**54**) and its desulfated derivative [43].



43

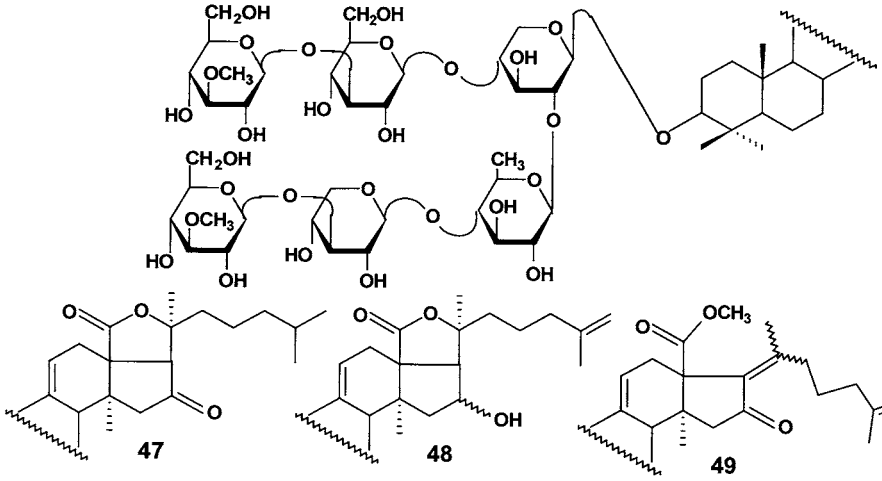


44

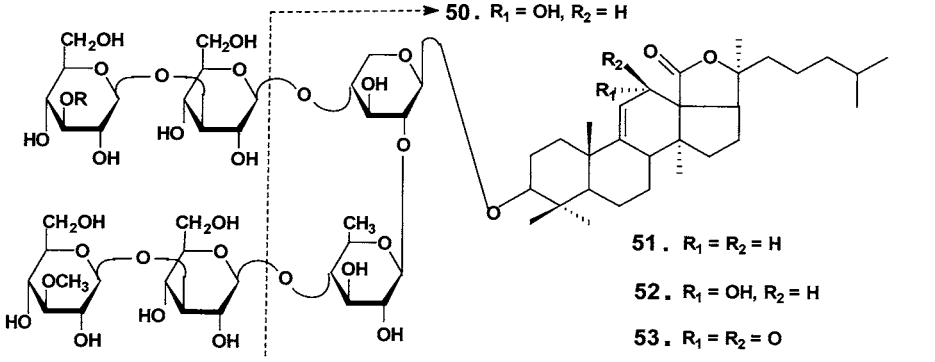


45. R =

46. R =



47 48 49


50. R1 = OH, R2 = H
51. R1 = R2 = H
52. R1 = OH, R2 = H
53. R1 = R2 = O

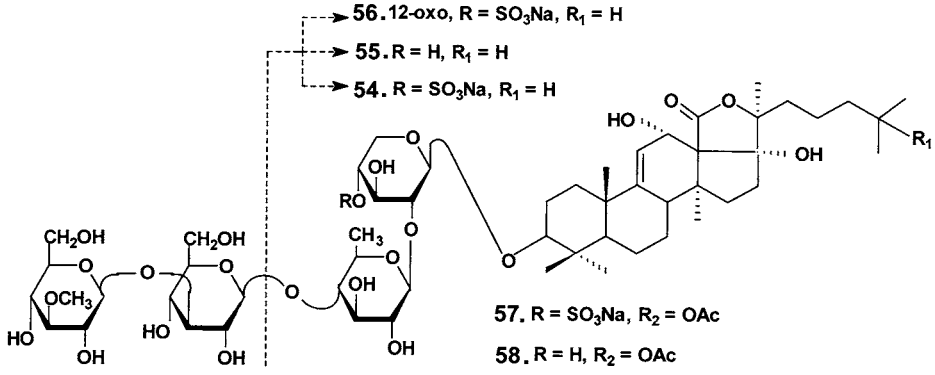

56. 12-oxo, R = SO3Na, R1 = H
55. R = H, R1 = H
54. R = SO3Na, R1 = H
57. R = SO3Na, R2 = OAc
58. R = H, R2 = OAc

Table 4. The antifungal properties of holothuroid glycosides: Minimal inhibitory concentration (μg/ml) (from [43, 44])

Substances	*Aspergillus niger*	*Aspergillus oryzae*	*Penicillium chryzogenum*	*Penicillium citricum*	*Mucor spinescens*	*Cladosporium herbatum*
35	>100	>100	>100	>100	>100	>100
36	>100	>100	>100	>100	>100	>100
33	0.78	1.56	1.56	12.5	>100	12.5
37	3.12	6.25	3.12	12.5	12.5	12.5
38	6.25	12.5	6.25	12.5	25	12.5
39	6.25	12.5	6.25	12.5	12.5	12.5
40	1.56	1.56	3.12	1.56	100	100
41	6.25	6.25	6.25	25	>100	>100
42	1.56	1.56	1.56	1.56	3.12	>100
43	12.5	12.5	6.25	12.5	>100	25
44	12.5	50	12.5	50	>100	>100
45	1.56	3.12	1.56	1.56	6.25	6.25
46	6.25	6.25	3.12	6.25	12.5	12.5
47	3.12	12.5	3.12	12.5	>100	25
48	50	50	50	>100	25	100
49	>100	>100	>100	>100	>100	>100
50	>100	6.25	6.25	>100	>100	>100
51	>100	>100	>100	>100	>100	>100
52	6.25	3.12	1.56	6.25	>100	50
53	25	25	12.5	>100	>100	50
54	3.12	3.12	3.12	3.12	>100	>100
55	25	25	50	>100	>100	>100
56	>100	6.25	>100	>100	>100	>100
57	3.12	3.12	3.12	3.12	50	25
58	12.5	12.5	6.25	6.25	12.5	50

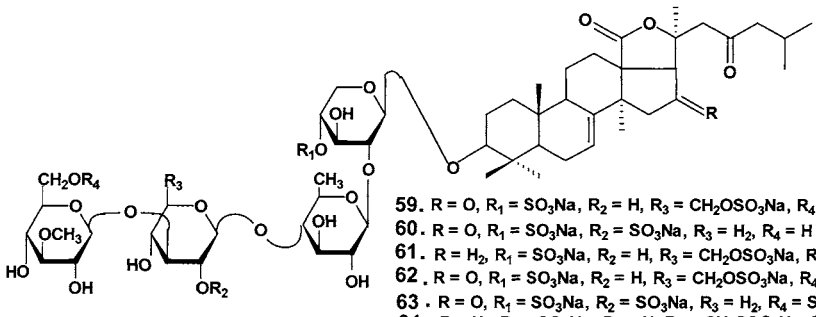
The importance of a linear tetrasaccharide fragment and an 18(20)-lactone for biological activity, the absence of a significant contribution of 4-O-sulfate at first xylose to activities of tetraosides and, in contrast, its important role for activity of biosides were shown for hemolytic action of triterpene glycosides from sea cucumbers [33]. It confirmed a common mechanism of their activity against membranes of both erythrocytes and fungal cells.

Miyamoto et al. have studied the antifungal activity of glycosides and their derivatives from *Cucumaria echinata* [49]. The results are shown in Table 6.

Cucumechinosides A (**59**), B (**60**), C (**61**), D (**62**), E (**63**) and F (**64**) are native glycosides. The substances **67** - **69** are desulfated derivatives of corresponding glycosides, and **65** and **66** are artificial aglycons.

Table 5. The antifungal properties of holothuroid glycosides: Minimal inhibitory concentration (μg/ml) (from [43, 44])

Substances	*Rhodotorula rubra*	*Tryhophyton mentagrophytes*	*Tryhophyton rubrum*	*Candida albicans*	*Candida utilis*
35	>100	>100	>100	>100	>100
36	>100	>100	50	>100	>100
33	25	12.5	12.5	>100	6.25
37	12.5	6.25	<1.56	12.5	3.12
38	12.5	1.56	0.78	6.25	3.12
39	12.5	12.5	6.25	25	12.5
40	3.12	6.25	3.12	50	3.12
41	>100	>100	12.5	>100	12.5
42	3.12	3.12	1.56	12.5	1.56
43	12.5	6.25	6.25	25	12.5
44	>100	>100	50	>100	50
45	3.12	1.56	1.56	6.25	6.25
46	6.25	6.25	6.25	12.5	12.5
47	6.25	6.25	0.78	12.5	3.12
48	>100	50	25	50	25
49	>100	>100	>100	>100	>100
50	>100	>100	25	>100	>100
51	>100	>100	>100	>100	>100
52	6.25	6.25	3.12	12.5	6.25
53	25	25	6.25	>100	12.5
54	12.5	12.5	6.25	>100	12.5
55	100	>100	100	>100	>100
56	>100	>100	>100	>100	>100
57	6.25	6.25	6.25	12.5	6.25
58	100	12.5	12.5	100	100



59. R = O, R_1 = SO_3Na, R_2 = H, R_3 = CH_2OSO_3Na, R_4 = H
60. R = O, R_1 = SO_3Na, R_2 = SO_3Na, R_3 = H_2, R_4 = H
61. R = H_2, R_1 = SO_3Na, R_2 = H, R_3 = CH_2OSO_3Na, R_4 = H
62. R = O, R_1 = SO_3Na, R_2 = H, R_3 = CH_2OSO_3Na, R_4 = SO_3Na
63. R = O, R_1 = SO_3Na, R_2 = SO_3Na, R_3 = H_2, R_4 = SO_3Na
64. R = H_2, R_1 = SO_3Na, R_2 = H, R_3 = CH_2OSO_3Na, R_4 = SO_3Na
67. R = O, R_1 = H, R_2 = H, R_3 = CH_2OH, R_4 = H
68. R = O, R_1 = H, R_2 = H, R_3 = H_2, R_4 = H
69. R = H_2, R_1 = H, R_2 = H, R_3 = CH_2OH, R_4 = H

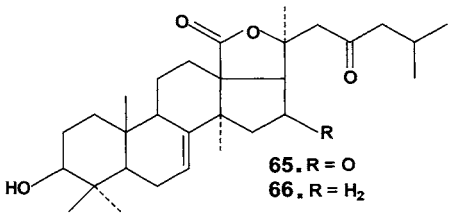


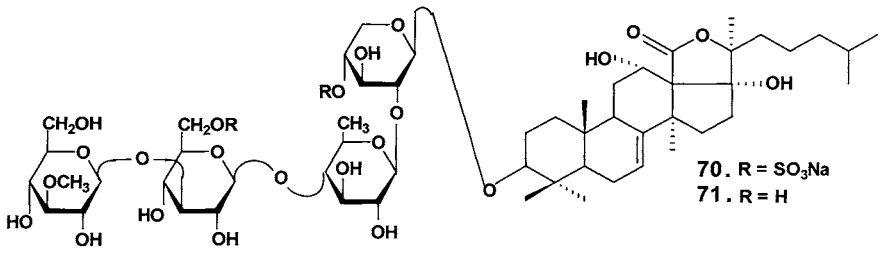
Table 6. Minimal growth inhibitory concentration (μg/ml) for glycoside and their derivatives from *Cucumaria echinata*

Fungi	59	60	61	62	63	64	67	68	69	65	66	33
Trichomonas foetus	-*	-	-	-	-	10	2.5	-	2.5	-	-	1.0
Candida albicans	-	-	-	-	-	-	5.0	-	5.0	-	-	1.0
Trichophyton mentagrophytes	-	-	-	-	-	-	2.5	-	2.5	-	-	1.0
Aspergillus niger (FA9959)	-	-	-	-	-	20	1.0	-	0.5	-	-	0.5
Aspergillus niger (FA24199)	-	-	-	-	-	20	1.0	-	0.5	-	-	0.5
Mucor hiemalis	-	-	-	-	-	-	-	-	-	-	-	-
Penicillum chrysogenum	-	-	-	-	-	-	5.0	-	2.5	-	-	2.5

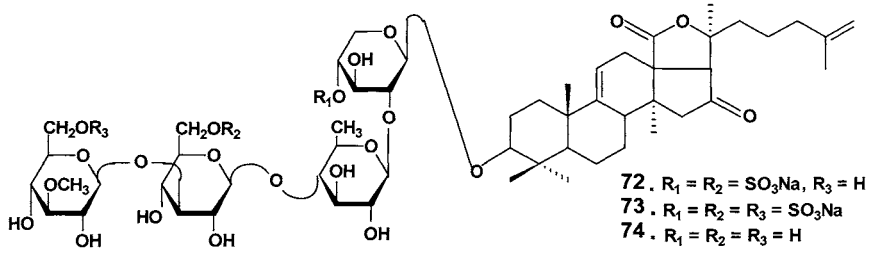
**No activity below the dose of 20 μg/ml

According to these data, only desulfated derivatives **67** and **69** have significant antifungal and antiprotozoan activity. Corresponding genuine glycosides, containing two or three sulfate groups are not active at doses below 20 μg/ml. Of the native glycosides, only cucumechinoside F (**64**) inhibited the growth of *Trichomonas foetus* and *Aspergillus niger* at rather high concentrations. Desulfated derivative **68**, distinguishing from **67** by the structure of a third monosaccharide unit, does not inhibit the growth of microorganisms at a concentration of 20 μg/ml. Aglycons **65** and **66**, obtained by acid hydrolysis of cucumechinosides A and C, also do not have antimicrobial activity. The sulfate group at C-4 of the first xylose does not influence the antifungal activity of tetrasaccharide derivatives. Therefore, the biological activity of native glycosides is greatly decreased by sulfated groups attached to C-6 of glucose or to C-6 of 3-O-metylglucose units or to C-2 of third monosaccharide residue (xylose).

Thus, the presence of the carbohydrate chain in the molecule is necessary for antifungal action. The quantitative and qualitative composition of the chain significantly influences antifungal activity.



Murray et al have studied antifungal activity of patagonicoside A (**70**) from *Psolus patagonicus* and its desulfated derivative **71** against patogenic fungus *Cladosporium cucumerinum* by a bioautographic technique [50]. Patagonicoside A (**70**) was more active than the desulfated derivative **71** showing inhibition zones of 8–19 mm at the tested concentrations (1.5–50 μg/spot). The desulfated derivative was inactive at the lowest concentrations (1.5 and 3 μg/spot) and weakly active (inhibition zones of 5–9 mm) at the highest tested concentrations (6–50 μg/spot). These results suggested that the presence of sulfate groups in the oligosaccharide chain of saponin **70** plays an important role in the antifungal activity.



Hemoiedemosides A (**72**) and B (**73**) from Patagonian sea cucumber *Hemoidema spectabilis* and their desulfated derivative **74** were evaluated for antifungal activity against *Cladosporum cucumerinum* [51]. Glycoside **72** showed inhibition zone of 8–33 mm at the tested concentration (1.5–50 μg/spot), while glycoside

73 differing from **72** in the presence of a third sulfate group at C-6 of terminal 3-O-methylglucose residue is less active than **72** (4–24 mm). The desulfated derivative **74** is less active than both of the glycosides (0 – 12 mm). Hence sulfate groups at C-4 of fist monosaccharide residue (xylose) and at C-6 of third monosaccharide residue (glucose) make a significant positive contribution into an antifungal activity while sulfate at C-6 of terminal monosaccharide residue (3-O-methylglucose) makes a negative contribution.

Antiprotozoal activity

"Holothurin" is active *in vitro* against *Amoeba proteus* [24], holotoxins from *Apostichopus japonicus* are active against *Trichomonas vaginalis* [52]. The data on antiprotozoal activity of glycosides of *Cucumaria echinata* [49] were discussed above. These data were correlated with antifungal activities of the glycosides.

Rats, intraperitoneally inoculated with crude holothurin and previously or simultaneously infected by *Tripanosoma lewisi* had lower parasitemia than in the controls [53]. But, on the contrary, a higher level of parasitemia was observed in rats treated after infection. Sen & Lin confirmed these results in mice inoculated with *Trypanosoma duttony* [54, 55].

Cytotoxic and antitumor activities

The action of triterpene glycosides from sea cucumbers on early embryogenesis of sea urchins is an indication of their cytotoxic action. Ruggierri & Nigrelli showed "holothurin" and "holothurin A" induced anomalies in the embryonic growth of the sea urchin *Arbacia punctilata* [56]. Holotoxin A_1 from *Apostichopus* (=*Stichopus*) *japonicus* [57, 38], "cucumarioside C" from *Eupentacta* (=*Cucumaria*) *fraudatrix* [58], the glycosides from *Holothuria mexicana* and cucumarioside G_1 (**7**) from *E. fraudatrix* [59] act analogously on fertilized eggs of the sea urchin *Strongylocentrotus intermedius*.

The dependence of cytotoxic action of triterpene glycosides from sea cucumbers on their structures has been studied [60]. The data are shown in Table 7.

Stichoposide A (**26**), with a bioside carbohydrate chain, is less active among the studied glycosides. Stichoposide C (**27**), a quinovose-containing hexaoside, is most active. Stichoposides D (**28**) and E (**29**) having no a quinovose residue are less active than **27**. Holotoxin A_1 (**33**), distinguished from stichoposide C (**27**) by aglycon structure, is slightly less active than **27**.

Table 7. Minimal concentrations of glycosides, stopping the division of fertilized eggs of the sea urchin *Strongylocentrotus intermedius* (μg/ml)

Substance	ED_{100}*	Substance	ED_{100}*
26	3.1	28	1.6
27	0.8	29	1.6
33	1.5		

*Minimal concentration stopping the division on zygote stage

Thus, the relationship between structures and activities found using sea urchin developing embryos is analogous with that of antifungal activity of the glycosides studied, but it is less distinct.

Frondoside A (**2**) from *Cucumaria frondosa* causes embryotoxic effect upon sea urchin developing embryos. EC_{100} and EC_{50} are 3.12 and 0.98 μg/m respectively [28].

Monosulfated pentaoside cucumarioside A_2-2 (**18**), and trisulfated pentaoside cucumarioside A_7-1 (**21**) with the same aglycons isolated from *Cucumaria japonica* showed cytotoxicity in a sea urchin *Strongylocentrotus nudus* embryo development test with EC_{50} values of 0.3 and 1.98 μg/ml. It is interesting that the trisulfated saponin was less active than the monosulfated one, while the pure aglycon was absolutely inactive in this test [61].

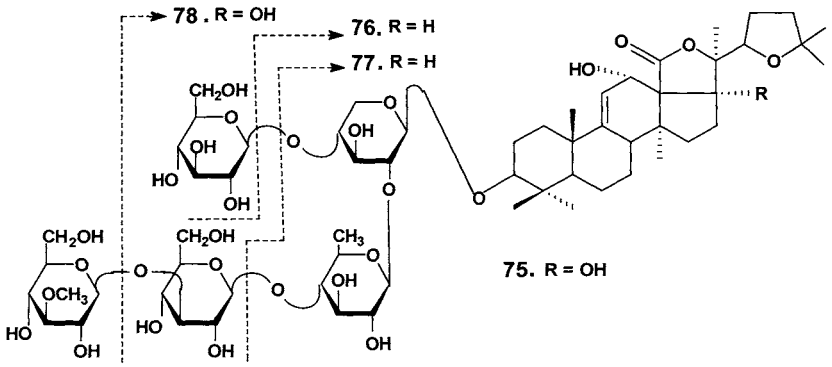
Zootoxicity of hemoiedemosides A (**72**) and B (**73**) from *Hemoiedema spectabilis* and their desulfated derivative **74** were evaluated using the brine shrimp *Artemia salina* larvae mortality bioassay [51]. Hemoiedemoside A (**72**) showed a noteworthy toxicity in this assay (LC_{50} 47.5 ppm). Hemoiedemoside B (**73**) was 2 times less active (LC_{50} 47.5 ppm) than glycoside **72** and nearly 10 times more active than the desulfated derivative **74** (LC_{50}

424.5 ppm). These results correlate with data concerning antifungal activity.

Antitumor action of the triterpene glycosides of sea cucumbers was discovered by Nigrelli [23]. He showed hypodermic injection of "holothurin" solution inhibited growth of tumor cells (Sarcoma-180) and induced regression of the tumor. Injection of ascytic tumor Krebs-2 cells treated with "holothurin" into healthy mice did not induce a marked tumor growth during 80 days [62]. "Holothurin", at the concentration 5 μg/ml, inhibited the growth of epidermal carcinoma (KB) tumor cells [63]. Cairns & Olmsted found significant activity of crude holothurin against mouse sarcoma 180 used in the form of ascytic tumor, but no effect was obtained on melanoma B 16 [64]. Pettit et al. (1976) showed the purified fractions of triterpene glycosides from *Stichopus chloronotus* and *Thelenota ananas* inhibited the development of lymphocyte leukemia P-388, and the so-called "actinostatin" from *Actinopyga mauritiana* had an inhibitory effect against cultures of KB cells and lymphoidal leukemia L 1210 [65]. The antitumor activity of glycoside fractions from sea cucumbers, collected near the Great Barrier Reef (Australia) and Western Samoa, was studied by Kuznetsova et al. [66] Glycosides from 19 species of the families Holothuriidae and Stichopodidae inhibited the growth of Sarcoma-37 cells at concentrations from 6.2 to 100 μg/ml *in vitro*.

Stichoposide A (**26**), C (**27**), D (**28**) E (**29**) affect survival of Ehrlich tumor cells. Minimal effective doses of glycosides ranged from 3.1 to 12 μg/ml. Stichoposide A, with two monosaccharide units, and stichoposide E, with the xylose residue as second monosaccharide unit, were less active [60].

Some triterpene glycosides from holothurians belonging to the family Stichopodidae show also antitumor activities [67, 60]. Holotoxin A_1 (**33**) from *Apostichopus* (=*Stichopus*) *japonicus* inhibited the growth of the solid form of Ehrlich tumor and Sarcoma-37 in mice on 37-65 and 13-53 %, respectively, at doses of 1.25 and 2.50 mg/kg. Triterpene glycosides from *Holothuria forskali* have cytotoxic activities against tumor cells [68].



Holothurinosides A (**75**), C (**76**) and D (**77**) are native substances, whereas (**78**) – is a derivative, obtained by partial acid hydrolysis of holothurinoside A. The data on cytotoxic activity against tumor cells of these glycosides and their derivatives are shown in the Table 8.

Table 8. Cytotoxic activity of holothurinosides (IC_{50} values(μg/ml)) against tumor cell growth *in vitro*

Tumor cell culture	75	76	77	78
P 388	0.46	0.34	2.00	2.00
A 549	0.33	0.16	2.50	5.00
HeLa	0.86	0.47		
B-16	0.71	0.93		

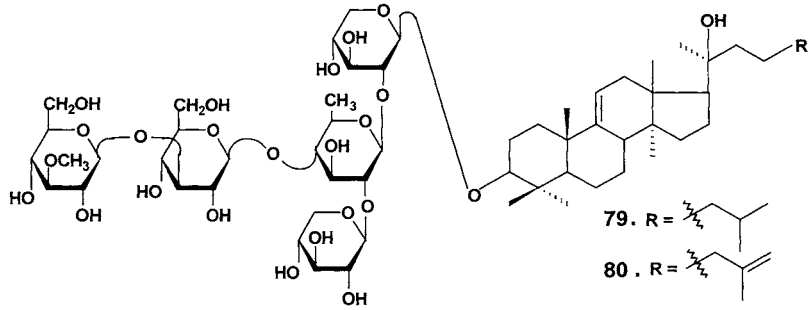
These data show a great significance of linear tetrasaccharide fragment in carbohydrate chains for cytotoxicity. Holothurinoside A (**75**), having a pentasaccharide carbohydrate chain with a linear tetrasaccharide fragment, and holothurinoside C (**76**), having a linear tetrasaccharide chain, have approximately equal and highest activities of all substances of the series studied. Bioside holothurinoside D (**75**) is the least active. It is interesting that the tetrasaccharide derivative **76**, with a branched carbohydrate chain is less active when compared with the bioside. Antifungal activity [42] depends on the architecture of the carbohydrate chain, particularly of a linear tetrasaccharide fragment. The number of monosaccharide units is not a sole important factor. This is true also for cytotoxic activities against tumor cells [68]. Triterpene

glycosides from *Cucumaria echinata* have cytotoxic activities against tumor cells L 1210 and KB [49] (Table 9).

Table 9. Cytotoxic activity of triterpene glycosides from *Cucumaria echinata* (IC50 (μg/ml)) against L1210 and RB cell growth *in vitro*

Tumor cell culture	59	60	61	62	63	64	67	68	69
L 1210	1.7	2.9	2.8	8.4	20.0	2.7	0.34	0.32	0.26
KB	4.0	6.3	4.0	7.6	36.0	3.6	1.2	0.7	1.1

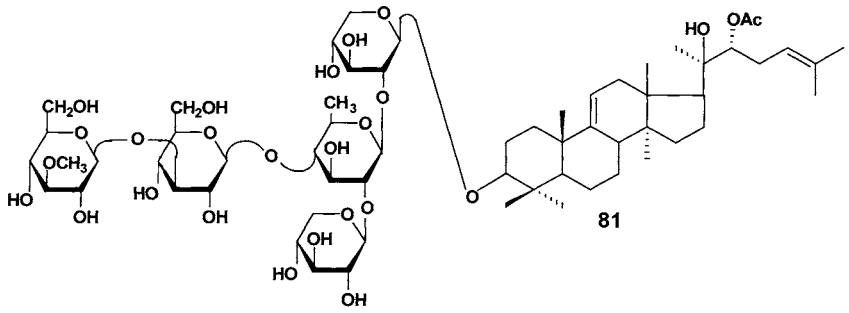
These data show that desufated derivatives **67**, **68** and **69** are about an order of magnitude more active than the native glycosides. It is interesting that glycosides **59**, **60** and **61**, with no sulfate groups at C-6 of terminal 3-O-metylglucoses, are more active than substances **62**, **63** and **64** with such groups.



DS-penaustrosides A (**79**) and B (**80**) revealed cytotoxic activities against murine lymphoma L1210 and human epidermoid carcinoma KB cell lines [IC_{50}=0.12 (L1210) and 0.62 (KB) μg/ml for **79**; 2.1 (L1210) and 4.6 (KB) μg/ml for **80**] [69]. Unfortunately, the authors did not show the data on activities of relative holostane derivatives in the same experimental conditions.

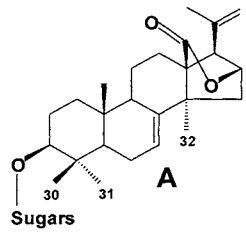
Thus, the data on antitumor activity of glycosides of sea cucumbers correlate, in general, with data on antifungal and hemolytic effects and also cytotoxic activities against sea urchin fertilized eggs.

Cucumariosides A_3 (**20**) and A_6-2 (**19**) from *Cucumaria japonica* showed IC_{50} = 1 μg/ml when tested *in vitro* against standard mouse and human tumoral cell lines (P-388, Schabel, A-549, HT-29 and Mel-28) [70].

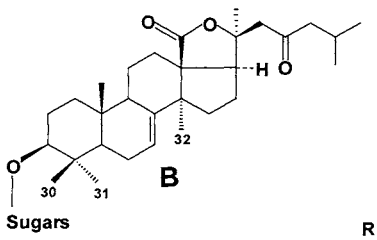


81

The desulfated derivative **81** obtained from frondoside C isolated from *Cucumaria frondosa* was found to demonstrate cytotoxic activity (IC_{50} = 1 μg/ml) when tested *in vitro* against several standard mouse and human tumoral cell lines (P-388, Schabel, A-549, HT-29, and Mel-28) [71].

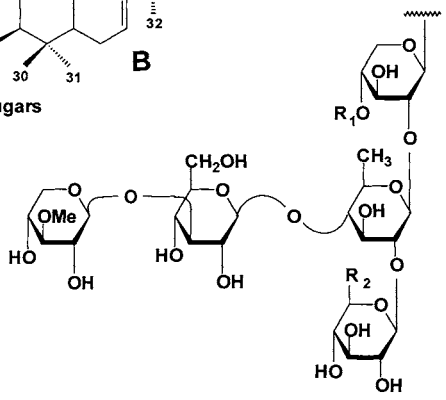


A

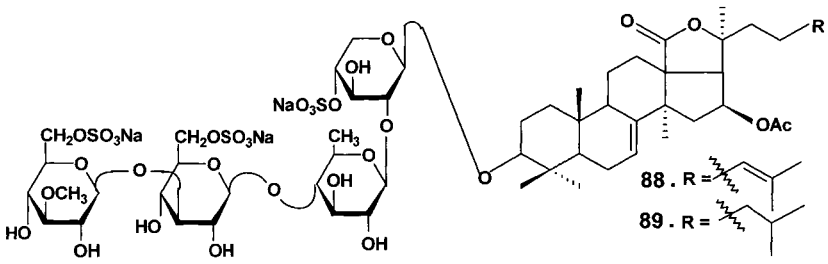


B

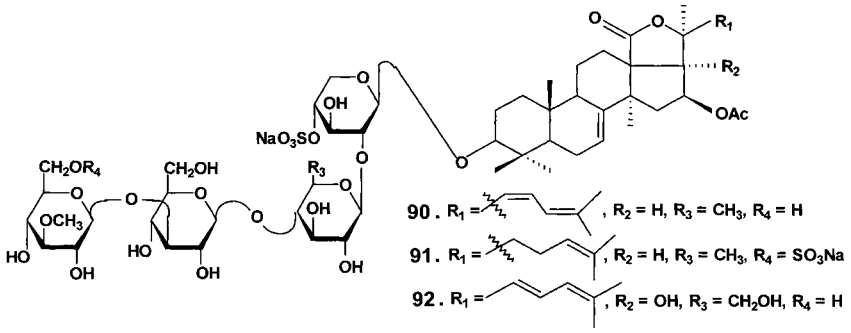
	Aglycon	R_1	R_2
82	A	SO_3Na	CH_3
83	A	SO_3Na	CH_2OH
84	B	SO_3Na	CH_2OH
85	A	H	CH_3
86	A	H	CH_2OH
87	B	H	CH_2OH



Three monosulfated pentaoside glycosides isolated from the Far Eastern sea cucumber *Pentamera calcigera*, calcigerosides B (**82**), C_1 (**83**) and C_2 (**84**) were not active *in vitro* against standard mouse and human tumor cell lines (P-388, A-549, HT-29, and Mel-28) while their desulfated derivatives (**85**, **86** and **87** respectively) showed moderate cytotoxic activity (IC_{50} = 5 μg/ml) [72].



Two trisulfated tetraoside glycosides isolated from the Antarctic sea cucumber *Staurocucumis liouvillei*, liouvillosides A (**88**) and B (**89**) showed little or no cytotoxicity at concentration ranging from 6.25 to 50 μg/ml against Vero cells within 8 h of cell exposure to the compounds but both saponins were cytotoxic following prolonged incubation periods [73].



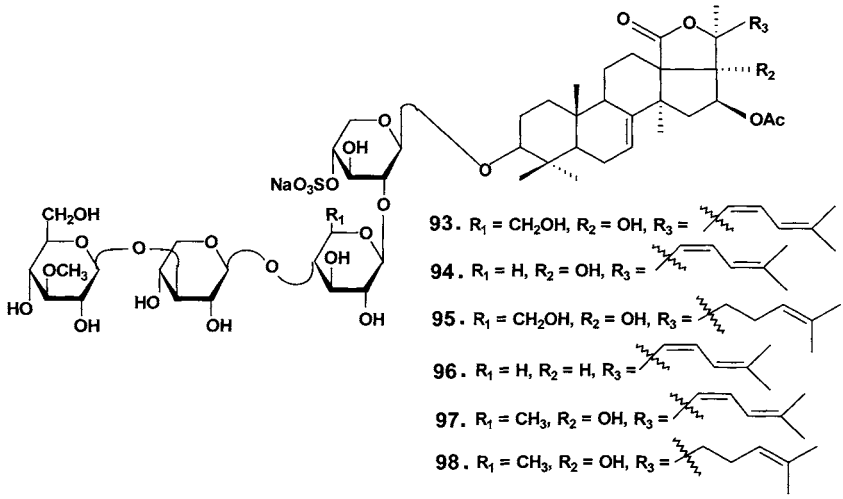
Intercedensides A (**90**), B (**91**) and C (**92**) from *Mensamaria intercedens* collected in South China Sea showed cytotoxicity against 10 human tumor cell lines with ED_{50} in the range 0.6–4.0 μg/ml (Table 10) [74]. Glycoside **90** exibited antineoplastic activity *in vivo* against mouse Lewis lung cancer and mouse S 180 sarcoma.

Table 10. ED_{50} values of substances 90, 91, and 92 against human tumor cells *in vitro* (μg/ml)

Substance	A 549	MCF-7	IA9	CAKI-1	U-87-MG	PC-3	KB	KB-VIN	SK-MEL-2	HCT-8
90	1.7	3.5	0.96	1.2	4.0	3.2	3.5	3.5	3.6	2.4
91	0.71	1.4	0.61	0.77	1.9	1.5	1.9	2.0	1.9	1.2
92	1.6	3.7	1.5	1.5	3.6	3.0	3.6	3.8	3.4	2.1

It seems to be uncommon that the substance **91** having a sulfate group at C-6 of the terminal monosaccharide residue was the most active, though it may be explained by negative contribution of a dienic group in the aglycon side chain in the glycosides **90** and **92** and glucose as the second monosaccharide residue in the carbohydrate chain of the glycoside **92**.

Intercedensides D (**93**), E (**94**), F (**95**), G (**96**), H (**97**) and I (**98**) were isolated from the same sea cucumber [75] and glycosides **93** – **97** were tested for *in vitro* cytotoxicity against 10 human tumor cell lines. The glycosides showed moderate cytotoxic activities without significant differences between each other. These data seems to be uncommon, inasmuch as many of these glycosides have characteristic structural features that have been shown to be important for cytotoxicity. Nevertheless, the glycosides **93** and **97** differing only in the structure of their second monosaccharide residue (glucose and quinovose respectively) have some differences in cytotoxicity while quinovose-containing glycoside **97** is more active. The saponins **93** and **95** differing only in structure of their aglycon side chain also have differences in cytotoxicity while glycoside **95** containing diene system in the aclycon side chain is less active. The substance **98** was not tested. The ED_{50} are values of these compounds are presented in the Table 11.



Phylinopsides A (**99**) and B (**100**) isolated from *Pentacta quadrangularis* were tested for *in vitro* cytotoxicity against ten human tumor cell lines [76]. These substances showed significant activities against all tumor cell lines. ED_{50} values are presented in the table 12. The glycoside **100** having an additional sulfate group at C-2 of the third monosaccharide residue (xylose) and the 25(26)-termimal double bond in the aglycon side chain was more active. Moreover phylinopside A (**99**) significantly inhibited the proliferation, migration, and tube formation of human microvascular endothelial cells (HMECs) in a dose-depend manner, with average IC_{50} values of 1.4±0.17, 0.89±0.23, and 0.98±0.19 μM, correspondingly.

Table 11. ED_{50} values of substances 93, 94, 95, 96 and 97 against human tumor cells *in vitro* (μg/ml)

Cell line	Substance				
	93	94	95	96	97
A549	1.8	1.4	1.7	1.6	1.4
MCF-7	2.4	1.4	2.1	2.0	1.8
1A9	2.4	1.7	1.7	1.9	0.96
CAKI-1	>5	1.6	1.7	3.8	1.0
U-87-MG	4.1	2.1	3.3	3.3	3.2
PC-3	3.3	1.7	2.3	2.0	2.2
KB	3.7	1.9	3.2	3.3	3.0
KB-VIN	4.3	2.0	3.2	3.9	3.7
SK-MEL-2	4.2	1.6	2.1	2.4	2.2
HCT-8	2.9	1.1	1.9	1.8	1.9

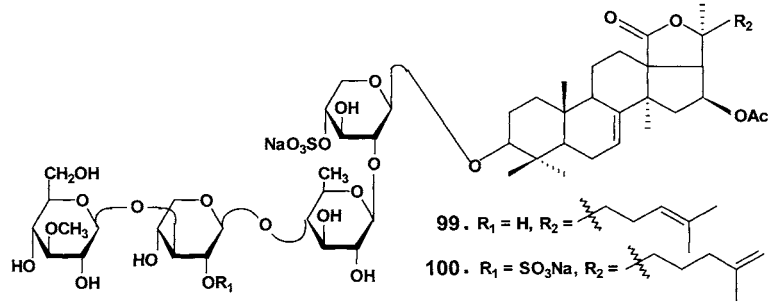


Table 12. ED_{50} values of substances 99 and and 100 against human tumor cells *in vitro* (μg/ml)

Substance	CAK1	HOS	KB-VIN	KB	SK-MEL-2	U87-MG	HCT-8	1A9	A549	PC3
99	3.00	1.80	3.30	3.50	3.20	3.20	1.70	1.79	1.70	1.70
100	2.00	1.10	3.00	3.00	2.60	2.40	0.93	0.90	0.75	1.70

Neurotoxic properties of sea cucumber glycosides

Friess et al. showed "holothurin A" irreversibly blocks neuromuscular transmission in bullfrog sciatic nerve fibers and phrenic nerve-diaphragm preparation of rat and cat [77, 78]. "Holothurin A" is comparable with such blocking agents as procaine, phisostigmine, cocaine in effectiveness of action but its effect is irreversible at 10^{-5} M. This toxin blocks twitch response through direct muscle stimulation as well as indirect stimulation of nerve. At very low concentrations (10^{-7} M) the effect is reversible. The irreversible effect of "holothurin" on mammalian neuromuscular synapse chemoreceptors is countered by trace concentrations (10^{-9}–10^{-10} M) of classical protectors - physostigmine and neostigmine [79, 80]. Friess et al. carried out a comparative study on neurotoxic action of "holothurin A" and its desulfated derivative [81-84]. While acting on different periferic neuromuscular receptors, both substances caused irreversible inactivation of excitement *in vitro* and *in vivo*. The active concentration of holothurin A was of an order of a magnitude lower than that of its desulfated derivative. Friess et al. considered this effect due to a negative charge of "holothurin A" [83, 84].

The action of "holothurin" on the membrane potential and conductivity of giant squid axon membrane was studied by De Groof & Narahashi [85]. The action of holothurin on the outside of the intact axon at a concentration of $2x10^{-4}$ M caused irreversible membrane depolarization, while the membrane potential approached zero. Elimination of Na^+ from outer side of membrane or from both sides led to partial depolarization. A possible mechanism, based on the biomembrane depolarization by glycosides, was proposed to be connected with the increasing membrane permeability for Na^+ ions.

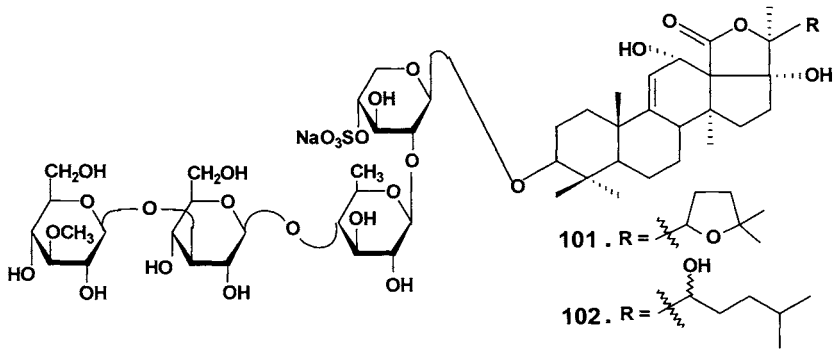
"Holothurin" ($2x10^{-4}$ M) irreversibly blocks a neural and direct response of stimulated monocellular electroplax preparation of *Electrophorus electricus* [86]. It also produced irreversible depolarization in a monocellular electroplax preparation of *Electrophorus electricus* [86]. These effects on the resting potential have been attributed to an initial K^+ efflux which then decreased steadily.

Ruggieri & Nigrelli indicated that "holothurin A" increased the conduction time through the atrioventricular node and decreased the automatic rate of spontaneously beating Purkinije cells [87]. They suggested that the "holothurin A" might be used for the treatment of sinus node at arhythmia and tachicardia. A griseogenin-derived saponin (probably holothurin A_1 (**77**)) from *Holothuria floridana* has a powerful, dose-related hypothermic activity [88].

Mechanisms of action of these effects have not been studied. They may be related to the toxic membrane properties of the glycosides and their destructive interaction, observed in excitable nerve and muscle tissues [89].

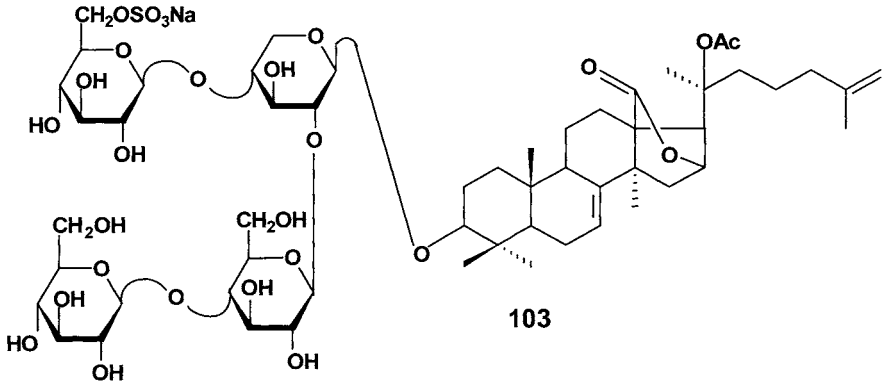
Inhibition of Na^+,K^+ -ATPase of rat brain

The dependence of Na^+,K^+-ATPase inhibition upon structures of triterpene glycoside at glycoside concentration of 10^{-4} M has been studied [90]. The glycosides – bivittoside A (**50**) and B (**44**), holothurin A (**101**) and A_2 (**57**), cucumariosides A_2-2 (**18**) and G_1 (**7**), stichoposide C (**27**), apostichoposide C (**43**), thelenotoside A (**30**), and holotoxins A_1 (**33**) and B_1 (**38**) inhibit Na^+,K^+-ATPase activity by 40–60 %.



The stichoposides D (**28**), E (**29**) and thelenotoside B (**31**), with no quinovose as second monosaccharide unit, were significantly less effective and inhibited the enzyme activity by 19–27 %. Holothurin A_1 (**102**) from the *Holothuria floridana*, containing a hydroxyl group on the side chain of aglycon, inhibited activity by

only 16 %. Apparently the absence of quinovose as the second monosaccharide unit for **28**, **29** and **31** or the presence of an additional hydroxyl group in the side chain of aglycon of holothurin A_1 make the penetration of glycosides into the membrane more difficult and, therefore, decrease the effect of the glycoside on Na^+,K^+-ATPase. Inhibition of Na^+,K^+-ATPase activity with echinoside A (holothurin A_2) (**57**) and its derivatives were also reported by Kitagawa [43]. The absence of a sulfate group and decreased number of monosaccharide units in glycosides sharply diminished the activity.



Psolusosides A (**16**) and B (**103**) isolated from *Psolus fabricii* inhibited rat brain Na^+,K^+-ATPase with I_{50} values of 1×10^{-4} M and 3×10^{-4} M respectively [91]. Glycoside **16** stimulated [^{3}H]ATP binding to Na^+,K^+-ATPase, weakly increased [^{3}H]ouabain binding to the enzyme, and inhibited K^+-phosphatase activity to a smaller degree than the total reaction of ATP hydrolysis. In contrast, glycoside **103** decreased [^{3}H]ATP binding to Na^+,K^+-ATPase, and had no effect on [^{3}H]ouabain binding to the enzyme. K^+-phosphatase activity was inhibited by glycoside **103** in parallel with Na^+,K^+-ATPase activity. The fluorescence intensity of tryptophanyl residues of Na^+,K^+-ATPase was increased by **16** and decreased by **103** in a dose dependent manner. The excimer formation of pyrene, a hydrophobic fluorescent probe, was decreased by **16** only. The different characteristics of inhibition mode for these substances may be explained by significant differences in their chemical structures and distinctive affinity to membrane cholesterol. Indeed,

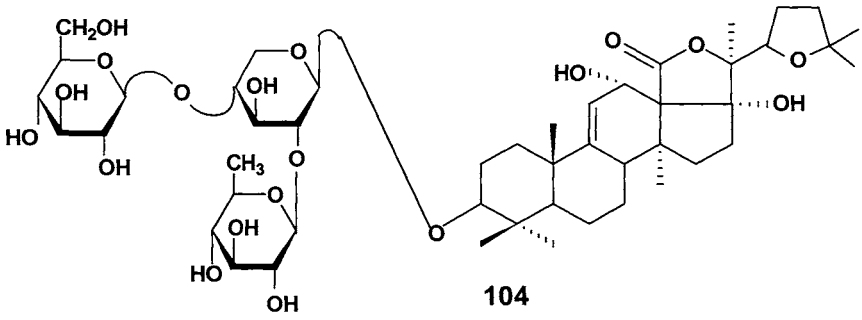
the glycoside **16** caused quick K^+ loss from human erythrocytes at 1×10^{-6} M while **103** was not active even at 1×10^{-5} M.

Psolusoside B (**103**) possesses the following structural features, which resulted in the decrease of membranolytic effect [9, 21, 34]: (1) the presence of a 18(16)-lactone instead of 18(20)-lactone in the aglycon moiety; (2) the presence of a sulfate group at C-6 of terminal glucose residue in the carbohydrate chain; (3) the absence of linear tetrasaccharide fragment which is important for the biological activities of holostane glycosides; (4) the presence of a glucose residue instead of quinovose. As a result, the glycoside **103** did not inhibit the activity of Na^+,K^+-ATPase by changing the state of lipid environment of the enzyme, probably because of its inability to form a complex with cholesterol, but it possibly influenced the protein properties.

Contraceptive activity of sea cucumber triterpene glycosides

Hypodermic injection into rats of a mixture of saponins from *Apostichopus japonicus* (at doses 0.1 and 1 mg/kg), in contrast with holothurin A (**101**), causes a contraceptive effect in 50 and 28 % cases, respectively [92].

Inhibition of Chemokine Receptor-5 (CCR 5)



104

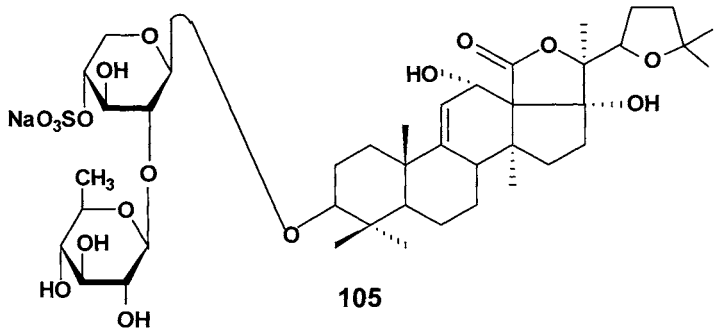
New saponin **104** and known bivittoside D (**52**) isolated from sea cucumber or a mixture of different sea cucumbers (not correctly identified) exhibited inhibitory activity (K*i*) of 30 and 5 μM, respectively, in a chemokine receptor subtype 5 (CCR 5) assay

[93]. The CCR 5 receptor is the most commonly used receptor by HIV-1 strains and is thought to be important in viral transmission. Glycoside **104** having only three monosaccharide residues was significantly less active than glycoside **52**, a hexaoside possessing by a linear tetrasaccharide fragment in the carbohydrate chain. These data correlate with dependence of cytotoxic action of sea cucumber triterpene glycosides upon their structures. The inhibitory doses are also comparable with cytotoxic ones.

Mitogenic activity

The crude "holothurin" from *A. agasssizi* stimulated hemopoesis in bone marrow of frog [24]. A minimal immunomodulatory dose (0.05 μg/kg) of crude cucumarioside from *Cucumaria japonica* induced delay of rat liver cell mitosis 28 to 32 hours after hepatoectomy but after 40 to 44 hours induced the compensatory increase in mitotic activity [94]. The authors concluded that the triterpene glycosides affect the regulation of proliferative processes.

Holothurin A (**39**), holothurin B (**105**), holothurin A_2 (**57**) isolated from sea cucumbers belonging to the family Holothuriidae and holotoxin A_1 (**33**) from *Apostichopus japonicus* stimulated both the incorporation of ^{3}H-Thd into mice spleen T- and B-spleenocytes *in vitro* in dose dependent manner in concentration range of 0.01–01 μg/ml and amplified the effect of spleenocytes blast-transformation induced by ConA or phytohemagglutinin [95]. Nevertheless, the preparation Cumaside based on complex of monosulfated glycosides from *Cucumaria japonica* with cholesterol did not so significantly affect proliferative activity of human blood lymphocytes compared to phytohemagglutinin. Namely Cumaside stimulated lymphocyte proliferation only in 1.2–2 times at concentration of 0.1 and 1 μg/ml [96, 97].



105

Immunomodulation properties of sea cucumber glycosides

The effects of sea cucumber triterpene glycosides injected into an organism at low concentrations are of scientific and applied interest. For instance, the fraction of glycosides from *Actinopyga agassizi* in experiments *in vitro* at concentrations of 0.1–6.0 μg/ml caused a stimulatory effect for leukocyte migration. This substance stimulated phagocytosis of *Staphylococcus aureus* by human blood leukocytes at concentrations of 4–100 μg/ml [98].

Sedov et al. found that intraperitoneal injection of the crude cucumarioside from *Cucumaria japonica* at doses 0.4–40 μg/mouse increased a resistance of mice to *Salmonella typhimurium* [99]. Moreover, the phagocytic activity of cells from mouse peritoneal exudate increased after an injection of the cucumarioside. Cucumariosides also had a nonspecific protective action against a series of gram negative microorganisms - representatives of both enterobacteria of the genera *Escherichia, Proteus, Salmonella* and gram negative coccies of the genus *Neisseria* [100]. Cucumariosides from *C. japonica* showed some adjuvant action, inducing the increase of antibodies in corpuscle whooping-cough vaccine and increasing the protective effect of the vaccine [101]. Cucumariosides demonstrate a restorative effect in mice with acquired immunodeficiency induced by radiation [102].

Cucumariosides from *C. japonica* showed also antiviral activity [103, 104]. Cucumariosides demonstrated a restorative effect in mink infected by Aleutian disease virus [103]. The authors suggested that the effect of the cucumariosides may be related to the increased interaction of T- and B- lymphocytes and humoral

immune response of the animals, favorably influencing proliferation of stem cells.

The immunomodulatory action of glycosides from *Cucumaria japonica* has an applied significance because the glycoside fraction from *Cucumaria japonica* is not mutagenic [27]. These glycosides were approved for veterinary use in Russia [103].

Immunomodulatory properties of cucumariosides A_2-2 (**18**), A_4-2 (**13**), A_3 (**20**), A_6-2 (**19**), A_7-1 (**21**) isolated from *Cucumaria japonica* as well as the desulfated derivatives **22** and **23** obtained from saponins **18** and **13** were also studied [105]. Extremely low doses (about 0.02 μg/mouse) of cucumariosides significantly increased mouse peritoneal macrophage lysosomal activity *in vitro*. Monosulfated cucumariosides A_2-2 and A_4-2 were among the most active compounds, while desulfation of their carbohydrate moiety completely abolished this activity. Both polysulfated and desulfated saponins lacked stimulatory properties. Hence the number and position of sulfate groups in the glycoside molecule were important to exert maximal stimulation of mouse peritoneal macrophages. Indeed, cucumariosides A_2-2 and A_4-2 containing only one sulfate group at C-4 of the xylose residue, were significantly more potent than glycosides containing an additional sulfate group at C-6 position of the glycose (cucumarioside A_3), or an additional sulfate at C-6 of terminal 3-O-methylglucose (cucumarioside A_6-2). Moreover cucumarioside A_7-1 having three sulfate groups showed dose-dependent immunosuppressory properties. In contrast to the *in vivo* effects, the studied triterpene glycosides exerted immediate and profound inhibition of latex bead phagocytosis by human granulocytes and LPS-induced TNF-α production by human monocyte/macrophages *in vitro*. It was also found that cucumarioside A_2-2 (**18**) showed more than 2-fold stimulation of lysosomal activity and induced a rapid short term reversible increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in mouse macrophage *in vitro*. [61]. Because macrophage lysosomal activity stimulation occurred at the same subtoxic concentration range (picograms to nanograms per milliliter) of cucumarioside A_2-2 that reversibly increased $[Ca^{2+}]_i$ it is quite possible that this stimulatory effect was Ca^{2+}-dependent and the stimulation of lysosomal activity was implemented via the regulation of cellular membrane permeability

for calcium ions. The toxic concentration of the saponin triggered a rapid irreversible prolonged lifting of the basal Ca^{2+} level into macrophages corresponding to reducing in lysosomal activity. The reversibility of rapid short term increase in cytosolic Ca^{2+} may be explained by a direct enhancement of the Na^{+}-Ca^{2+} exchange induced by the saponin (because of its ability to form non-specific ion channels) and secondary augmentation of the Na^{+}-Ca^{2+} exchange due to an increased $[Ca^{2+}]_i$ as it was suggested for the plant saponin, digitonin, applied at sub-skinning concentrations [106]. Therefore, immunomodulatory properties of cucumarioside A_2-2 may be caused by its membranotropic properties.

In order to decrease hemolytic properties of glycosides, a special preparation, containing some glycosides of *Cucumaria japonica* and cholesterol and having immunomodulatory properties was created and named "Cumaside" [96, 97]. The influence of Cumaside on mouse macrophages in low doses was accompanied by more then two-fold stimulation of lysosomal activity. This preparation was found to increase significantly the host resistance against bacterial infections elicited by various pathogens. Cumaside stimulated phagocytosis, reactive oxygen species (ROS) formation, IL6 and TNF-α production in lymphocytes, increased the number of antibody producing cells and amplified the expression of several cell surface molecules (CD3, CD4, CD8) in the limphocytes preliminary cultured with hydrocortisone. At the same time, the preparation did not affect the delayed-type hypersensitivity, proliferate activity of lymphocytes, cytotoxic activity of NK-cells and cytokine IFNγ and IL12p70 release.

Frondoside A (**2**), a major triterpene glycoside from the North Atlantic commercially harvesting sea cucumber *Cucumaria frondosa* also possesses strong immunomodulatory properties in subtoxic doses [28, 107]. Frondoside A stimulates lysosomal activity of mouse macrophages *in vivo* at a maximal effective stimulatory dose of 0.2 μg/mouse. The stimulatory effect of one injection of frondoside A is maintained over 2 weeks. Frondoside A exhibits 30% stimulation of lysosomal activity of mouse macrophages *in vitro* at the effective concentrations of 0.1–0.38 μg/ml. Frondoside A stimulates phagocytosis of bacteria *Staphylococcus aureus* by macrophages *in vitro* at a maximal

effective concentration of 0.001 μg/ml. Frondoside A stimulates ROS formation in macrophages *in vitro* at maximal effective concentration of 0.001 μg/ml. Frondoside A stimulates the increase in the number of antibody plaque-forming cells (normally B-cells in spleen) *in vivo* with maximal stimulatory effect at concentration of 0.2 μg/mouse (stimulatory index 1.86). Frondoside A has a weak effect upon IgM production after mouse immunization with sheep erythrocytes *in vivo*. Hence frondoside A is a specific immunostimulant of cell immuninty including phagocytosis without a significant stimulation of humoral immune system amplification activity and adjuvant action. Probably, it may be applied as a mean against diseases in which immunosuppression contributes to the pathology.

Radioprotective action

As it was already mentioned above, glycosides from *Cucumaria japonica* demonstrate a restorative effect in mice with acquired immunodeficiency induced by radiation [102]. Individual triterpene glycoside cucumarioside A_2-2 (**18**) from *C. japonica* and glycoside preparation "trepangin" from the Far Eastern sea cucumber *Apostichopus japonicus* showed radioprotective properties while cucumarioside A_2-2 was significantly more active in this respect. Survival of lethally radiated mice (γ-rays, ^{137}Cs at 7.7 GR, 5–10 min) after glycoside **18** administration at the dose of 1 μg/kg was about 40 % over control and after 60 min radiation was about 20 %. The restoration of hemopoesis was observed in the radiated glycoside-dosed animals [108].

Antiviral action

A mechanism of antiviral activity of sea cucumber triterpene glycosides may be connected with antiviral protection at the stage of virus-cell interaction. This type of protection was confirmed by experiments on inhibition of the cytopathic effect of vesicular stomatite, poliomyelitis and other viruses in cell culture by cucumariosides [104]. Cucumarioside A_2-2 (**18**) was shown to be

more active than cucumarioside A_4-2 (**13**) whereas cucumarioside G_1 (**7**) was not active in the same assay.

Holothurinosides A (**75**), C (**76**) and D (**77**) from *Holothuria forskali* and the desulfated derivative **78** at a concentration 20 μg/ml caused an inhibition of cytopathic effect induced by vesicular stomatitis virus (VSV) in cell culture (20% inhibition of VSV in baby hamster kidney cell line) [68].

Two trisulfated tetrasaccharide glycosides, liouvillosides A (**88**) and B (**89**) isolated from the Antarctic sea cucumber *Staurocucumis liouvillei* were found to be antiviral against herpes simplex virus type 1 (HSV-1) at concentrations below 10 μg/ml. Glycoside **88** produced a weak inactivation of HSV-1 since at the maximum concentration tested, the residual ineffectivity was 24 % with respect to the control virus sample, whereas after treatment with **89** in the same experimental conditions, the remaining inactivity was 10-fold lower (2.5%). Glycoside **89** is distinguished from **88** only by the absence of a 24(25)-double bond in the side chain of the aglycon [73].

Inhibition of root growth

Triterpene glycosides from *Cucumaria japonica* have demonstrated inhibition of the main root growth in *Cucumis sativus* seedlings [109]. The fraction A_2 (monosulfated pentaosides having glucose as third monosaccahride unit in carbohydrate chains), total fraction of monosulfated glycosides, the fractions A_4 (monosulfated pentaosides having glucose instead of 3-O-methylglucose as terminal monosaccharide unit), A_6 (disulfated pentaosides having additional sulfate group at terminal 3-O-methylglucose residue) and A_7 (trisulfated pentaosides) were tested. The ED_{50} were 53.1±3.6 μg/ml, 127.4±5.8 μg/ml, 346.5±15.2 μg/ml, 375.7±17.2 μg/ml and 539.4±11.5 μg/ml correspondingly. The average length of the main root decreased with the increase of glycoside concentrations.

PHYSICO-CHEMICAL BASIS OF THE MEMBRANOTROPIC ACTION

Studies on sea cucumber glycoside membranotropic action have being continued during the past 20 years due to the isolation and structural elucidation of several dozen substances [110, 6, 22] and the growth of investigation on biological and model membranes [111, 112]. It was reasoned that the interaction of glycosides with sterols of plasmatic membranes are the main, but not a sole factor determining various biological activities of sea cucumber glycosides [14]. This has been confirmed by a series of experiments.

Most triterpene glycosides form insoluble complexes with sterols in aqueous and ethanol solutions. These complexes have been used for the isolation of glycosides [113] and analyses of cholesterol content in biological solutions [114].

The comparative study of IR spectra of holotoxin A_1 (**33**), cholesterol, their complexes and mechanical mixtures showed the formation of a hydrogen bond between 3β-OH group of cholesterol and CO-group of the lactone cycle of holotoxin A_1 in corresponding complexes [115].

Sterol-dependent properties of sea cucumber glycosides were found in experiments with different test systems. Studies of antimicrobial and cytotoxic activities of glycosides showed that their toxic properties occur only in test systems (yeast, fungi, eggs and embryos of sea urchins, experimental tumor cells), which contain significant amounts of sterols in membranes. On the contrary, the glycosides were not active in relation to bacteria, eggs and embryos of sea cucumbers, cellular membranes of which do not contain, or contain only trace amounts, of free sterols [14]. Addition of cholesterol into the medium decreased the hemolytic action of "holothurin" [24] and antifungal properties of glycosides from *Apostichopus* (=*Stichopus*) *japonicus* [115]. Addition of cholesterol and sea urchin embryo lipids to incubation medium also led to the decrease of cytotoxic action of holotoxin A_1 [116]. Cholesterol inhibited the cytotoxic action of holotoxin A_1 against tumor cells, whereas Δ^7-sterols, Δ^5-sterol sulfates and Δ^7-sterol xylosides had significantly less effect on this activity [117].

Cytotoxic activity of these glycosides against tumor cells was significantly increased after the treatment of the cells with liposomes saturated with cholesterol [118]. The glycosides have a high affinity to liposomes containing sterols [117].

Sea cucumber triterpene glycosides may form complexes with sterols both in solution and in membranes. Likhatskaya et al., using a microcalorimetry method, demonstrated that holothurin A_2 (**57**) forms complexes with liposomal membrane cholesterol [119].

Popov has demonstrated general correlation between hemolytic, cytotoxic against tumor cell lines activities and possibility to induce the [^{14}C]glucose loss from liposomes containing cholesterol (Table 13) for holothurin A (**101**), holothurin B (**105**), holothurin A_2 (**57**), holothurin B_1 (**56**) holotoxin A_1 (**33**) and cucumarioside G_1 (**7**) [120].

Table 13. A comparative study of hemolysis, cytotoxic, and liposomal activities of sea cucumber triterepene glycosides (ED_{50} (μg/ml))

Substance	Hemolysis, ED_{50}	Cytotoxic activity, ED_{50}		[^{14}C]glucose loss from liposomes*, ED_{50}	
		Erlich ascite cells	L1210	I	II
101	1.50	1.50	1.50	20.0	>100
105	3.00	2.50	3.00	35.0	>100
57	1.00	1.25	1.25	15.0	>100
56	2.50	2.50	2.50	25.0	>100
33	3.50	2.00	2.50	35.0	>100
7	0.75	0.75	0.75	10.0	>100

* - liposomes content: I, egg PCh + Cholesterol (33 molar %); II, egg PCh. Incubation at 20°C, pH 7.2

X-ray analysis of complexes of holothurin A_2 (**57**) and astichoposide C (**43**) with cholesterol confirmed the molecular interaction of these substances in membranes. X-ray showed that membrane preparations of Na^+,K^+-ATPase, treated by glycosides produced identical reflexes with those of model glycoside-cholesterol complexes. The results of such X-ray analyses were characteristic for each of the glycosides studied [121, 122]. In the interaction with membranes, sea cucumber trirerpene glycosides "pull" cholesterol which has a great affinity to phosphatidylcholine in the native membrane. As a result, it significantly influences the physico-chemical properties of membranes: stability [123] and microviscosity of lipid bilayers [121, 124], as well as lipid-protein

interactions and conformations of membrane proteins [121]. These properties determine the activities of membrane enzymes and the functional status of whole membranes in many respects. For instance, activities of membrane Na^+,K^+-ATP-ase and Mg^{2+}-ATP-ase *in vitro* decrease in the presence of sea cucumber triterpene glycosides [123]. Moreover, the glycosides change the parameters of active Ca^{2+} transportation in vesicles of sarcoplasmatic reticulum of rabbit skeletal muscles [126].

Triterpene glycosides from sea cucumbers influence the active transport of substances through biological membranes and induce the efflux of low molecular weight compounds (nucleotides, nitrogen of amines, K^+, inorganic phosphorus) from cells [127, 128, 39, 129]. Echinosides A (**57**) and B (**56**) and holothurins A (**101**) and B (**105**) stimulate the passive Ca^{2+} transport from vesicles of heart sarcolemma [130]. Temperature and ion content of a medium cause important changes in the effect of glycosides on barrier biomembrane properties. For instance, the permeability of sea urchin embryo plasma membranes and yeast cells is sharply increased in the presence of glycosides. Glycosides of sea cucumbers demonstrated membranotropic action on permeability at lower temperatures in comparison with plant glycosides. Membranotropic activity of glycosides was minimally expressed in media containing no ions. Glycosides were more active in the presence monovalent ions (K^+, Na^+). As a rule, Ca^{2+} and Mg^{2+} effectively protect cells against the destructive effect induced by glycosides [39, 129].

Triterpene glycoside activity differs in the effect upon the permeability of biological membranes containing cholesterol and cells containing ergosterol such as yeast. At a concentration of 2.5–5 μg/ml glycosides and amphoterecin B induced a loss of K^+ from fertilized eggs of sea urchins. However at higher concentrations of cucumarioside G_1 (**7**), holotoxin A_1 (**33**) and holothurin A_2 (**57**) not only the efflux of K^+ from cells was increased, but also the loss of UV-absorbing substances (nucleotide pool) was induced [128]. These experiments confirm the formation of larger pores in cells when compared with the effects of polyene antibiotics. The action of sea cucumber glycosides against erythrocyte and liposomal membranes was similar [131]. Efflux of K^+ , nucleotide pool and

proteins were also shown from tumor cells [67]. The glycoside also induced the simultaneously efflux of both K^+ and UV-absorbing substances from yeast. In this case holothurin A_2 induced the formation of large pores immediately [128]. Continuous loss of K^+ from a cell led to the increase in osmotic pressure that consequently could influence transport properties of cell membranes.

Holotoxin A_1 inhibits the RNA biosynthesis in *Candida albicans* and *Saccharomyces carlsbergensis*, as indicated by the decrease in incorporation of ^{14}C-uridine to the acid-insoluble fraction of the cells. Similar results were obtained for glycoside fractions of 14 species of Pacific sea cucumbers [132]. Apparently the inhibition of RNA biosynthesis in *Saccharomyces carlsbergensis* is related to nucleotide loss from yeast cells after treatment with glycosides. Holotoxin A_1 also inhibits biosyntheses of squalene, lanosterol and ergosterol in *S. carlsbergensis* [133]. Mitosis is arrested and DNA synthesis inhibited in onion root bulbs by crude holothurin [134].

Radioactive precursor experiments have shown holotoxin A_1 inhibited biosyntheses of DNA, RNA and proteins in sea urchin embryos. The inhibition was not a result of a direct effect on corresponding enzyme systems [135, 136]. Actually, holotoxin A_1 does not inhibit DNA biosynthesis in isolated nuclei and mitohondria of sea urchin embryos, whereas it increases activities of acid and basic nucleases in subcellular fractions of sea urchin embryos. It was concluded that cytotoxic activities of sea cucumber glycosides against sea urchin embryos are related to biopolymer synthesis inhibition, induced by the decrease in nucleic acid and protein precursor concentrations resulting from disturbance of membrane transport. At higher concentrations of glycosides, such inhibition is the result of the "loss" of precursors through plasmatic membranes [136].

The inhibition of biosyntheses of nucleic acids and proteins in rat spinal cord cell cultures were also studied to ascertain the mechanism of action of sea cucumber glycosides [137]. Addition of holotoxin A_1 (**33**) to incubation media (2.5–5 μg/ml) inhibited ^{14}C-alanine incorporation into the acid-insoluble cell fraction more than ^{14}C-thymidine and ^{14}C-uridine [138]. Incorporation of ^{14}C

alanine into aminoacyl-t-RNA was not inhibited [139]. A decrease of radioactivity in the intracellular amino acid pool indicates the significant inhibition of aminoacid transport into cells.

More detailed studies on peculiarities of glycoside-sterol interaction were carried out using bilayer membranes (BLM). Holothurin A_2 (**57**) caused a sharp increase in ion conductivity in bilayer membranes containing sterols [140-142]. BLM conductivity is dependent on glycoside concentration in this case, showing the possibility of ion pore formation in membranes. At low concentrations of holothurin A_2 (0.1 ng/ml) on both sides of the lipid-cholesterol bilayer, the discrete sudden change of conductivity is related to the formation of solitary channels. The conductivity of these channels had a distinct maximum at 28±4 pSm and a mean life time of 12 milliseconds. Bilateral mode of holothurin A_2 effect on BLM shows that newly generated channels consist of two semipores. Conductivity levels ranged from 4 to 8 pSm at holothurin A_2 concentration from 10 to 100 ng/ml. The formation of "big" channels (pores) apparently results from structural cooperative transitions in bilayers due to the fusion of solitary channels or the inclusion of new subunit complexes into holothurin channels. Holothurin A_2 affects the functioning lipid-ergosterol bilayers. At concentration of 0.1 ng/ml, single steps on the conductivity curve occur. The amplitude was two times less and the mean life time in the open position was two times longer than those of single channels of holothurin A_2-cholesterol. An increase in glycoside dose from 0.1 to 1.0 ng/ml caused the sudden transition into spontaneous fluctuations of high-amplitude current in contrast with effects against BLM containing cholesterol. This means a more rapid transition from the conductivity of solitary channels, having "small" sizes, to that of larger channels (pores) in ergosterol containing membranes. Results of these investigations and those on biological membranes show that holothurin A_2 induces greater damage to the ergosterol-containing membranes than to cholesterol-containing ones [128]. Thus the effects of glycosides in hemolytic, antifungal and antitumor activities and a cytotoxic action on early embryogenesis of sea urchins are similar, as they are related to the membranotropic action of these substances on sterol-containing membranes of target cells.

A greater dependence of hemolytic activity upon glycoside structures was observed in comparison with other properties. This may be related to the direct result of glycoside activity detected on the membrane of target cells, namely the efflux of K^+ and hemoglobin from erythrocytes with hemolysis. We observed an indirect effect through disturbance of membrane permeability and inhibition of active membrane transport, followed by disturbance of cell metabolism and, finally, cessation of cell proliferation of sea urchins embryos and tumor cells. More significant changes of antifungal activities from changes of glycoside structures may be related to the presence of ergosterol in membranes of fungi.

BIOLOGICAL ROLE OF SEA CUCUMBER TRITERPENE GLYCOSIDES

Triterpene glycosides in organism-producer

To begin discussion about the endogenous regulatory role of sea cucumber triterpene glycosides, we would like to consider the resistance of sea cucumber cells against endotoxins - triterpene glycosides, possessing high membranolytic action. Cell membranes of sea cucumbers are resistant to both their own glycosides and those of terrestrial plant. Oocytes, unfertilized eggs, and embryos (40 hours after fertilization) of *Apostichopus* (=*Stichopus*) *japonicus* are not sensitive to holotoxin A_1 at concentrations up to 100 μg/ml [143-144] and to triterpene and steroid glycosides of higher plants [144]. The resistance of sea cucumber cells to endotoxins is connected to low content of free sterols in membrane lipids and the presence of Δ^7-sterols and sulfated and glycosylated sterols instead of Δ^5-sterols [145, 8]. The content of sterols in the total lipid fraction of the sea cucumber eggs is 80-times less than in sea urchin eggs [143]. The cytotoxic effect of holotoxin A_1 on early embryogenesis in sea urchins is neutralized by Δ^7-sterols, β-xylosides of Δ^7-sterols to a significantly less degree than by cholesterol. Sulfates of sterols have no effect [143].

Therefore, the steroid components of membrane lipids of sea cucumbers actually interact minimally with triterpene glycosides. Artificially produced lipid membranes containing Δ^7-sterols, β-

xylosides of Δ^7-sterols and sulfated Δ^5-sterols are also more resistant against holotoxin A_1 than cholesterol-containing membranes [143, 145]. Holotoxin A_1 greatly increases ion permeability of model lipid bilayers containing cholesterol and has little effect on bilayer lipid membranes containing Δ^7-sterols, β-xylosides of Δ^7-sterols, and sulfated Δ^5-sterols [146]. Unusual sterols having a 9(11)-double bond and additional methyl groups in steroid nuclei occur in cellular membranes of some sea cucumbers [147]. Bilayer artificial lipid membranes containing such sterols, namely 14α-methylcholest-9(11)-en-3β-ol and 4α,14α-dimethylcholest-9(11)-en-3β-ol from *Eupentacta* (=*Cucumaria*) *fraudatrix*, are resistant against cucumarioside G_1 from this sea cucumber [148]. Thus, the resistance of cellular membranes of sea cucumbers against endotoxin (triterpene glycoside) is related to the chemical nature of membrane steroid components.

A change of triterpene glycoside content in several tissues of sea cucumbers with both season and age suggest a basis for the contributory role of glycosides in reproductive processes. For instance, the glycoside content, determined by hemolytic index, in body walls of *Holothuria leucospilota* was constant in different seasons, but was greatest in the gonads at their largest size before decrease prior to the beginning of spawning. The glycoside content in the Cuvierian tubules changes likewise but less sharply [149]. A similar change in glycoside content occurs seasonally in ovaries of *Apostichopus* (=*Stichopus*) *japonicus* [150]. The content of glycosides in body walls of *Eupentacta* (=*Cucumaria*) *fraudatrix* is not seasonally changed [151], whereas the glycoside content in the gonads is significantly decreased from December to May. The content of glycosides in body walls in the initial period of sea cucumber growth up to maturation was increased and after it remained constant. The most detailed investigation on sea cucumber glycosides as regulators of reproduction showed that the glycoside content in *A.* (=*S.*) *japonicus* ovaries was increased 40-fold during the period prior spawning [152]. The accumulation of triterpene glycosides in ovaries of sea cucumbers during this period reflects their participation in gametogenesis. Holotoxin A_1 (**33**) inhibits both "spontaneous" maturation of oocytes and that induced by $CaCl_2$, Ca-ionophore A_{23187} and pronase with dithyotreitol

[153]. Holotoxin A_1 decreases the rate accumulation of $^{45}Ca^{2+}$ in oocytes and consequently inhibits the meiotic maturation [153]. Holotoxin A_1 increases the microviscosity of oocyte "ghosts" and model liposomal membranes containing Δ^7-sterols, β-xylosides of Δ^7-sterols and sulfates of Δ^5-sterols [124]. It was showed model bilayer lipid membranes, composed of phospholipids of *A.* (=*S.*) *japonicus* contain a calcium ionophore that is inhibited by holotoxin A_1. This process was associated with an increase of membrane microviscosity [146].

Thus glycosides from sea cucumbers play an important role in regulation of sea cucumber reproduction, synchronizing processes of oocyte maturation [154].

Hamel and Mercier showed that *Cucumaria frondosa* secretes a biologically active mucus that helps maintain gametogenic synchrony among conspecifics [155]. Such freshly collected mucus was able to initiate gametogenesis in conspecifics that were in the gametogenesis recovery stage when other envinronmental conditions were stable. The mucus was active after retention in seawater 3 hours prior its use, but after retention of 6 hours, had lost the activity. The authors postulated that their experimental results and the abundance of saponin detected in the body wall of *C. frondosa* suggest these substances potentially diffuse from the body wall toward the water column along with abundant mucus and may be mediators of hametogenesis. However such hypothesis seems to be confirmed by direct experiments with saponins. Because the excretion of mucus is maximal just before spawning period and the contents of saponins in gonads sharply decreases just before the spawning, it may be proposed that biosynthesis of saponins may be regulated by such a feedback mode.

Hence saponins may play two regulatory roles during reproduction: 1) to inhibit oocyte maturation; 2) and as a mediator of gametogenesis.

External function of sea cucumber triterpene glycosides

A strong membranotropic action and high solubility of most triterpene glycosides from sea cucumbers, associated with the presence of monosaccharide units, sulfate groups and functional

oxygen groups in a aglycon moiety, suggest an important "external" role of these substances. A high ichthyotoxic activity due to damage of gills was known from the earliest investigations (see the corresponding part of this review). Bakus developed the hypothesis of an ecological cause for the origin of toxicity in tropical marine invertebrates, and related the evolution of sea-cucumber glycosides to their protective action against fish predators [16, 156, 157]. Attack by a predator leads some species of the family Holothuriidae to expel Cuvierian tubules containing an extremely high concentration (up to 10–20 % dry weight) of toxic triterpene glycosides [113] that may lead to deterrence of predation or death of the predator [158]. Sea cucumbers that expel their Cuvierian tubules are venomous while those that contain toxic glycosides in their body are poisonous. The adaptiveness of venoms and poisons is the same, although their mode of effect is different.

In his review on the biological role of echinoderm glycosides, Levin disagreed with this explanation of the biological role of sea-cucumber glycosides [19]. He referred to sea cucumber species which do not expel their Cuvierian tubules when aggravated. The Cuvierian tubules of *Bohadschia* species are expelled easily with aggravation while those of *Actinopyga* species are expelled with only a very great aggravation. Those of *Pearsonothuria graeffei* and some *Holothuria* species are not expelled at all. Some species, e.g. *Holothuria atra*, do not have these organs. VandenSpiegel & Jangoux showed that Cuvierian tubules of *Actinopyga mauritiana, A. echinities* and *A. miliaris* are not expelled [159]. Those and similar occasions were also noted by Lawrence [160].

We do not believe these objections change the traditional ideas about the defensive role of glycosides and Cuvierian tubules. Actually, glycosides from the *Bohadschia* species, which have well developed and easily expelled Cuvierian tubules, contain less oxygenated aglycon moieties than glycosides of other species of the family Holothuriidae [161, 162, 6]. Moreover, the glycosides of species of this genus do not contain a sulfate group. In contrast, glycosides from *Holothuria*, *Pearsonothuria* and *Actinopyga* have additional oxygen functional groups in aglycon moieties and a sulfate group in a carbohydrate chain (except *Holothuria forskali* [68]) that lead to higher hydrophilicity and therefore to a higher

rate of excretion. The concentration of glycosides in the water surrounding these animals must be high without the involvement of the Cuvierian tubules. Fish placed in an 8 m^3 aquarium with two individuals of *Holothuria sp.* quickly died (Kalinin, personal observations). This was not due to poor water conditions, as the commensal carapid fish associated with the sea cucumbers were not affected. Mosher showed secretion of toxic substances in an aquarium tank by skin of *Actinopyga agassizi* after removal of the Cuvierian tubules [163]. Nigrelli noted higher resistance of carapid fishes against holothurin than other fishes [23]. Cuvierian organs gradually lose their defensive functional significance in the evolutionary transition from *Bohadschia* to *Actinopyga*, *Pearsonothuria* and *Holothuria*, and are even reduced in some *Holothuria* species. The additional oxygen groups in aglycon moieties and a sulfate group in carbohyrate chains of glycosides increased the solubility of the saponins and changed the mode of delivery of this toxins from the sea cucumber to the fish predator. These peculiarities of glycoside molecular structure superseded the defensive function of Cuvierian tubules. Thus, the presence of Cuvierian tubules is a plesiomorphic character within the Holothuriidae.

Moreover, Levin (1989) noted numerous cases of predation of sea cucumbers by starfish and the gastropod *Tonna perdix* [19]. *Tonna galea* even ate the Cuvierian tubules ejected by *Holothuria forskali* and *Holothuria sanctori* attacked by the gastropod [164]. The explanation is obvious in the case of the starfish predators. They contain membranolytic steroid glycosides (distinguished from triterpene glycosides of sea cucumbers in structure and biosynthetic origin) and, like sea cucumbers, Δ^7- and sulfated Δ5-sterols instead of Δ^5-sterols that make their cell membranes resistant to sea-cucumber glycosides [12]. Many gastropods have highly active carbohydrases that may allow tonnid mollusks to neutralize the toxic effects of sea cucumber glycosides by cleaving the carbohydrate chains [165-167]. Another possible explanation may be in the biochemistry of the membrane components of specific predators and commensals of toxic sea-cucumbers. It will be very interesting to study steroid components of tonnid mollusks, carapid fishes, and other predators and symbionts from this point of view.

Thus sea cucumber glycosides are strong but not absolute defenses against various predators.

Glycosides of sea cucumbers can inhibit the growth of larvae of marine organisms, particularly sea-urchin embryos, containing cholesterol in the cell membranes (see corresponding part of this review). Sea cucumbers and sea urchins occupy quite different niches as Levin correctly noted, and are not competitors [19]. Therefore, it is not appropriate to attribute inhibition of sea-urchin development as a specific function of glycosides. However, sea-urchin embryos may be a good model for investigation of allelopathic action of sea cucumber glycosides. The glycosides of *Cucumaria echinata* demonstrated an action on shellfish in culture [168].

Sea cucumber glycosides may play an important role in defending the body surface from fouling by other organisms. To confirm this function, Levin noted surfaces of most sea cucumbers are not fouled [19] but *Psolus* species, covered with overlapping calcareous plates have only slightly toxic glycosides [5, 91] and very often are fouled with sponges, algae etc. The high antifungal activity of sea cucumber glycosides may provide a protective action against some fungi. Apparently biological functions of triterpene glycosides, which initially were only regulatory ones, later became broader. Therefore, external functions have appeared as a secondary evolutionary response of sea cucumbers to fish predation [161].

GRAPHICAL INTERPRETATION OF BIOLOGICAL ACTIVITIES AND BIOLOGICAL ROLE OF SEA CUCUMBER TRITERPENE GLYCOSIDES

The structure-function relationships in sea cucumber triterpene glycosides were graphically analyzed by system-theoretical approach developed by Van der Klaauw [170] and Dullemeijer [170, 172] in comparative anatomy (morphology) [173]. The method describes a subdivision of morphological-functional complexes of living organisms into functional components, which may be represented as any group of morphological (structural) elements sharing one function. The notion of "element" is used for each part which may be separated without applying criteria of function, system or tissue. Morpho-functional complexes may be

presented as a network diagram, where structural elements and activities are connected by different kinds of relations. The different functional components are often overlapped by their structural elements. Moreover, the functional components may be directly connected if one activity springs directly out another. The presence of a functional connection is shown by making corresponding elements non-functional. The results of theoretical experiments are compared with the real experimental data. The most significant connection for any activity are marked by bold lines. Components or elements which are most significant for any function and determining the structure of the other ones are termed "dominant". Dominant elements, as a rule, are spatially separated and form the so-called "center of realization" while subordinate elements and components are referred to as the "functional periphery". A general view of the diagram will be determined by isolating corresponding "activities" and "elements". Such network diagram may be useful for demonstrating a very complicated picture of structural-functional relations.

Good example of an application of this approach to sea cucumber glycosides is a diagram depicting hypothetical interrelations of functional components and structural elements for the triterpene glycoside holothurin A_2 (=echinoside A) isolated from several sea cucumbers belonging to the family Holothuriidae (Fig. **1**) [6]. The network is based on the data from the mechanism of sea cucumber glycoside activities already discussed in this review. The diagram of the interrelation of morphological and functional indexes of the different levels of structural organization of the muscle-skeleton complex designed by Iordansky [174] has been used as a prototype.

The left part of the scheme shows the structural elements of a glycoside as blocks connected by chemical bonds and the support connections depicted by arrows with triangle tops, directed to blocks which are "mainstays" for other blocks. The blocks located between other blocks as first, second and third monosaccharide units, for example, support neighboring blocks and rest on them (bi-directional arrows). The right part of the diagram depicts the activities connected with the shared structural elements. The direction of the arrows showing connections between structural

elements and activities are also bi-directional because it is necessary to show both the contribution of structural elements to the activity and functional "demand" determining this element evolutionarily. These activities are related to the corresponding biological or adaptive roles and determine the contribution of the glycoside in the fitness of the organism-producer.

An important function of the triterpene glycosides of sea cucumbers is probably the inhibition of oocyte maturation due to increase in microviscosity of the oocyte membrane by glycoside effect on the inhibition of Ca^{2+} transport. The biological significance of such inhibition probably consist in the synchronization of sea cucumber oogenesis and regulation of reproduction [154]. Sea cucumber cellular membranes rarely contain Δ^5-sterols which form insoluble complexes with triterpene glycosides, but the concentration of other types of free sterols is also very low in the membranes. Therefore, the adaptive role is not determined by the formation of a complex, but modification of oocyte membrane structures due to incorporation of glycoside aglycon into the membrane. Penetration of glycoside into the oocyte membrane depends on the hydrophobic part (lanostane nucleus of the aglycon) of the molecule. Besides, in high membranotropic effect of glycosides having quinovose as the second monosaccharide unit (and not glucose or xylose) the quinovose unit helps glycoside penetration into the target cellular membrane. This is strongly supported by a similar dependence for inhibition of Na^+,K^+-ATPase from rat brain [90, 121]. This activity is not due to the formation of single channels or nonselective pores, but due to the formation of a complex of cholesterol from the Na^+,K^+-ATPase membrane preparation and the glycoside [90, 121]. Therefore, the similarity in dependence of an activity from the structure may be connected with glycoside penetration into the membrane preparation. As a result, two structural elements, viz., lanostane nucleus and quinovose residue, produce a functional component, which determines the penetration of glycoside into the membranes of target cells and also overlaps the component "breeding regulation". For rapid reduction of inhibition in Ca^{2+} transport, i.e. reversibility of the action, the regulator must have hydrophilic nature. Therefore, the block "breeding regulation"

connects with a functional component determining the hydrophilic nature.

The most important biological functions are those that are needed for an organism in its daily life [175]. Therefore, the adaptive role of glycosides of sea cucumbers, i.e. deterrence of predators, is the most important for the fitness of the organism (shown by a bold arrow). For deterrence of predation, it is necessary that the glycosides have a membranotropic action against capillaries of fish gills, i.e. (a) formation in the membranes of the corresponding target cells of single selective ion channels at low concentrations, (b) formation of non-selective large pores at higher concentrations and (c) lysis of the cells at even higher concentrations (functional components "deterrence of predators" and "formation of channels, pores, followed by lysis" are connected by the bold arrows). The channels and pores are made by formation of a glycoside complex with membrane Δ^5-sterols (functional components "formation of channels and pores, followed by lysis" and "formation of a complex with membrane sterols" are connected by the bold arrow). At very high concentrations of the glycosides, membrane lysis may occur without the formation of a complex with sterols due to detergent properties of glycoside (the blocks "penetration into membrane" and "formation of channels, pores, following lysis" are connected directly by an arrow).

The formation of a complex with membrane sterols requires penetration of the glycoside into the membrane (corresponding blocks are connected by an arrow). Owing to the ability of an aglycon to form a complex [175] the corresponding block is connected with the structural elements related to an aglycon part of the molecule. The stereochemistry of lanostane nucleus plays a major role in the formation of this complex, besides, the presence of 18(20)-lactone is also important [9, 42] (these functional relations are depicted by bold arrows).

The carbohydrate chain is important for the formation and maintenance of the channels and pores and it determines their size and form [176]. Therefore, all four monosaccharide units are connected with the block "formation of channels, pores, following lysis".

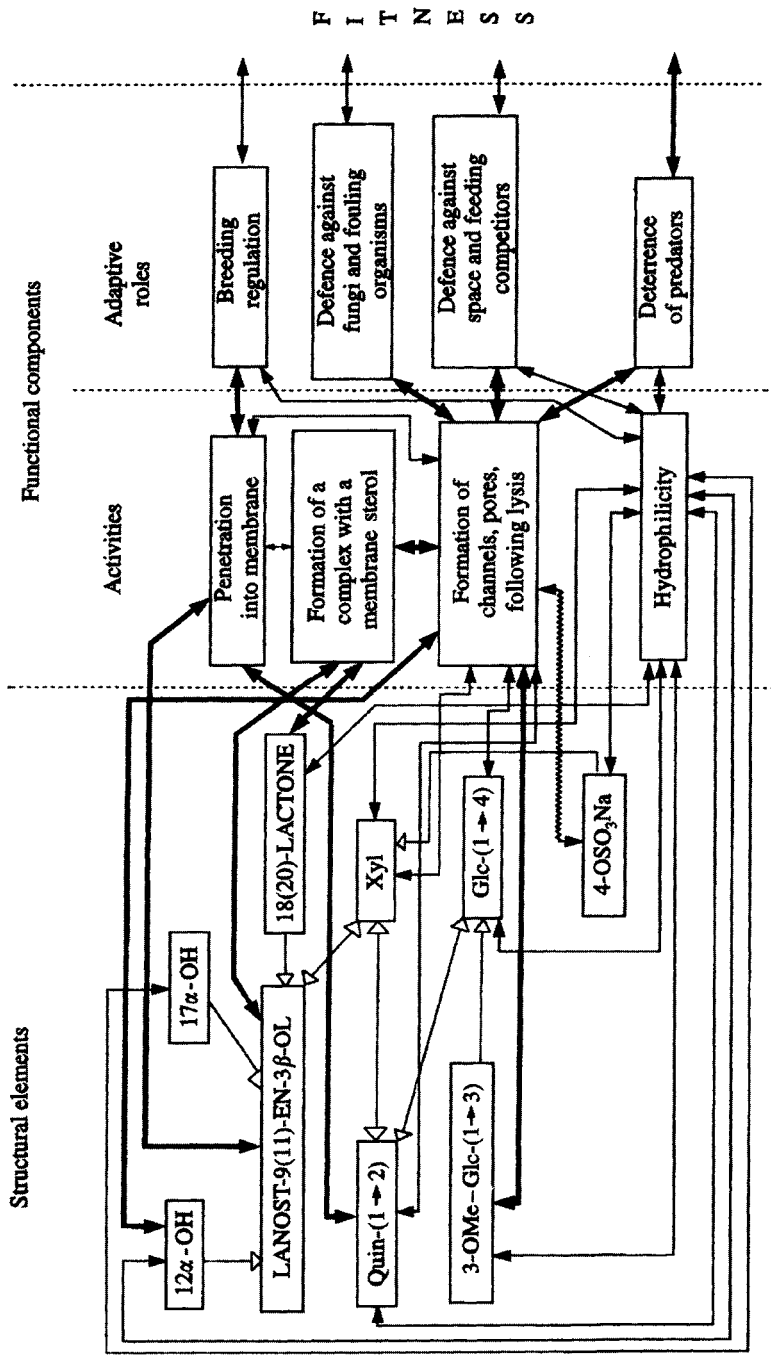


Fig. (1). A network diagram of structural-functional relationships for holothurin A_2 (**57**)

The linear tetrasaccharide fragments are also important for the formation and maintenance of the channel. The holothurin A_2 (**57**) carbohydrate chain is such tetrasaccharide fragment. As shown above, all the branched tetraosides, trioside, bioside and monooside derivatives have low activity. Hence, all monosaccharide units are connected with this block by arrows. To show the special significance of linear tetrasaccharide unit, the connecting arrow from the terminal 3-O-methylglucose is indicated by a bold line.

A 4-O-sulfate group, attached to the first monosaccharide unit (xylose) has a little effect on membranotropic activity. Under desulfation, the activity of such glycosides does not significantly change, but this sulfate group has a specific compensatory or reserve function. Thus, after "elimination" of the last two monosaccharide units from holothurin A_2 molecule, the activity of the derivative obtained, corresponding to the natural product holothurin B_1 (**56**) (echinoside B) [110, 1], is not significantly decreased. But after desulfation of holothurin B_1 or a similar bioside, the activity is decreased by an order of magnitude [9, 42]. Therefore, the sulfate group in holothurin B_1 compensates for the absence of the two monosaccharides and the difference in activities between holothurin A_2 (**57**) and B_1 (**56**) is not great. The connection between this sulfate group and the block "formation of channels, pores following lysis" is depicted by the wavy line showing a different "reserve" character of the connection.

The presence of a 12α-hydroxyl group is also significant for membranotropic action [42]. Probably, this oxygen functional group has an effect on size and as a result affects the maintenance of single channel or nonselective pores [176]. The corresponding connection is shown by a bold line.

To deter a predator, the toxic substance must be mobile, i.e. exhibit quick secretion into the sea water that is connected with an hydrophilicity (arrows connecting functional components "hydrophilicity" and "deterrence of predators"). All polar structural elements such as monosaccharides, oxygen functional groups in aglycon, and sulfate are also connected with "hydrophilicity" by corresponding arrows.

The defensive action of sea-cucumber glycosides against feeding competitors connects with the same functional components as

deterrence of predators, i.e. "formation of channels, pores, following lysis" and "hydrophilicity". Defense against fungi and fouling organisms connects with the block "formation of channels, pores, following lysis" only, as mobility is not necessary.

The dominant functional component (adaptive role) on such a diagram is "deterrence against predators"; other adaptive roles have subordinate significance. Among the activities, dominant functional components are "formation of channels, pores and following lysis" and "formation of the complex with membrane sterols". They do not form any "center of realization", as they have too many connections with too many structural elements and functional components and high degree of overlapping of these connections.

The scheme described above is not only the scheme of structural-functional relationships for holothurin A_2 (**57**), but for holothurin B_1 (**56**) also. That is enough to eliminate "excessive" structural elements and their functional connections and change reserve connection "4-O-sulfate" – "formation of channels, pores, following lysis" with a common one. However it seems to be pointless to create similar scheme for all the known glycosides of sea cucumbers, but more rational to design schemes for the structurally closed groups of substances. Such diagrams were designed for glycosides from the sea cucumbers of the families Holothuriidae, Stichopodidae and genera *Eupentacta* and *Cucumaria* [173]. Such modeling may satisfactory predict different kinds of biological activities of sea cucumber glycosides based on their membranotropic action.

Thus, the glycoside is a heterogeneous morpho-functional adaptive complex sharing general adaptive roles (by Iordansky [177]): the main role (deterrence against predators) and subordinate role (inhibition of oocyte maturation). Natural selection affects the structural elements of glycoside through an "entrance" of the system (network). Selection does not "press" on the structural elements directly, but on their adaptive roles. The adaptive roles then influence, by different ways, the activities and structural elements. Therefore, similar functional demands may be realized by very different structural ways. A plurality of structural solutions to the same biological task characteristic for sea cucumber triterpene glycosides seems to be one of the most important causes of their

very impressive structural diversity. Nevertheless, a significant degree of overlapping of functional components may explain general "constructive plan" stability for all the sea cucumber glycosides.

CONCLUSIONS

The triterpene glycosides from sea cucumbers have strong membranotropic effects against cellular and model membranes containing Δ^5 and $\Delta^{5,7}$-sterols. This explains the wide spectrum of their biological activities. For instance, the formation of complexes with sterols of cell-target membranes followed by the formation of single ion channels and more large pores are the basis of hemolytic, antifungal, antitumor, cytotoxic activities of sea cucumber triterpene glycosides. At low concentration of glycosides, the formation of a complex with cholesterol may lead to the efflux of ions from cells and, as a result of this process, the increase of microviscosity of membranes followed by inhibition of membrane transport systems.

The presence of an 18(20)-lactone in most of cases and additionally at least one oxygen functional group near it (for compounds with 9(11)-double bonds in aglycon moiety) are very important for the membranotropic activity of sea cucumber triterpene glycosides. A linear tetrasaccharide fragment is a determining structural feature of carbohydrate chains, which is very important for membranotropic action. Moreover, glycosides containing quinovose as the second monosaccharide unit are the most active. The sulfate group at C-4 of the first xylose residue in non-branched glycosides containing a linear tetrasaccharide fragment does not greatly influence activities, whereas the absence of a sulfate group at C-4 of xylose residue in biosides decreases the activities more than one order of magnitude. The presence of sulfate at C-4 of first xylose of branched pentaosides having a 3-O-methyl group in a terminal monosaccharide increases the activity. Such sulfate decreases the activity of branched pentaosides having no the 3-O-methyl group. The sulfate groups attached to a C-6 of terminal 3-O-methylglucose residues greatly decrease activity. The sulfate at C-6 of glucose (it is the third monosaccharide residue)

generally increase activity. These rules are quite correct for most kinds of membranolytic activities of sea cucumber glycosides, but in the tests, where the time of incubation is great (different kinds of cytotoxic activities) these regularities are not so evident.

Internal, the regulatory functional role of the glycosides (the inhibition of oocyte maturation) also is connected with the modifying action of the glycosides on the membranes of sea cucumber eggs. The absence or very low concentration of Δ^5-sterols in their membranes suggests another nature of the modifying action in comparison with Δ^5-sterols-containing membranes.

The biological activities of sea cucumber glycosides and physical properties of these substances indicate an important external defensive function. Their functions must correlate with membranotropic action of glycosides against model biological test-systems.

Immunomodulatory action of sea cucumber glycosides at very low concentrations has the greatest interest from the pharmacological point of view. It reasonable, that this action is also related to the modifying effect on membranes of target cells.

Therefore, the ability to modify the structural organization of cellular membranes by different modes is the basis of the wide spectrum of biological activities as well as biological roles of sea cucumber triterpene glycosides.

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BIOACTIVE METABOLITES FROM MARINE MICROORGANISMS

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Abstract: Diverse arrays of new bioactive secondary metabolites have been isolated from marine microorganisms; and the number of publications in this area has greatly increased in recent years. In this review, the emphasis is placed on new compounds with antitumor, enzyme inhibitors, antivirus, and other bioactive metabolites from fungi, bacteria, actinomycetes, and cyanobacteria reported between 2000 and 2005. Supply is a major limitation in the development of marine bioproducts, and the methods for supplying these products are important. So in this review, the secondary major point is placed on the chemical synthetic studies. References of 390 structures and 263 citations are overall presented in this review.

Keywords: Marine microorganism; Bioactive Metabolite

1 Introduction

Microorganisms have been the source of many valuable compounds in medicine, industry, and agriculture; most are derived from terrestrial habitats. After intensive studies on terrestrial microorganisms, consequent attentions have been focused on other ecosystems, especially those subject to extreme environmental conditions such as the desert, hot springs and the sea. Microorganisms living in "sea environments" are subject to high salinity, high pressure, poor nutrition, low temperature (or local high temperature), and complex relations with other different creatures. These factors have resulted in the development of unique metabolisms, which provide the opportunity to produce metabolites that differ from the

terrestrial ones. Thus, sea microorganisms offer a wonderful resource for the discovery of new compounds and compounds with new activity.

Although various bioactive compounds from marine microorganisms have been studied, antitumor agents and antibiotics remain the main research target in the last five years. Many new compounds with strong bioactivities have been isolated from marine microorganisms. For example, a highly cytotoxic peptide ester, from marine cyanobacterium of *Symploca hydnoides*, showed IC_{50} values of **0.2-0.5 ng/mL** against P-388, A-549, and HT-29 (human colon carcinoma) cell lines and 0.7 nM against the MEL-28 cell line, and two novel antibiotics, YM-266183 and YM-266184, [157] from the culture broth of *Bacillus cereus* QN03323, both exhibited potent antibacterial activities against staphylococci and enterococci including multiple drug resistant strains with MICs between 0.02 and 1.56μg mL^{-1}.

On primary investigation, the research results included by this review were showed in Table 1.

Table 1

Marine Microorganism Species	Numbers of Compounds with different Type of Bioactivities					Numbers of Literatures
	Anti-tumor agents	Anti-microbial agents	Enzyme inhibition agents	Antivirus agents	Miscellaneous bioactive agents	
Fungus	62	38	11	5	26	130
Bacterium	3	23	1		5	23
Cyanobacterium	47	2	4		33	70
Actinomycete	11	16	0			40

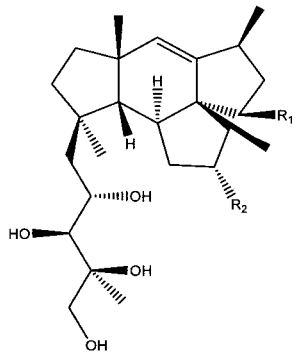
This review covers the literature published between 2000 and 2005 for bioactive metabolites from marine microorganisms.

2 Antitumor agents

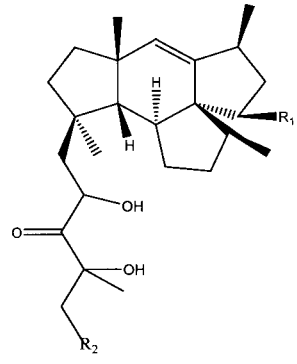
2. 1 From fungus

A new marine fungal, tentatively identified as *Fusarium heterosporum*, produced a series of compounds with spirotricyclic framework, mangicols A-G **1-7** The stereochemistry determination of **1** was based on spectral data from both the natural product and derivatives synthesized.[1] The mangicols **1-7** showed only weak to modest

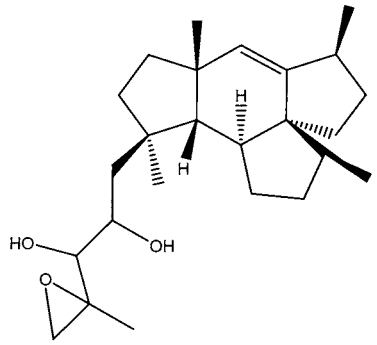
cytotoxicities IC_{50} values ranging from 18 to 36 μM in a study conducted in the National Cancer Institutes 60 cell line panel. Mangicols A **1** and B **2** also showed significant anti-inflammatory activity in the PMA (phorbol myristate acetate)-induced mouse ear edema model. A biosynthetic pathway for the neomangicol and mangicol carbon skeletons is proposed on the basis of the incorporation of appropriate radiolabeled precursors. Enantioselective synthesis of the spirotetracyclic carbon core **8** of mangicols were succeeded, key steps in the synthesis include a transannular Diels-Alder reaction, a novel chlorination and an intramolar Nozaki-Hiyama-Kishi coupling reaction.[2] New aspects in ring closing metathesis reactions studied toward the synthesis of mangicol A were reported.[3] Lithium-iodine exchange initiated intramolecular additions was applied to the annulation protocol of the total synthesis of (+/-)-mangicol F.[4]



1 R_1=OH, R_2=H
2 R_1=H, R_2=OH
4 R_1=H, R_2=H



3 R_1=OH, R_2=H
5 R_1=H, R_2=OH
6 R_1=H, R_2=H



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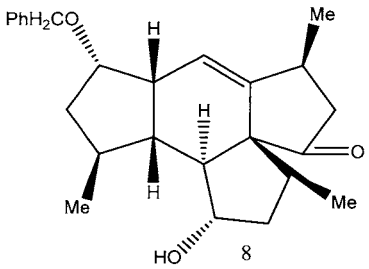

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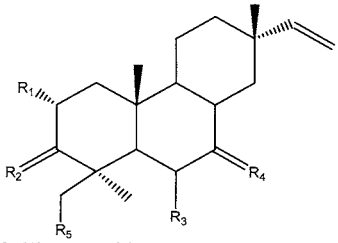
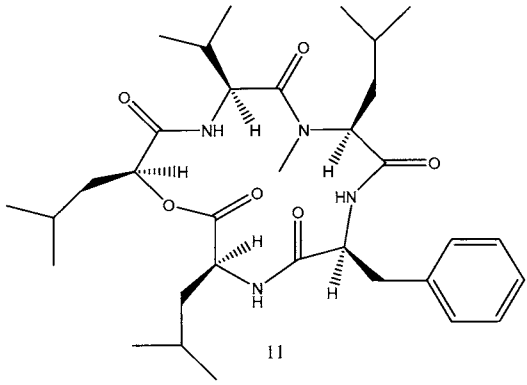


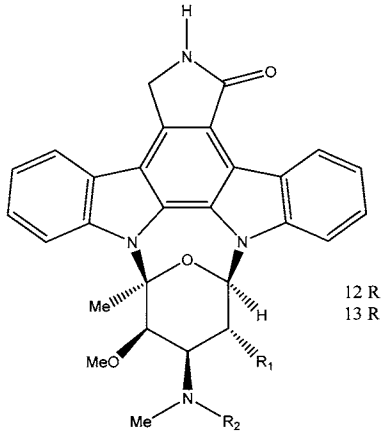
9 Virescenosides M: R_1=OH, R_2=H,beta-OH, R_3=H, R_4=O, R_5=beta-D-altropyranosyl-
10 Virescenosides N: R_1=OH, R_2=H,beta-OH, R_3=beta-H, R_4=H,H,R_5=beta-D-ltropyranosyl-

Two new diterpenic glycosides, virescenosides M **9** and N **10**, were isolated from the marine fungus *Acremonium striatisporum* KMM 4401 which was associated with the holothurian *Eupentacta fraudatrix*.[5] They both exhibited cytotoxic action against tumor cells of *Ehrlich carcinoma* in vitro, and showed a cytotoxic effect on developing eggs of the sea urchin *Strongylocentrotus intermedius*.

N-Methylsansalvamide **11**, a new cyclic depsipeptide, was isolated from extracts of a cultured marine fungus, strain CNL-619.[6] The fungus was identified as a member of the genus *Fusarium*. Compound **11** exhibited weak in vitro cytotoxicity in the NCI human tumor cell line screen (GI_{50}, 8.3 μM). Synthesis towards N-methylsansalvamide was reported.[7]



11

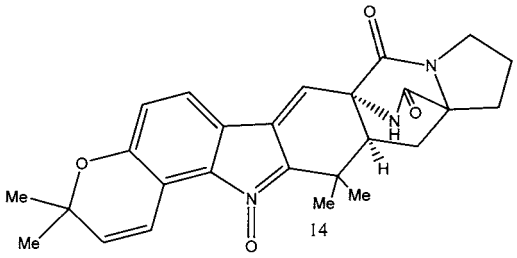


12 R_1=OH, R_2=Me
13 R_1=OH, R_2=H

Two new indolocarbazole alkaloids, 4'-*N*-Methyl-5'-hydroxystaurosporine **12** and 5'-hydroxystaurosporine **13**, were isolated from the culture broth of a marine *Micromonospora* sp.[8,9] (strain L-31-CLCO-002); both showed cytotoxic activities against various tumor cell lines.

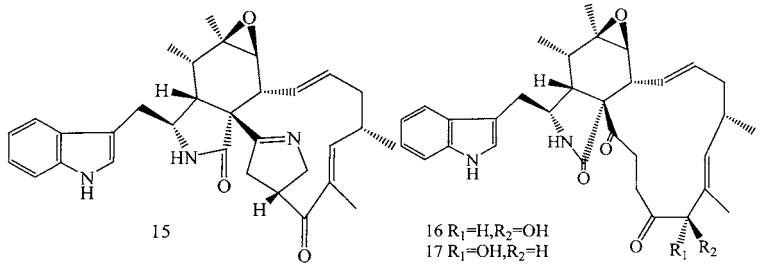
Avrainvillamide **14** was produced from fermentation of the marine fungus *Aspergillus* CNC358, and also from the fermentation broth of *Aspergillus ochraceus*, CL41582.[10,11] **14** demonstrated to be active against the human colon tumor cell line HCT-166, and showed significant selective in vitro cytotoxicity towards the melanoma cell line

MALME-3M and the the human breast cancer cell lines βT-49 and T-470. The 3-alkylidene-3H-indole-1-oxide moiety of **14** was successfully synthesized by a cross coupling-reductive condensation sequence.[12] And its total synthesis was accomplished by 17 reaction steps with 4.2% yield. [13] However a comparatively simple scheme towards its total synthesis was achieved by starting with the analogue of **14**, which gave **14** with a higher yield.[14]



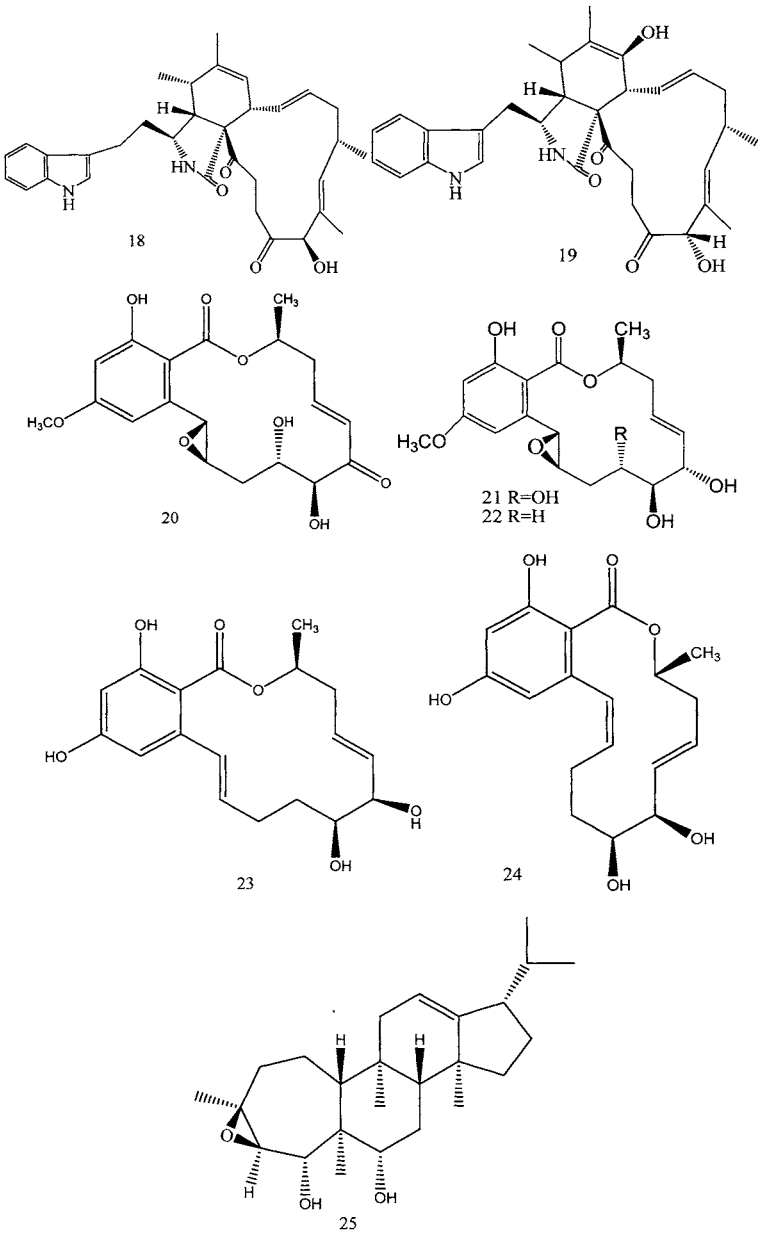
14

New cytochalasans, penochalasins D-H **15-19**, were isolated from a strain of *Penicillium* sp.[15] originally separated from the marine alga *Ebteromorpha intestinealis*, and their stereostructures were established based on spectral data. All compounds **15-19** exhibited significant cytotoxic activity (ED_{50} 3.2, 2.1, 1.8, 1.9, 2.8 μg mL^{-1}, respectively) against cultured P388 cells.



15

16 R_1=H,R_2=OH
17 R_1=OH,R_2=H

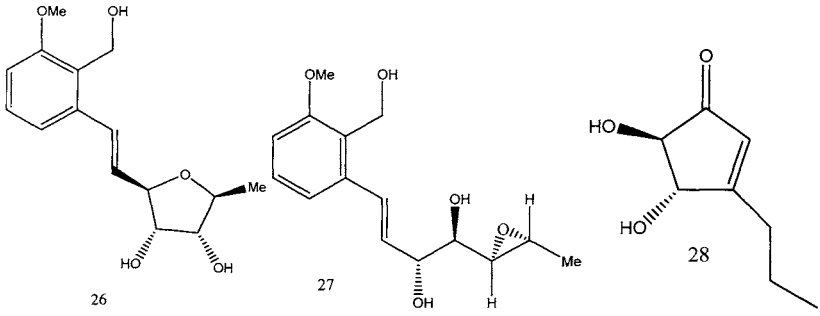


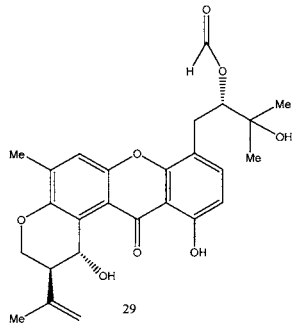
Aigialomycins A-E **20-24**, new 14-membered resorcylic macrolides, were isolated from the mangrove fungus *Aigialus parvus* BCC 5311.[16]

Aigialomycin D **23** exhibited in vitro anti-malarial activity with IC_{50} value of 6.6 μg mL^{-1} and also showed cytotoxicity with IC_{50} values of 3.0 μg/mL for KB cells, 18 μg/mL for BC-1 cells, and 1.8 μg/mL for Vero cells; and **23** was successfully synthesized by different methods.[17-20] The resorcinylic moiety of **23** was constructed by a highly efficient Diels-Alder reaction using a disiloxydiene and a 14-membered ynolide as the dienophile synthesized by ring-forming olefin metathesis.[17] The key feature of another synthetical scheme involved cobalt-complexation-promoted ring-closing metathesis (RCM) to generate ynolides, followed by Diels-Alder reaction with dimedone-derived bis-siloxy dienes to elaborate the benzo system.[18]

A new sesterterpene epoxide-diol, aspergilloxide **25**, was isolated from the extract of a cultured marine-derived fungus (strain CNM-713), a member of the genus *Aspergillus*.[21] **25** showed little in vitro cytotoxicity toward HCT-116 human colon carcinoma.

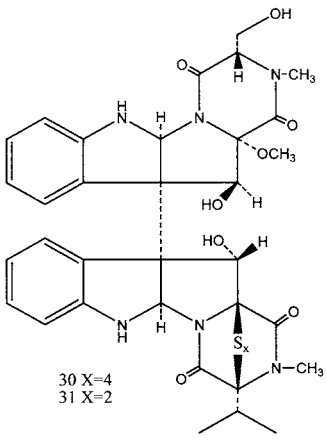
From a marine-derived strain of the fungus *Emericella variecolor*, varitriol **26**, varioxirane **27**, dihydroterrein **28**, and varixanthone **29** were found. In the NCI's 60-cell panel, **26** displayed potency toward selected renal, CNS, and breast cancer cell lines. **29** showed anti-microbial activity. Studies toward the total synthesis and absolute configurational determination of (+)-**26** were achieved.[22, 23]



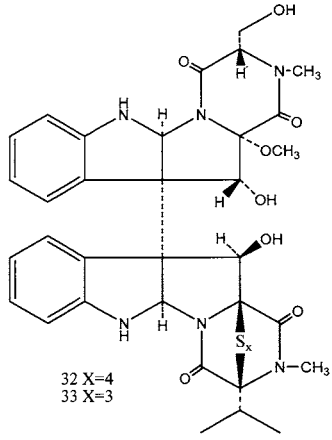


29

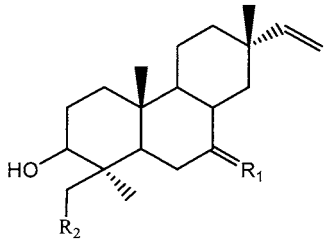
Leptosins M **30**, M_1 **31**, N **32**, and N **33** were isolated from a strain of *Leptosphaeria* sp. originally separated from the marine alga *Sargassum tortile*[24] All compounds exhibited significant cytotoxicity against cultured P388 cells. In addition, leptosins M **30** proved to exhibit significant cytotoxicity against human cancer cell lines and to inhibit specifically two protein kinases PTK and CaMKIII, and human topoisomerase II.

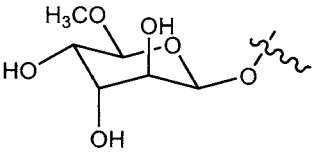


30 X=4
31 X=2



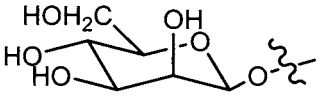
32 X=4
33 X=3





R1		R2
34	H, α-OH	β-D-altropyranosyl-
35	O	β-D-altropyranosyl-
36	H	β-D-mannopyranosyl-

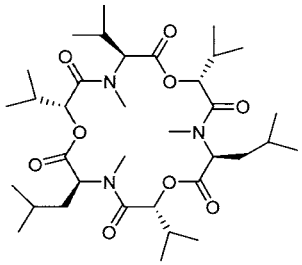
β-D-altropyranosyl-



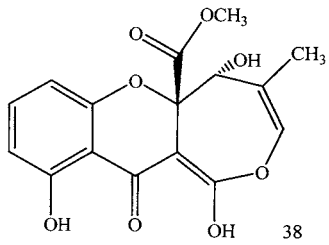
β-D-mannopyranosyl-

Three new diterpene glycosides, virescenosides O **34**, P **35**, and Q **36**, were isolated from a marine strain of *Acremonium striatisporum* KMM 4401 associated with the holothurian *Eupentacta fraudatrix*.[25] All of them exhibited cytotoxic action against tumor cells of Ehrlich carcinoma (IC_{50} = 20-100 μM) in vitro. **35** also showed cytotoxic effects on developing eggs of the sea urchin *Strongylocentrotus intermedius* (MIC_{50} = 5.0 μM).

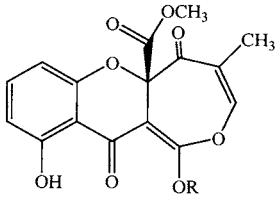
A novel cyclic depsipeptide, enniatin G **37**, was isolated from the mangrove fungus *Halosarpheia sp* (strain 732) from the South China Sea.[26] Compound **37** exhibited the activity against Heps 7402, ED_{50} = 12 μg mL^{-1}. This compound was also obtained from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449.[27,28]



37



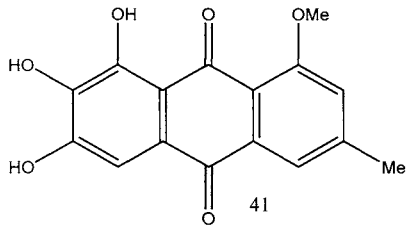
38



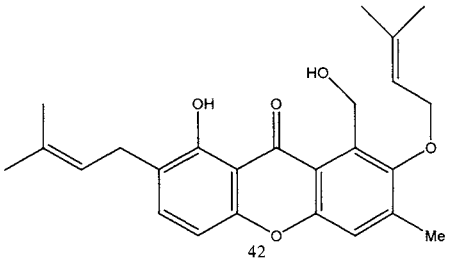
39 R=H
40 R=CH_3

Chemical investigation of a *Penicillium brocae*, obtained from a tissue sample of a Fijian *Zyzyya* sp. sponge, yielded three novel polyketides brocaenols A-C **38–40**.[29] All contain an unusual enolized oxepine lactone ring system. They were tested in an HCT-116 cell line use MTT assay; all showed moderate cytotoxicity. The IC_{50} values for **38–40** were 20, 50, and > 50 μg mL^{-1}, respectively.

In the extract of fungus *Emericella variecolor*, derived from the marine sponge *Haliclona valliculata*, two new natural products, evariquinone **41** and isoemericellin **42**, were isolated.[30] Compound **41**, which also can be obtained from terrestrial fungus, showed anti-proliferative activity towards KB (60% inhibition) and NCI-H460 cells (69% inhibition) at a concentration of 3.16 mg mL^{-1}.[31]

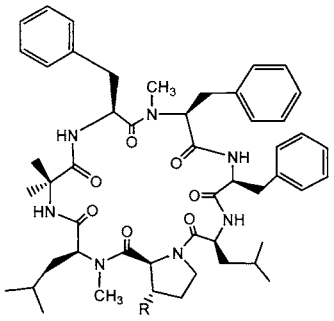


41

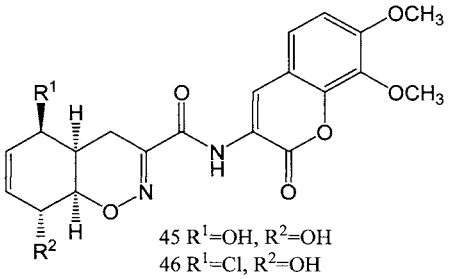


42

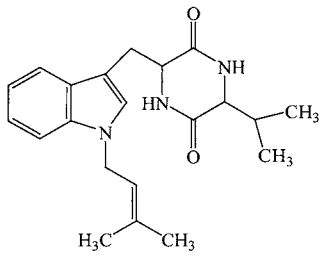
Two new cyclic heptapeptides, scytalidamides A **43** and B **44**, were isolated from the culture broth of a marine fungus, *Scytalidium* sp., collected from the Bahamas.[32] Compounds **43** and **44** showed moderate in vitro cytotoxicity toward HCT-116 human colon adenocarcinoma with IC_{50} values of 2.7 and 11.0 μM, respectively. Both compounds displayed moderate cytotoxicity in the NCI 60 cell-line panel with mean GI_{50} values of 7.9 and 4.1 μM for **43** and **44**, respectively. The most sensitive cell lines were MOLT-4 leukemia (3.0 μM) for **43** and Uacc-257 melanoma (1.2 μM) for **44**. The total syntheses of scytalidamide A **43** was achieved on solid phase using two different resins, a phenylalanine silane resin and a 4-methoxybenzaldehyde backbone linker resin.[33-35]



43 R=H
44 R=CH_3



45 R^1=OH, R^2=OH
46 R^1=Cl, R^2=OH

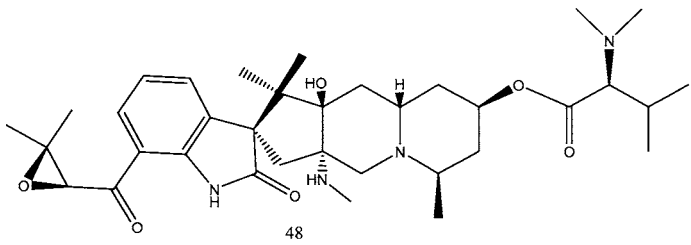


47

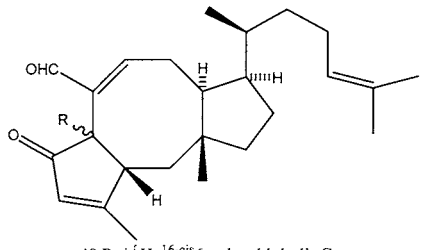
Trichodermamides A **45** and B **46**, two modified dipeptides possessing a rare cyclic *O*-alkyl-oxime functionality incorporated into a six-membered ring, were isolated from cultures of the marine derived fungus *Trichoderma virens*.[36] Compound **46** displayed significant in vitro cytotoxicity against HCT-116 human colon carcinoma with an IC_{50} of 0.32 μg mL^{-1}. And they were also obtained from marine fungus *Aspergillus unilateralis* (MST-F8675) and *Spicaria elegans*.[37]

07H239-A **47**, a new eremophilane sesquiterpene was isolated from a marine-derived xylariaceous fungus, showed cytotoxicity toward a variety of cancer cell lines (mean IC_{50} = 3.2 μg mL^{-1}) and with some selectivity for a CCRFCEM leukemia line (IC_{50} = 0.9 μg mL^{-1}).[38]

A novel pentacyclic alkaloid, citrinadin A **48**, was isolated from the culture broth of the fungus *Penicillium citrinum,* which was isolated from a marine red alga.[39] **48** exhibited cytotoxicity against murine leukemia L1210 and human epidermoid carcinoma KB cells (IC_{50} 6.2 and 10 μg mL^{-1}, respectively). The absolute stereochemistry of citrinadin A was determined. [40]

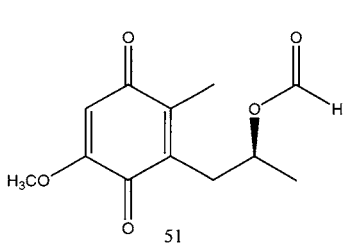


48

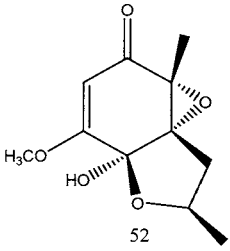


49 R=¦ÁH, $^{16\text{-cis}}$6-epi-ophlobolinG
50 R=¦ÁH, 6-epi-ophlobolinN

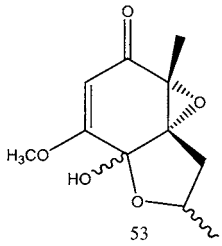
Two new sesterterpenes, 6-epi-ophiobolin G **49** and 6-epi-ophiobolin N **50**, were isolated from the extracts of the fungus, *Emericella variecolor* GF10 obtained from marine sediment.[41] These compounds showed cytotoxicity against the neuroblastoma cell line, Neuro 2A.



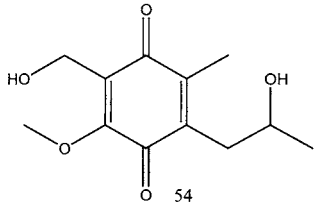
51



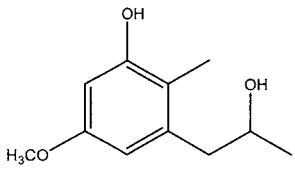
52



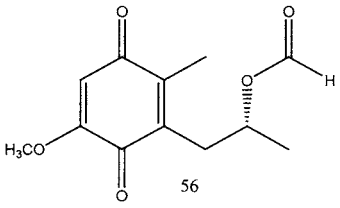
53



54



55

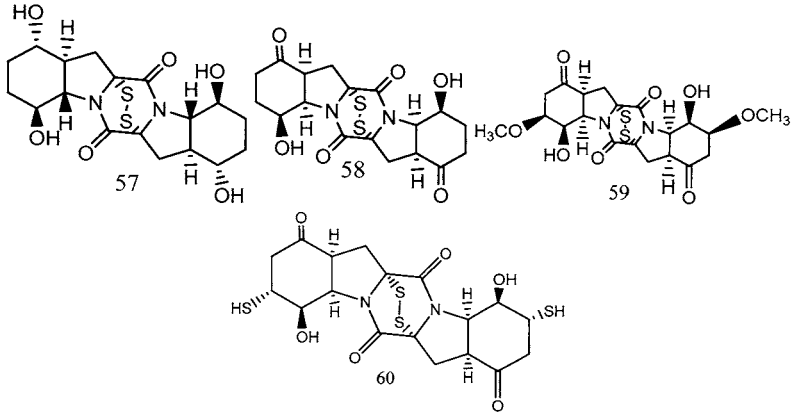


56

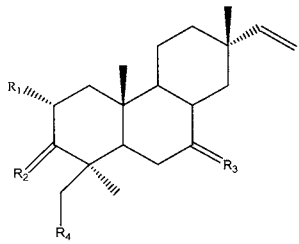
Three previously unknown pentaketides, (+)-formylanserinone B **51**, (-)-epoxyserinone A **52**, and (+)-epoxyserinone B **53**, along with two constituents, hydroxymethylanserinone B **54** and deoxyanserinone B **55**,

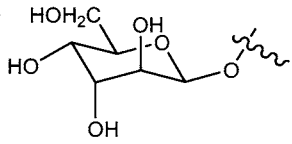
were isolated from saltwater culture broth of a deep sea (4380 ft below) fungus.[42] Bioactivity was explored in two separate cell-based assays, and all compounds except **52** showed significant inhibition of murine cancer cell lines with IC_{50} values of 2 to 4μg mL^{-1}. **51** showed highest electivity against Leukemia but only exhibited modest activity against the MDA-MB-435 cell line. The stereochemistry for compound **51** was later revised as **56**.[43]

Four new cytotoxic disulfides, rostratins A-D **57-60**, were isolated from the broth of the marine-derived fungus *Exserohilum rostratum* (Drechsler), a fungal strain found associated with a marine cyanobacterial mat.[44]These compounds showed in vitro cytotoxicity against human colon carcinoma (HCT-116) with IC_{50} values of 8.5, 1.9, 0.76, and 16.5 μg mL^{-1}, respectively.

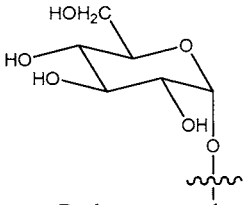


Four new diterpene glycosides, virescenosides R **61**, S **62**, T **63**, and U **64**, were isolated from a marine strain of *Acremonium striatisporum* KMM 4401 associated with the holothurian *Eupentacta fraudatrix*.[45] Compound **61-64** exhibited cytotoxic activity against tumor cells of *Ehrlich carcinoma* (IC_{50} = 25-60 μM) in vitro, and showed a weak cytotoxic effect on developing eggs of the sea urchin *Strongylocentrotus intermedius* (IC_{50} = 100-150 μM).





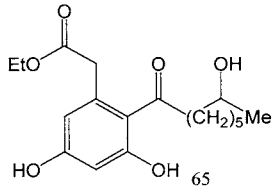
β-D-altropyranosyl-



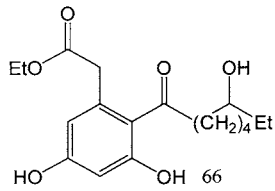
α-D-glucopyranosyl

	R_1	R_2	R_3	R_4	
61	OH	H, β-OH	H	α-D-glucopyranosyl(1-6)-β-D-altropyranosyl-	7,8
62	H	O	H,α-OH	β-D-altropyranosyl-	8,14
63	H	O	O	β-D-altropyranosyl-	8,9
64	H	O	O	β-D-altropyranosyl-	8,14

In a study of mangrove endophytic fungi metabolites, four new compounds, dothiorelone A-D **65-68**, were isolated from an endophytic fungus, *Dothiorella* sp. HTF3.[46, 47] They all exhibited cytotoxic activities.



65



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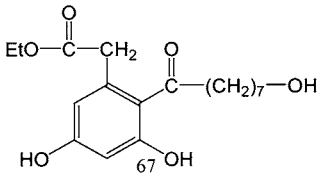
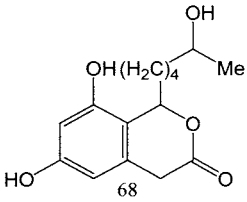
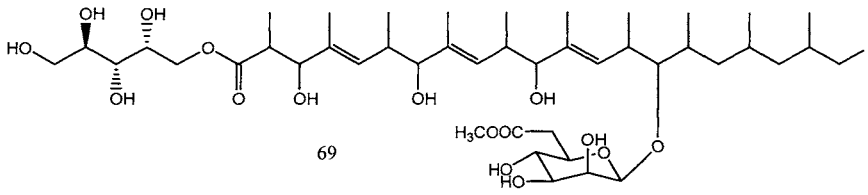


67



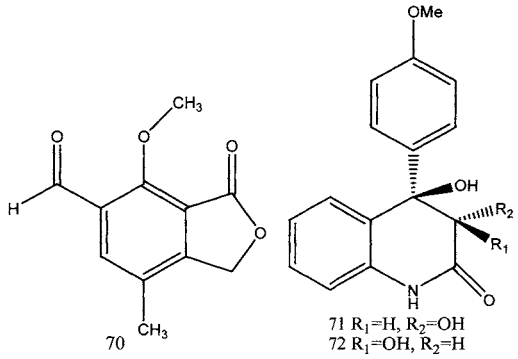
68

A new polyketide glycoside, cladionol A **69**, was isolated from the cultured broth of a fungus *Gliocladium* sp.,which was separated from sea grass *Syringodium isoetifolium*.[48] Cladionol A exhibited cytotoxicity against murine leukemia L1210 and human epidermoid carcinoma KB cells with IC_{50} values of 5 and 7 μg/mL, respectively.



69

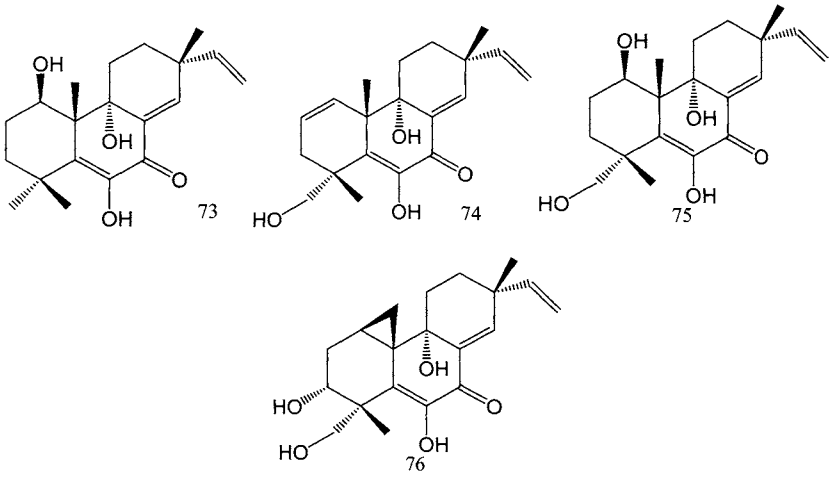
A new compound named diaporthelactone **70** was isolated from the culture of *Diaporthe* sp., a marine fungus growing in the submerged rotten leaves of *Kandelia candel* in the mangrove nature conservation areas of Fugong, Fujian Province of China.[49] It was identified to be 1, 3-dihydro-4-methoxy-7-methyl-3-oxo-5-isobenzofuran-carboxyaldehyde, which showed cytotoxic activity against KB and Raji cell lines (IC_{50} 6.25 and 5.51μg mL^{-1}, respectively). It also showed inhibitory activity against *Aspergillus niger* with MIC 50μg mL^{-1}.



70

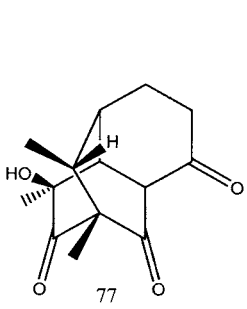
71 R_1=H, R_2=OH
72 R_1=OH, R_2=H

From marine-derived fungus *Penicillium janczewskii*, two new diastereomeric quinolinones, 3*S**, 4*R**-dihydroxy-4-(4'-methoxyphenyl)-3, 4-dihydro-2(1*H*)-quinolinone **71** and 3*R**, 4*R**-dihydroxy-4-(4'-methoxyphenyl)-3, 4-dihydro-2(1*H*)-quinolinone **72**, were identified.[50] These two new compounds **71** and **72** showed a low to moderate general toxicity against MDA-MB 231, DU-145, HT-29, A549, CAKI-1, SK-MEL 2, and K562 cells, with **72** being slightly more potent. In addition, a significantly stronger cytotoxicity against SKOV-3 cells was found for **72**.



The induction of biosynthesis of four new diterpenoids libertellenones A-D **73-76**, was observed following the addition of a marine α-proteobacterium to an established culture of the marine-derived fungus *Libertella* sp.[51] The fungal strain and the marine bacterium, cultured alone, do not produce diterpenoid metabolites. The libertellenones show varying levels of cytotoxicity against the HCT-116 human adenocarcinoma cell line with libertellenone D being the most potent (IC_{50} = 0.76 μM).

Two novel polyketides, penicillones A **77** and B **78**, with tricyclo [5.3.1.$0^{3,8}$] undecane skeleton, were isolated from marine-derived fungus *Penicillium terrestre*.[52] It is the first time that compounds with such a skeleton have been isolated from microorganisms. Compound **77** showed weak cytotoxicities against P338 and A-549 cell lines with IC_{50} values of 83.0 and 68.4 μM, respectively, while **78** showed IC_{50} values of 97.6 μM to A-549 cells, but was inactive against P388.



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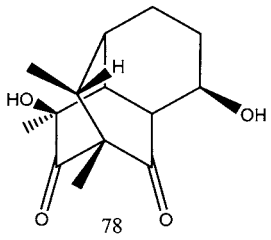

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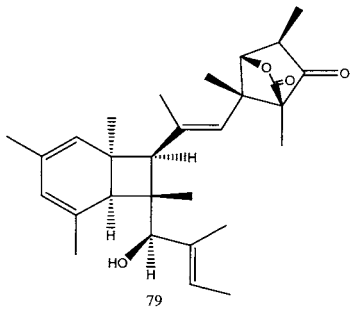
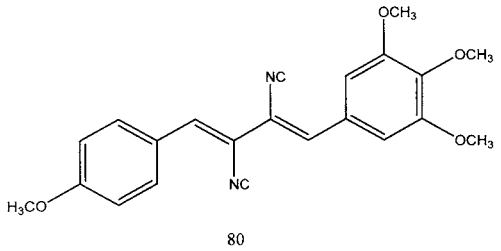


79

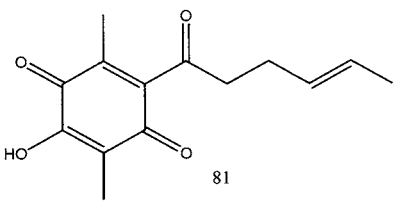
Shimalactone A **79**, a novel polyketide having bicyclo[4.2.0]octadiene and oxabicyclo[2.2.1]heptane units, was isolated from a cultured marine fungus of *Emericella variecolor* GF10.[53] Shimalactone A **79** induced neuritogenesis at 10 mg/mL against neuroblastoma Neuro 2A cells. At higher concentration of 20 mg/mL, it showed cytotoxicity against the same cell line.

A new xanthocillin **80** was isolated from marine fungus *Basipetospora* sp. as thrombopoietin (TPO) mimics.[54] It promoted the proliferation of a TPO-sensitive human leukemia cell line, UT-7/TPO, and UT-7/EPO-mpl, genetically engineered to express c-Mpl, a receptor for TPO in dose-dependent manners. However, the proliferation of UT-7/EPO, a parental cell line of UT-7/EPO-mpl that was devoid of TPO receptor, was not affected by it. These data indicated that xanthocillin **80** is putative agonists for c-Mpl, as its cellular actions was analogous to those of TPO.

Four new compounds were isolated from a marine-derived fungus *Penicillium terrestre*, namely 2-(2', 3'-dihydrosorbyl)-3,6-dimethyl-5-hydroxy-1, 4-benzoquinone **81**, 3-acetonyl-2,6-dimethyl-5-hydroxy-1,4-benzoquinone **82**, dihydrobisvertinolone **83**, and tetrahydrobisvertinolone **84**.[55] Their cytotoxic effects on P388 and A-549 cell lines were preliminarily examined by the MTT method.



80



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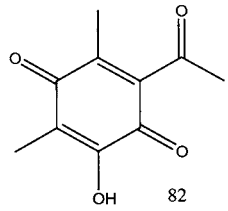

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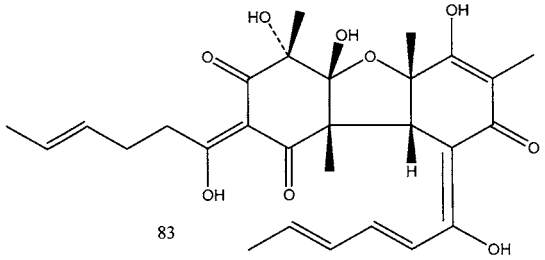

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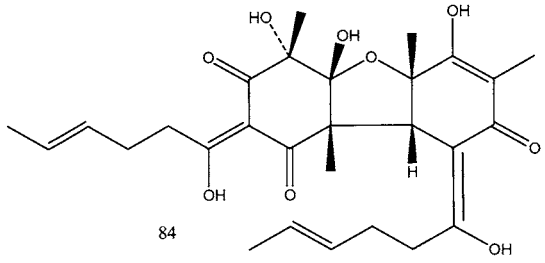
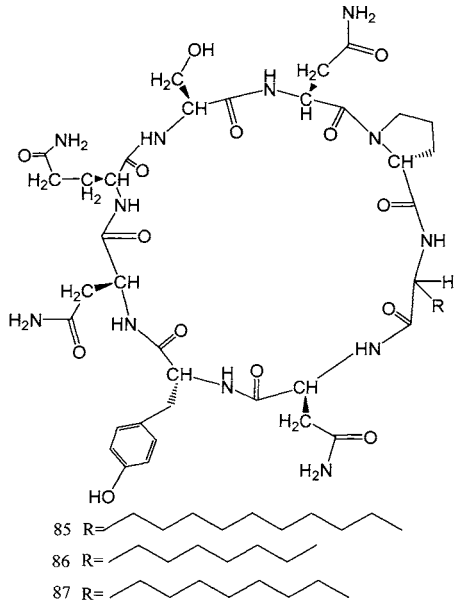


84

2. 2 From bacteria

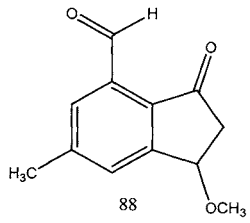
Three new cytotoxic cyclopeptides, mixirins A **85**, B **86**, and C **87**, of iturin class were isolated from marine bacterium *Bacillus* sp.[56] These three compounds inhibited the growth of human colon tumor cells (HCT-116) with IC_{50} of 0.68, 1.6, 1.3 μg mL^{-1}, respectively. Solid-phase synthesis of the mixirins were also reported.[57]



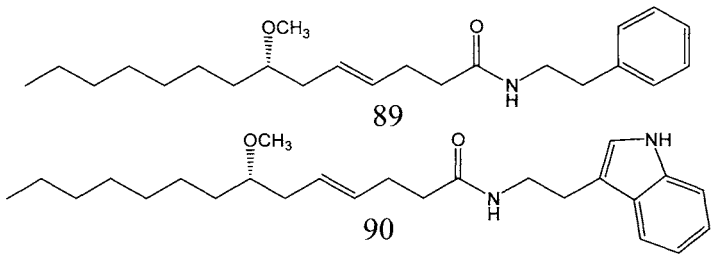
2. 3 From cyanobacteria

Various collections of the marine cyanobacterium *Lyngbya* sp. had afforded many novel metabolites with significant antitumor activities and their syntheses were also reported.

A new indanone **88** was isolated from the filamentous marine cyanobacterium *Lyngbya majuscula*.[58] Compound **88** inhibited hypoxia-induced activation of the VEGF genepromoter in Hep3B human liver tumor cells *in vitro*. VEGF (vascular endothelial growth factor) is an important regulator of tumor angiogenesis.



88



89

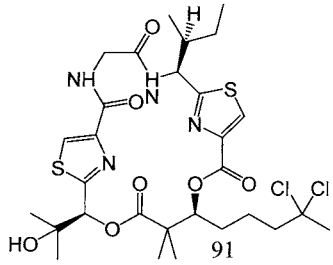
90

A Papua New Guinea collection of the marine cyanobacterium *Lyngbya majuscula* yielded two new toxic natural products, hermitamides A **89** and B **90**.[59] Hermitamides **89** and **90** exhibited LD_{50} values of 5 μM and 18 μM, respectively, in the brine shrimp (*Artemia salina*) bioactivity experiment, and IC_{50} values of 2.2 μM and 5.5 μM to Neuro-2a neuroblastoma cells in tissue culture. Hermitamide A was mildly ichthyotoxic to goldfish, with an LD_{50} value of 19 μM, while hermitamide B was inactive at 25 μM.

Lyngbyabellin A **91**,[60, 61] a cytotoxic compound with unusual structural features, was isolated from a strain of the Guamanian marine cyanobacterium *Lyngbya majuscula*. **91**exhibited cytotoxicity against KB cells and LoVo cells with IC_{50} values of 0.03μg mL^{-1} and 0.50μg mL^{-1} respectively. In vivo trials revealed that lyngbyabellin A was toxic to mice; the lethal dose varied from 2.4 to 8.0 mg kg^{-1}. **91** also showed as a potent disrupter of the cellular microfilament network, which disrupted the cellular microfilament network in A-10 cells at concentrations of 0.01-5.0 μg mL^{-1}. Its structure was confirmed by syntheses.[62,63] Both functionalized thiazole carboxylic acid units were synthesized using chemical manganese dioxide oxidation from the corresponding thiazolidines. The asymmetric synthesis of the dichlorinated β-hydroxy acid unit was achieved by the chiral oxazaborolidinone mediated aldol reaction. Finally, fragment condensation followed by the macrolactamization to provid the lyngbyabellin A.

Lyngbyabellin B **92**, an analogue of lyngbyabellin A **91**, was also

isolated from marine cyanobacterium *Lyngbya majuscula*.[62,65] Compared with **91**, compound **92** was slightly less cytotoxic in vitro with IC_{50} values of 0.10 and 0.83 μg mL^{-1} against KB and LoVo cells respectively.[62] **92** also displayed potent toxicity toward brine shrimp and the fungus *Candida albicans*.[65] The structure was also confirmed by synthesis using the similar method by the same group of chemists in the synthesis of lyngbyabellin A **91**.[64]



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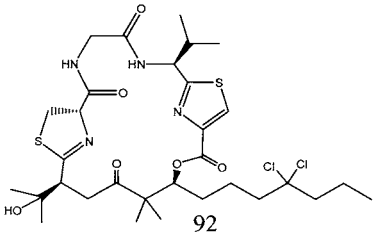
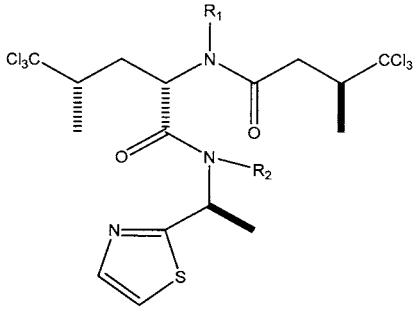
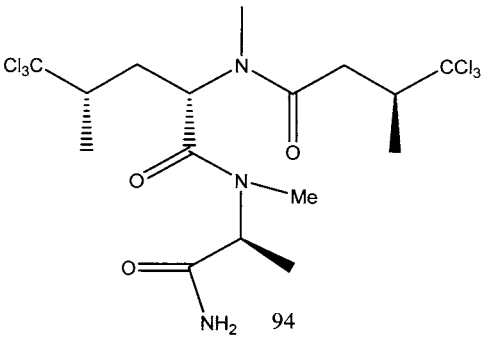


92

Four new metabolites, named pseudodysidenin **93**, dysidenamide **94**, nordysidenin **95**, and dragonamide **96**, were isolated from a marine red cyanobacterium, *Lyngbya majuscula*, collected at Boca del Drago Beach, Bocas del Toro, Panama.[66] Both compounds exhibited cytotoxycity against P-388, A-549, HT-29, and MEL-28 (IC_{50} > 1 μg mL^{-1}). The total synthesis of dragonamide was reported and led to a reassignment of the configuration at the stereogenic centre on the alkyne-bearing fragment of **96** to **97**.[67]



93 R_1=H R_2=CH_3
95 R_1=H R_2=H



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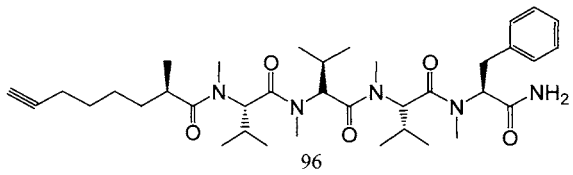

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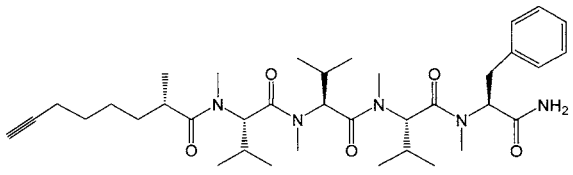

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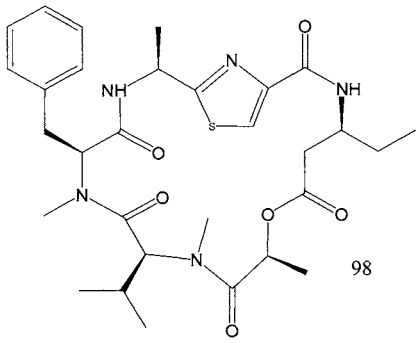

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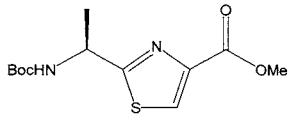
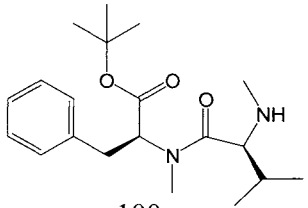


99

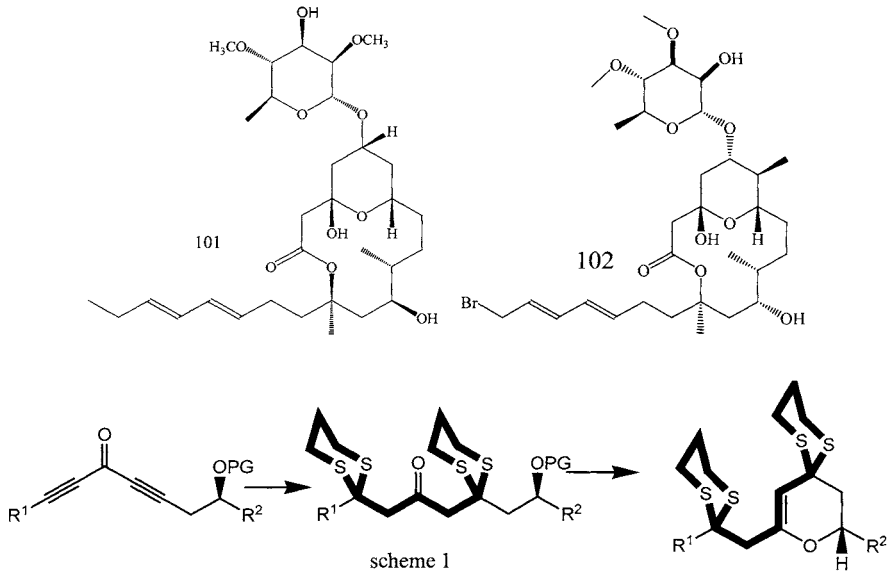


100

A variety of the marine cyanobacterium *Lyngbya confervoides* collected in Saipan, Commonwealth of the Northern Mariana Islands, yielded obyanamide **98**, and this compound exhibited moderate cytotoxicity against KB and LoVo cells with IC_{50} values of 0.58 and 3.14 μg mL^{-1} respectively.[68] Only segments of **98,** so far as it is known, were synthesized as following. Methyl

2-(1-tert-butoxycarbonylaminoethyl)thiazole-4-carboxylate **99**, the chiral unit containing thiazole of obyanamide, was synthesized from (S)-serine and N-Boc-(S)-alanine in an overall yield of 21.4% through seven steps. The ee value of **99** was up to 98%.[69] Hindered N-methyldipeptide (MeVal-MePhe-OBu-t) **100**, another segment of obyanamide, was synthesized from L-valine and L-phenylalanine, overall yield 48.2%.[70]

A glycosidic macrolide, lyngbouilloside **101**, was isolated from the marine cyanobacterium *Lyngbya bouillonii* collected from Papua New Guinea.[71] Lyngbouilloside was modestly cytotoxic to neuro-2a neuroblastoma cells (IC_{50} value of 17 μM). The conjugate addition of dithiols to bis-ynones generates a versatile masked 1,3,5-triketone platform (scheme 1) to synthesize the tetrahydropyranyl and β-keto 1,3-dithiane fragments of **101**.[72,73]

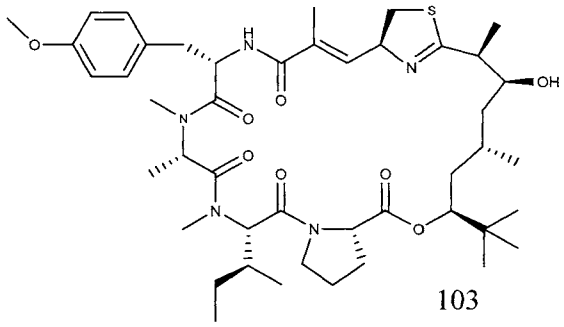


scheme 1

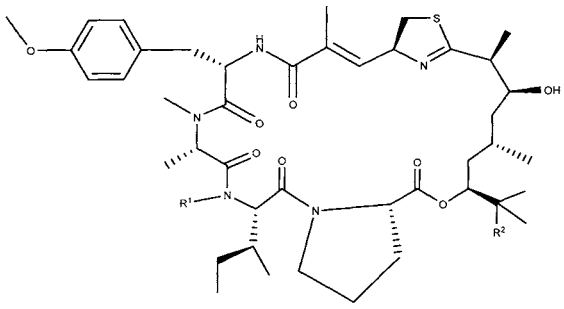
A Palauan collection of the marine cyanobacterium *Lyngbya* sp. yielded a new glycoside macrolide, lyngbyaloside B **102**.[75] Lyngbyaloside B was weakly cytotoxic against KB cells with an IC_{50} value of 4.3μM and exhibited a considerably smaller effect on LoVo cells ($IC_{50} \approx 15$ μM). The

tetrahydropyranyl fragment of **102** was synthesized *via* the similar methodology as used for **101**.[74]

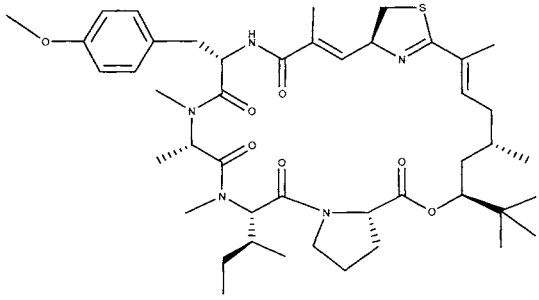
Apratoxin A **103**, a potent cytotoxin with a novel skeleton, was isolated from the marine cyanobacterium *Lyngbya majuscula* Harvey ex Gomont from Finger's Reef, Apra Harbor, Guam.[76] **103** exhibited potent cyto toxicity in vitro with IC_{50} values of 0.52 nM against KB and 0.36 nM against LoVo cancer cells; however, it was only marginally active in vivo against a colon tumor and ineffective against a mammary tumor. Syntheses of apratoxin A, its analogue and segments were reported.[77-83]The total synthesis of **103** featured stereocontrolled access to the novel polyketide domain and the late-stage installation of the sensitive 2,4-disubstituted thiazoline moiety using Staudinger reduction-intramolecular aza-Wittig process.[76] In the synthesis of an oxazoline analogue of apratoxin A, the success in macrocyclization at the *N*-Me-Ile-Pro site demonstrated again that this site is a suitable macrolactamization site for synthesizing apratoxins and their analogues.[78] In the synthetic approaches to the polyketide segment 3-OTBS-7-OPMB-2, 5, 8, 8-tetramethylnonanoic acid, key steps involved asymmetric allylation, crotylation, and asymmetric conjugate additions.[80] Both strategies are very efficient and proceed with high levels of stereocontrol throughout.



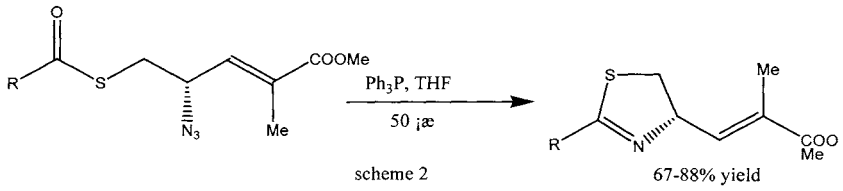
103



104 R^1=H, R^2=CH_3
105R^1=CH_3, R^2=H



106



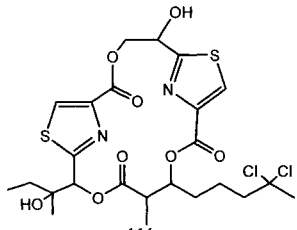
scheme 2

67-88% yield

Two collections of the marine cyanobacterium *Lyngbya* sp. from Guam and Palau both afforded the potent depsipeptide, cytotoxin apratoxin A **103**; however, they yielded different structural analogues of low degree of methylation. The new apratoxins, termed apratoxins B **104** and C **105**, were evaluated for their in vitro cytotoxicity along with semisynthetic E-dehydroapratoxin A **106** to identify key structural

elements responsible for the cytotoxicity and to initiate SAR studies on this novel family of depsipeptides IC_{50} in nMKB: Apratoxin A **103**: 0.52, Apratoxin B **104**: 21.3, Apratoxin C **105**: 1.0, E-Dehydroapratoxin A **106**: 37.6; IC_{50} in nMLoVo:Apratoxin A **103**: 0.36, Apratoxin B **104**: 10.8, Apratoxin C **105**: 0.73, E-Dehydroapratoxin A **106**: 85.1.[82] All analogues **104–106** displayed weaker cytotoxicity than **103**. The cytotoxicity of compound **105** was very close to compound **103**, while compounds **104** and **106** exhibited a much lower activity; the reduced activity was possibly related to the conformation change of the molecules. The 16S rRNA genes of the different apratoxin producers were partially sequenced and compared. Studies towards synthesis of the thiazoline-containing domain of apratoxin natural products were reported.[79-83] In developing a synthetic entry to it, vicinal azido-thiolesters were converted into 2,4-disubstituted thiazolines *via* Staudinger reduction/aza-Wittig reaction (scheme 2).

The apratoxin-producing marine cyanobacterium *Lyngbya* sp. from Palau afforded four new compounds **107-110**.[84] Lyngbyabellin C **107**, exhibiting activities with IC_{50} values of 2.1 μM against KB and 5.3 μM against LoVo cells, was structurally related to other lyngbyabellins, to dolabellin, to lyngbyabellin A **91**, and to hectochlorin. Conformationally, through its decomposition, **107** tends to form homohydroxydolabellin and thus suggested that the sea hare isolate, dolabellin, could be an isolation artifact. Lyngbyapeptins B **108** and C **109**, two new modified tetrapeptides, and palau'imide **110**, a new cytotoxic N-acylpyrrolinone, were also isolated. Compound **110** exhibited in vitro cytotoxicity with IC_{50} values of 1.4 and 0.36 μM against KB and LoVo cells respectively.



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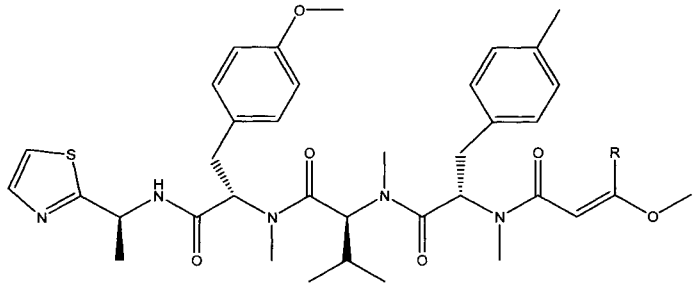
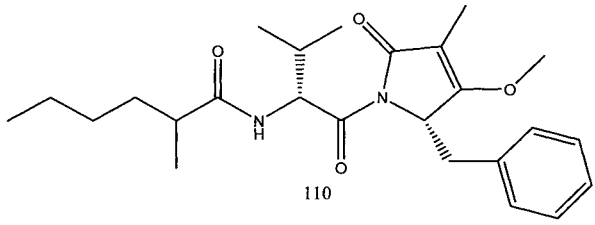
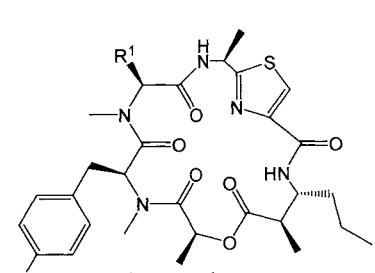


108 R=Me
109 R=Et

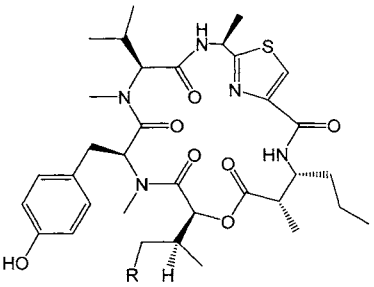


110

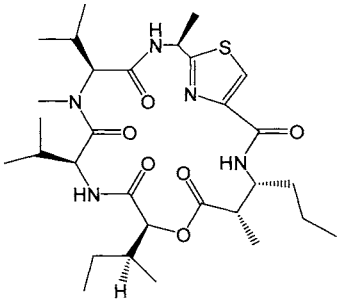
Six new β-amino acid-containing cyclic depsipeptides, termed ulongamides A-F **111-116**, had been isolated from collections of apratoxin-producing cyanobacteria from Palau.[85] All compounds except **116**, which lacked an aromatic amino acid moiety, displayed weak in vitro cytotoxicity with IC_{50} values of ca.1 μM and ca. 5 μM against KB and LoVo cells, respectively.



111 R^1=*i*-Pr R^2=H
112 R^1=*i*-Pr R^2=OH
113 R^1=Bn R^2=OH

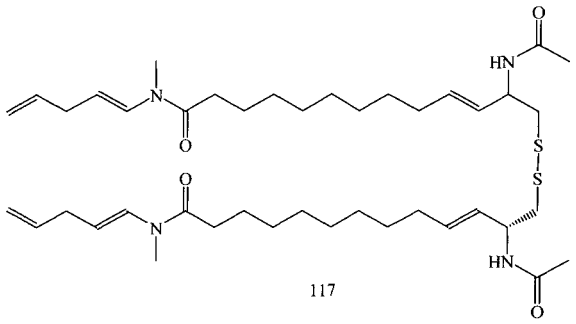


114 R=H
115 R=Me



116

Bioactivity-guided investigation of the extract from a *Lyngbya majuscula/Schizothrix* sp. assemblage of marine cyanobacteria led to the discovery of somocystinamide A **117**, an extraordinary disulfide dimer of mixed PKS/NRPS biosynthetic origin. [86] Somocystinamide A is highly acid sensitive, and rapidly and completely converted in acidic medium into a characterizable derivative **118**. Compound **117** exhibited significant cytotoxicity against mouse neuro-2a neuroblastoma cells (IC_{50} = 1.4 μg mL^{-1}), whereas **118** had no activity.



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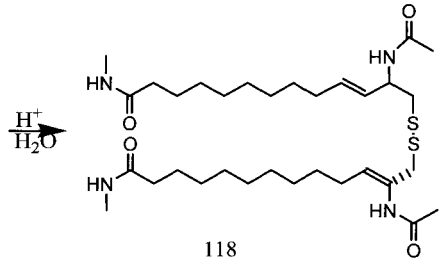
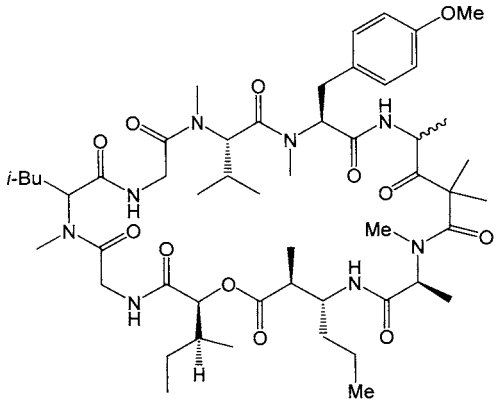


118

Lyngbyastatin 3 **119**, a new lyngbyastatin 1 homologue, was isolated by bioactivity-guided fractionation from *Lyngbya majuscule* Harvey ex Gomont (Oscillatoriaceae) strains collected from Guam. Pure compound **119** had IC_{50} values of 32 and 400 nM against KB and LoVo cell lines, respectively.[87]

Novel cyclic depsipeptides, guineamides A-F **120-125**, were isolated from a Papua New Guinea collection of the marine cyanobacterium *Lyngbya majuscule*.[88] Guineamides B **121** and C **122** possessed moderate cytotoxicity to mouse neuroblastoma cell line with IC_{50} values of 15 and 16 μM respectively.



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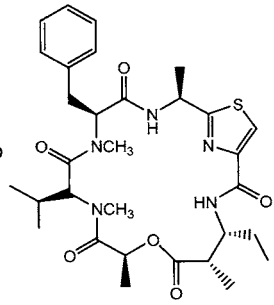

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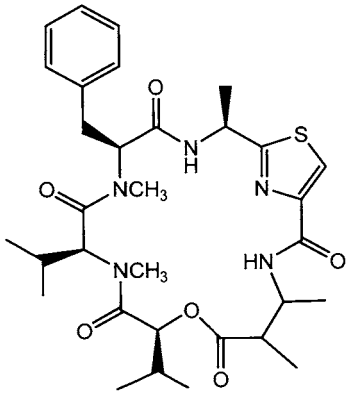

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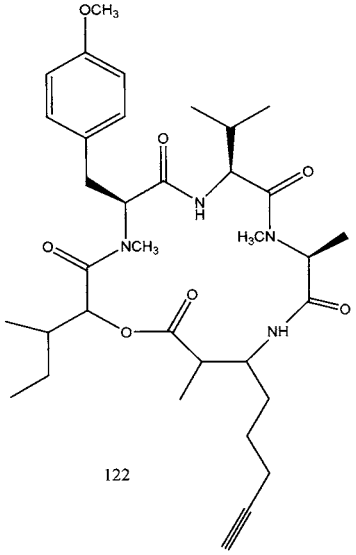

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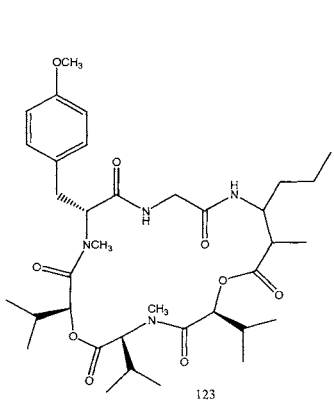

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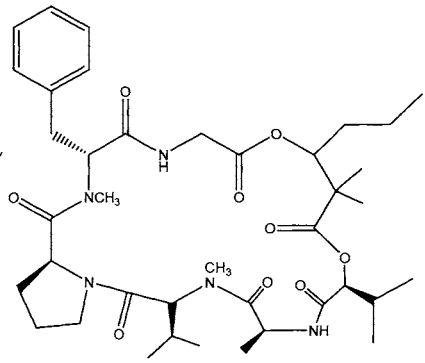

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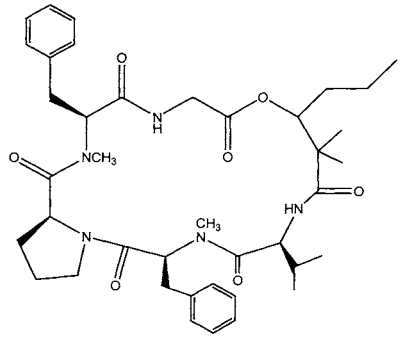

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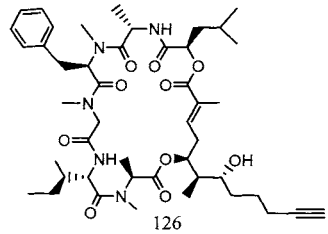

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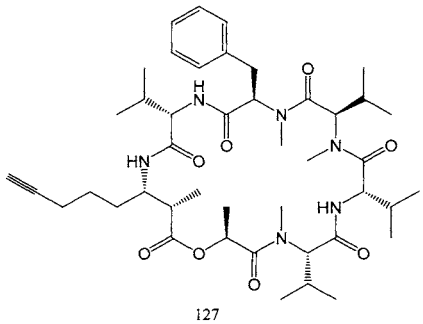


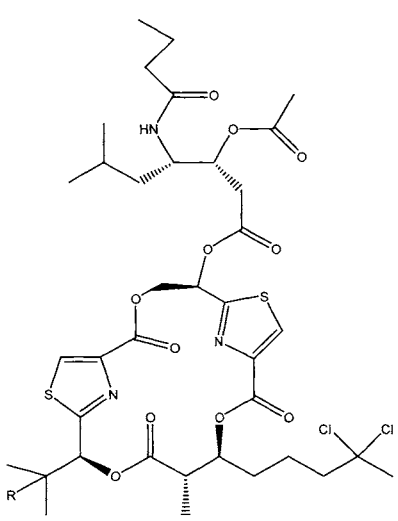
127

Bioactivity-guided fractionation of the extract of a species of *Lyngbya* from Palau yielded Palau'amide **126**, which had an IC_{50} value of 13 nM against KB cells.[89] Total synthesis of a cyclic depsipeptide with the proposed structure of **126** was achieved, which showed different rotation and NMR data compared to those reported for palau'amide. It implied that

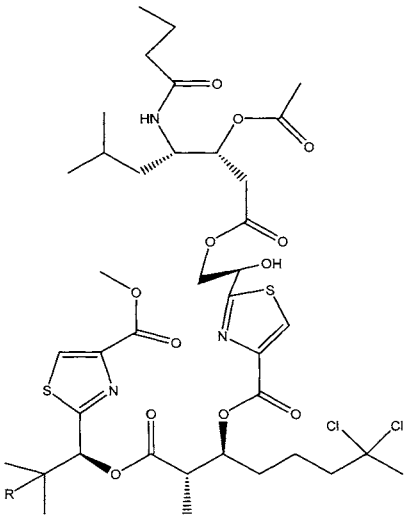
the conformation of synthetic compound was close to that of the palau'amide, but some stereochemistry in the structure of the palau'amide was incorrectly assigned.[90]

Palauan marine cyanobacteria, *Lyngbya* sp., produced a cyclic depsipeptide, ulongapeptin **127**, and the ulongapeptin is cytotoxic against KB cells with IC_{50} value of 0.63 μM.[91]

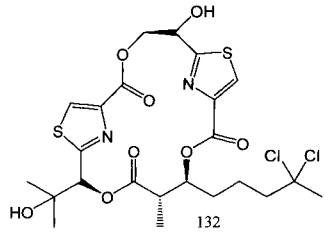
Five new lyngbyabellin analogs lyngbyabellins E–I **128-132** were isolated from the marine cyanobacterium *Lyngbya majuscula* collected from Papua New Guinea.[92] All five lyngbyabellins showed cytotoxicity to NCI-H460 human lung tumor and neuro-2a mouse neuroblastoma cell lines with LC_{50} values between 0.2 and 4.8 μM. Lyngbyabellin E **128**, at concentrations of 0.01–6.0 μM, disrupted the cellular microfilament network in A-10 cells.



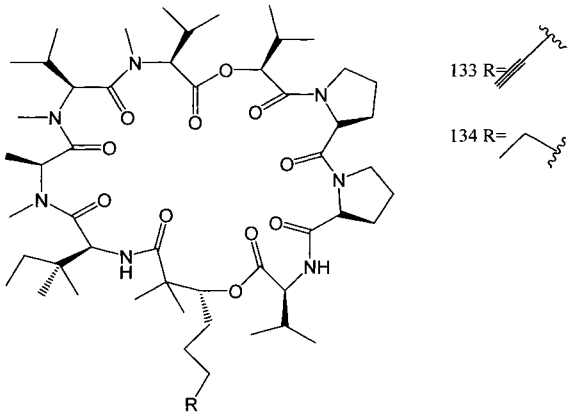
128 R=OH
129 R=H

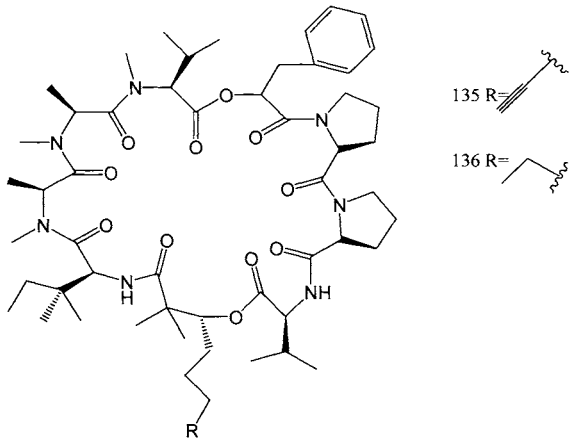


130 R=OH
131 R=H



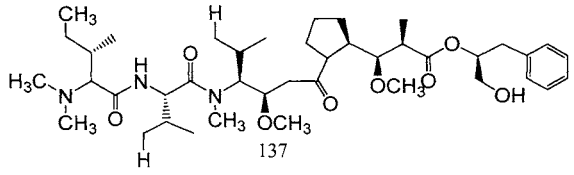
Four new depsipeptides wewakpeptins A-D **133-136** have been isolated from the marine cyanobacterium *Lyngbya semiplena* collected from Papua New Guinea.[93] The wewakpeptins represent an unusual arrangement of amino and hydroxy acid subunits relative to known cyanobacterial peptides and possess a bis-ester, a 2, 2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya) or 2, 2-dimethyl-3-hydroxyoctanoic acid (Dhoaa) residue, and a diprolyl group reminiscent of dolastatin 15. Wewakpeptin A and B were the most cytotoxic among these four depsipeptides with an LC_{50} of approximately 0.4 μM to both the NCI-H460 human lung tumor and the neuro-2a mouse neuroblastoma cell lines.





In addition, chemical investigations of the marine cyanobacterium of *Symploca* sp. led to the discovery of several new cytotoxic compounds.

Malevamide D **137**, a highly cytotoxic peptide ester from a Hawaiian marine cyanobacterium of *Symploca hydnoides*, showed IC_{50} values of 0.3-0.7 nM (0.2-0.5 ng/mL) against P-388 (mouse lymphoma), A-549 (human lung carcinoma), and HT-29 (human colon carcinoma) cell lines and 0.7 nM against the MEL-28(human melanoma) cell line.[94,95]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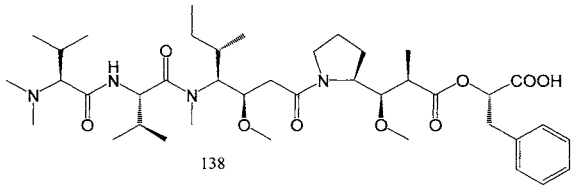
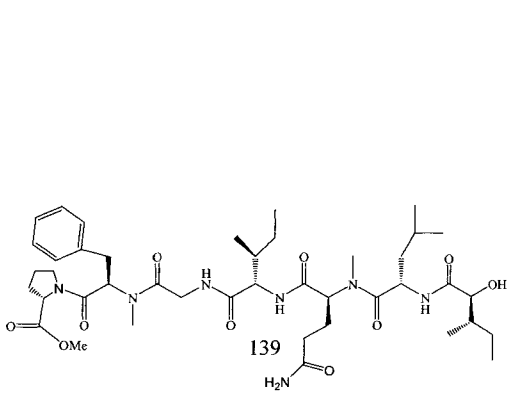

138

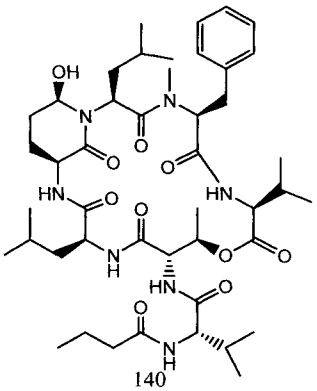
Symplostatin 3 **138**, a new analogue of dolastatin 10, was isolated from extracts of a variety of Hawaiian marine cyanobacterium *Symploca* sp. VP452.[96] Compound **138** possessed in vitro cytotoxicity with IC_{50}

values of 3.9 and 10.3 nM against KB and LoVo cell lines respectively. It required a higher concentration than dolastatin 10 to disrupt microtubules for its weaker in vitro cytotoxicity.

The acyclic peptide tasiamide **139** was isolated from the marine cyanobacterium *Symploca* sp.[97] collected at Short Drop-off in Palau. Tasiamide was cytotoxic against KB and LoVo cells with IC_{50} values of 0.48 and 3.47μg mL^{-1}, respectively.



139



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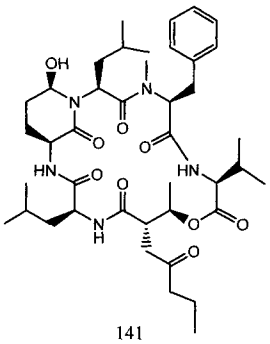
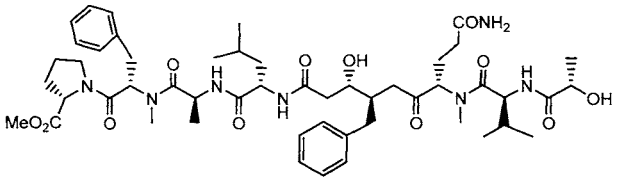


141

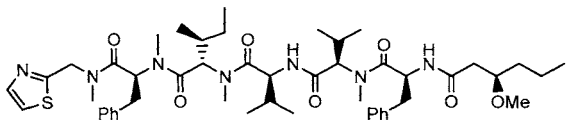
Two new depsipeptides, tasipeptins A **140** and B **141**, were isolated from a *Symploca* sp. collected from Palau, and both were cytotoxic toward KB cells with IC_{50} values of 0.93 and 0.82 μM, respectively.[98]

A new cytotoxic peptide named tasiamide B **142**, which displayed an IC_{50} value of 0.8 μM against KB cells in bioactivity, was isolated from the marine cyanobacterium *Symploca* sp.[99]

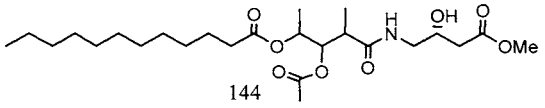


142

Micromide **143** and guamamide **144**, two new cytotoxins were isolated from marine cyanobacterium belonging to genus *Symploca* collected in Guam.[100] They both exhibited activities against KB cells with IC_{50} values at 260 and 1200 nM, respectively.



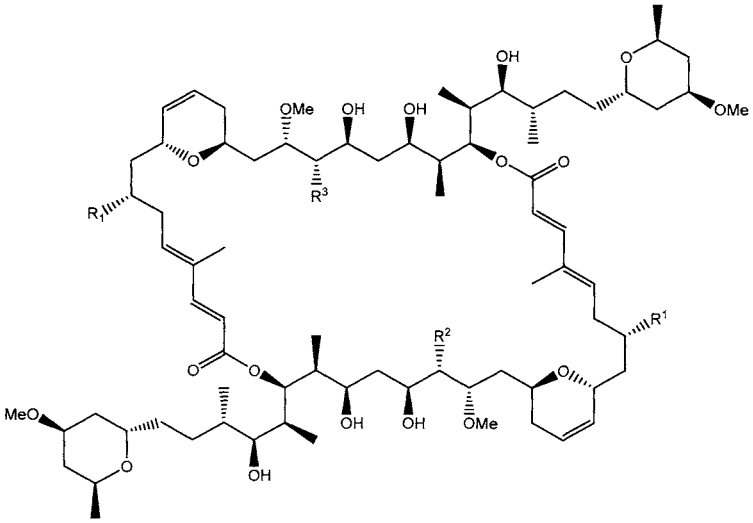
143



144

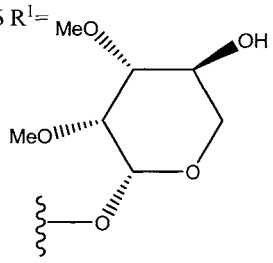
Bioassay-guided investigation of cyanobacterium *Geitlerinema* sp., from a Madagascar field collection, resulted in the isolation of two new glycosylated swinholides, ankaraholides A **146** and B **147**, along with the known compound swinholide A **145**.[101] Since swinholide-type compounds were previously only found from the heterotrophic bacteria of sponges, these findings raise intriguing questions about their true metabolic source. Ankaraholide A inhibited proliferation (IC_{50} values) in NCI-H460 (119 nM), neuro-2a (262 nM), and MDAMB-435 (8.9 nM) cell lines. Furthermore, in A-10 cells, **146** caused the complete loss of the filamentous (F)-actin at 30 and 60 nM, which is coincided with the dramatic changes in cell. Hence, the mode of action as well as the

biological activity of ankaraholide A **146** is comparable to that of swinholide A **145**. The additional sugar moieties in ankaraholide A do not affect its biological properties.



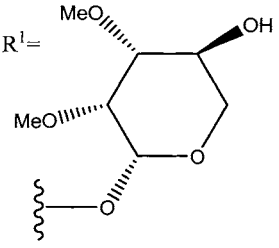
145 R^1 OH,R^2=R^3=Me

146 R^1=



R^2=R^3=H

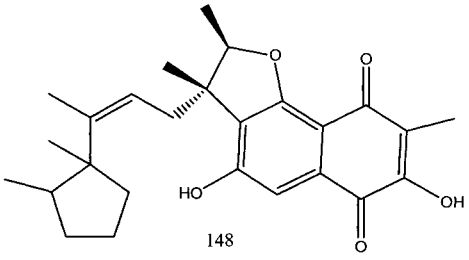
147 R^1=



R^2=H,R^3=Me

2. 4 From actinomycete

Neomarinone **149** which was originally assigned as **148** is a novel metabolite possessing a new sesquiterpene- and polyketide-derived carbon skeleton; it was isolated from fermentation broth of a taxonomically-novel marine actinomycete (strain #CNH-099).[102] Compound **149** displayed moderate in vitro cytotoxicity, IC_{50}= ca. 8 μg mL^{-1} against HCT-116 colon carcinoma and a mean IC_{50} value of 10 μM in the NCI's 60 cancer cell line panel. The biosynthesis of **149** was probed through feeding experiments with ^{13}C-labeled precursors (scheme 3), and the structure was confirmed *via* NMR characterization of the [U-$^{13}C_6$] glucose-enriched neomarinone and extensive 2D NMR spectrometry with the unlabeled one.[103]



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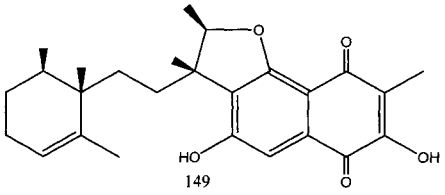
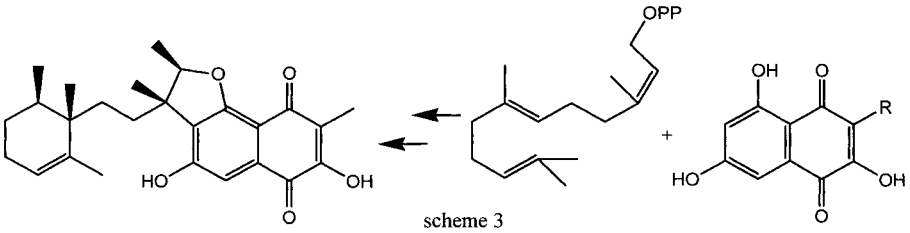
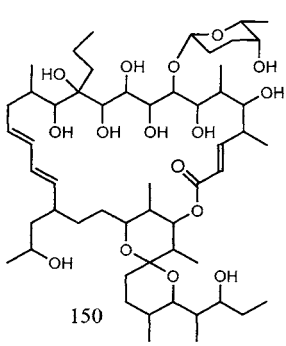


149



scheme 3

A novel bioactive macrolide, IB-96212 **150**, was isolated from the fermentation broth of a marine actinomycete, L-25-ES20-008.[104] The strain was isolated from homogenates of a sponge belonging to the genus *Microomnospora*. The macrolide showed a very strong cytotoxic activity against P-388, and low but significant activity against A-549, HT-29, and MEL-28 cell lines; it showed antibiotic activity against *Micrococcus luteus* as well.



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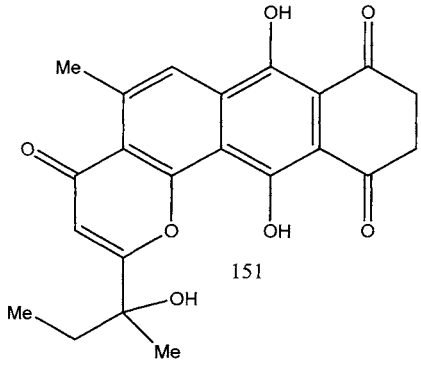

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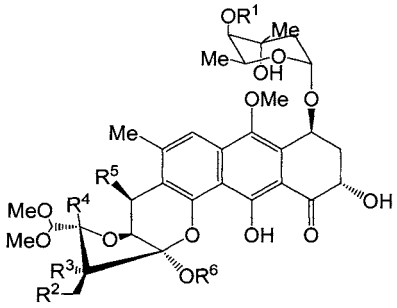
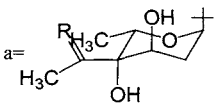
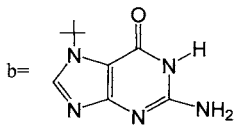


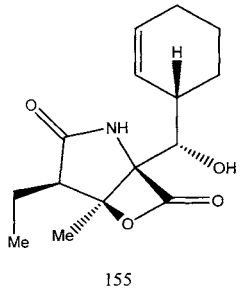




152 R^1=H; R^2-R^3=R^4-R^5=O; R^6=a:R=O
153R^1=$COCH_3$; R^2=R^3=R^4=R^5=OH; R^6=H
154 R^1=$COCH_3$; R^2=R^3=R^4=R^5=OH; R^6=a:R=O

A new antibiotic parimycin **151** with a novel structure was obtained from the culture broth of *Streptomyces* sp. isolate B8652.[105]In addition to the antibacterial activity, **151** also showed anti-tumor activity against human tumor cell lines GXF 251L (stomach cancer), H460, LXFA 629L, and LXFL 529L (lung cancer models), MCF-7 and MAXF 401NL (breast

cancer); MEXF 462L and MEXF 514L (melanomas) with IC_{70} values ranging from 0.9 to 6.7 μg mL^{-1}. Later, the ethyl acetate extract of the same strain delivered three new derivatives designated as trioxacarcins D-F **152-154**.[106] Compound **152** exhibited high anti-tumor and anti-malaria activities with IC_{70} values of 0.033 to 1.553μg mL^{-1}. In addition, **152** and **153** both showed high antibacterial activity.



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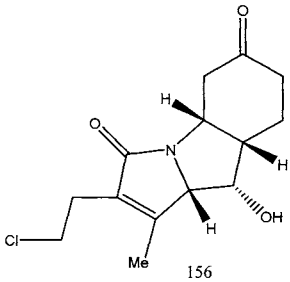

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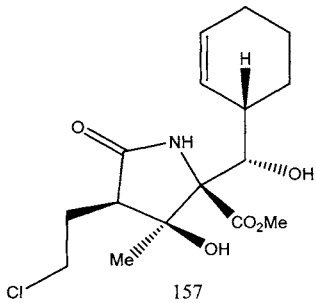

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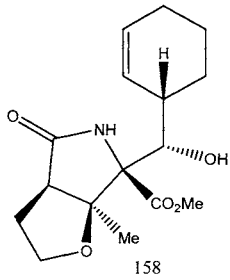

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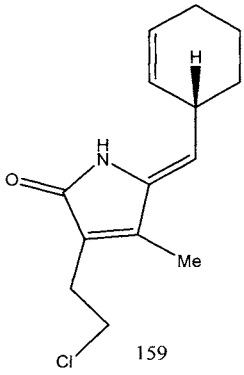

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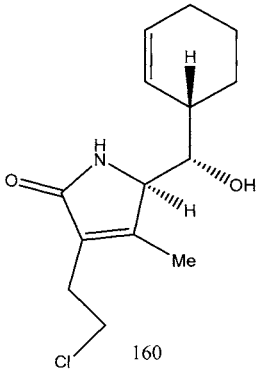

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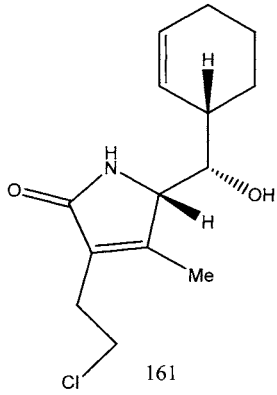

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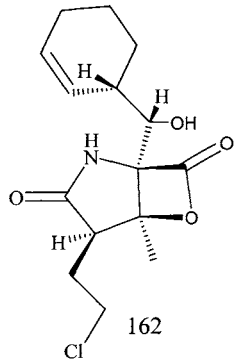
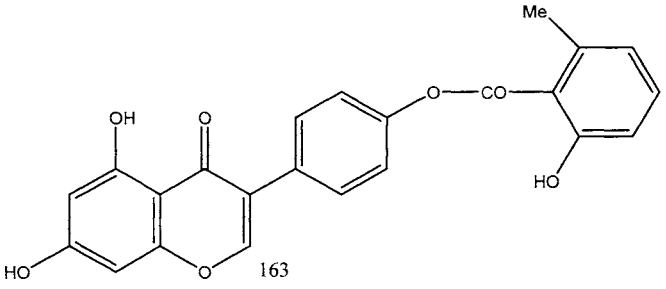


162

Cytotoxicity-guided fractionation of the crude extract of obligate marine actinomycete *Salinispora tropica* (strain CNB-392), isolated from a heat-treated marine sediment collected at depths of more than 1,000 meters, led to the isolation of salinosporamide A **157**.[107,108] Compound **157** displayed potent in vitro cytotoxicity against HCT-116 human colon carcinoma with an IC_{50} value of 11 ng mL^{-1}; it also displayed potent and highly selective activity in the NCI's 60-cell-line panel with a mean GI_{50} value (the concentration required to achieve 50% growth inhibition) of less than 10 nM and a greater than 4 log LC_{50} differential between resistant and susceptible cell lines. The greatest potency was observed against NCI-H226 non-small cell lung cancer, SF-539 CNS cancer, SK-MEL-28 melanoma, and MDA-MB-435 breast cancer (all with LC_{50} values less than 10 nM). When tested against purified 20S proteasome (Calbiochem, cat. no. 539158), **157** inhibited proteasomal chymotrypsin-like proteolytic activity with an IC_{50} value of 1.3 nM. Its significant bioactivities attracted lots of attention from chemists. The first stereo-controlled total synthesis of salinosporamide A **157** reported by Professor E. J. Corey confirmed its structure.[109] And the synthesis towards its intermediates, its analogues and **157** itself were accomplished by several schemes.[110-118] Structure-activity relationship studies of salinosporamide A found that marked reductions in potency in cell-based assays accompanied replacement of the chloroethyl group with un-halogenated substituents, while substitution of chlorine atom with Br

or I do not showed any change in potency. Slight stereochemical modifications significantly attenuated the activity.[119] An extensive study of the secondary metabolites produced by the same strain CNB-392 led to the isolation of seven related cytotoxic γ-lactams **155-161**.[120] The most important compounds are salinosporamide B **155**, which is the non-chloride-analogue of **162**, and salinosporamide C **156**, which is a decarboxylated pyrrole analogue. New SAR data for all eight compounds, derived from extensive testing against the human colon carcinoma HCT-116 and the 60-cell-line panel at the NCI, indicate that the chloroethyl moiety plays a major role in the enhanced activity of **162**.

A strain of *Streptomyces aureoverticillatus* (NPS001583) isolated from a marine sediment was found to produce a novel 22-atom macrocyclic lactam, aureoverticillactam **163**, which incorporates a conjugated triene and tetraene moieties.[121] Compound **163** had moderate activity against various tumor cell lines with EC_{50} values of 3.6±2.6 μM against HT-29 cells, 2.2±0.9 μM against B16-F10 cells, and 2.3±1.1 μM against Jurkat cells. Its acetyl derivative was prepared, which showed anti-tumor activity in an HT-29 assay with EC_{50} = 0.2625 μg mL^{-1}.[122]



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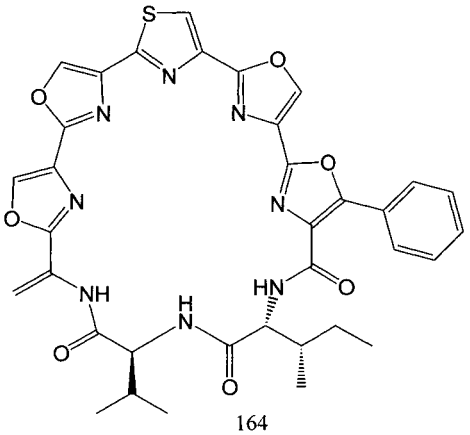

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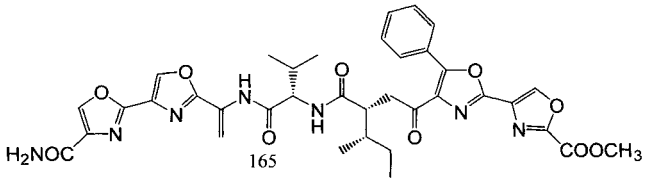


165

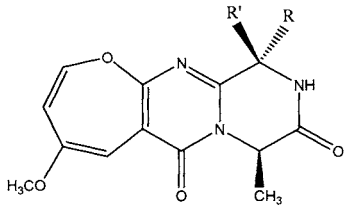
A new cytotoxic substance named mechercharmycin A **164**, and its linear congener mechercharmycin B **165** were isolated from marine-derived Thermoactinomyces sp. YM3-251.[123] **164** exhibited relatively strong antitumor activity, whereas **165** exhibited almost no such activity.

3 Antimicrobial agents

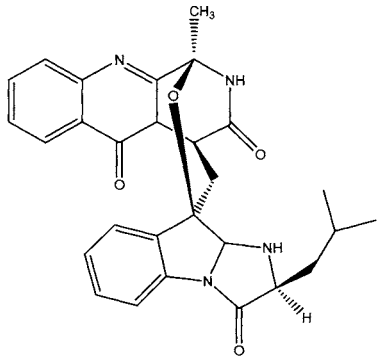
3. 1 From fungus

Three new oxepin-containing natural products oxepinamides A-C **166-168** and two new fumiquinazoline metabolites fumiquinazolines H-I **169-170** were isolated from organic extracts of the culture broth and mycelia of *Acremonium* sp., a fungus obtained from the surface of the Caribbean tunicate *Ecteinascidia turbinata*.[124] Compound **166** exhibited good anti-inflammatory activity in a topical RTX-induced mouse ear edema assay. Compounds **169** and **170** both exhibited weak anti-fungal activity towards Candida albicans in a broth microdilution assay. Total synthesis of (-)-fumiquinazolines H and I were succeeded.[125-127] Synthesis

of (-)-fumiquinazoline H *via* a 15-step reaction path, using $FmocNHCH(CH_2SePh)CO_2H$ as a dehydroalanine precursor that spontaneously eliminated benzeneselenol without oxidation under the cyclization conditions were reported.[127]



166 R=$CH(CH_3)CH_2CH_3$, R'=OH
167 R=OH, R'=$CH(CH_3)CH_2CH_3$
168 R=$CH_2CH(CH_3)_2$, R'=OCH_3



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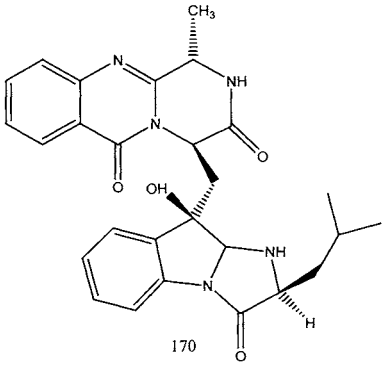
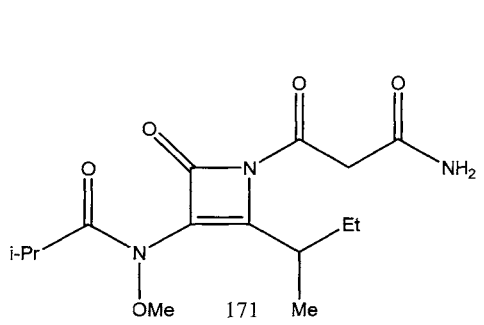


170

Kasarin **171**, a novel azetinone compound from a marine fungus, exhibited antibacterial activity and weak cytotoxicity.[128]



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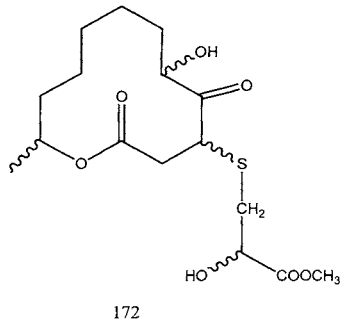

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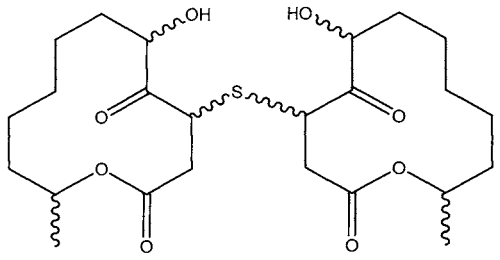

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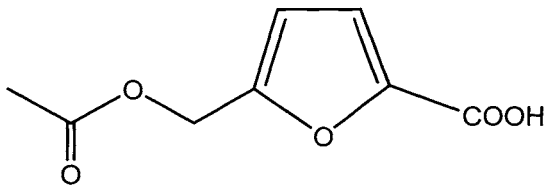
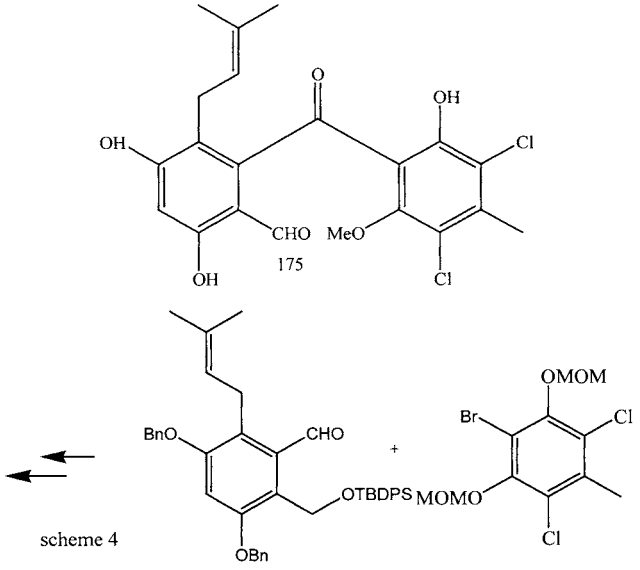


174

Bioactivity-guided fractionation of organic extracts of *Cladosporium herbarum,* isolated from the marine sponge *Callyspongia aerizusa,* yielded two new macrolide metabolites, pandangolide 3 **172** and 4 **173**, and a anti-microbial active (against *Bacillus subtilis* and *Staphylococcus aureus*) new furan carboxylic acids, acetyl Sumiki's acid **174**.[129]

A *Pestalotia* species of fungus, isolated from the surface of the brown alga *Rosenvingea* sp. from Bahamas, produced a chlorinated benzophenone antibiotic pestalone **175** only when a unicellular marine bacterium was co-cultured in the fungal fermentation.[130] This compound

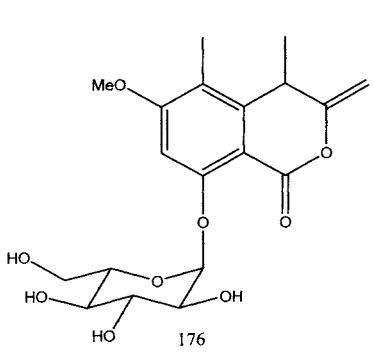
was not detected when either organism was cultivated individually. Pestalone showed potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MIC = 37 ng mL^{-1}) and vancomycin-resistant *Enterococcus faecium* (MIC = 78 ng mL^{-1}). It was also bio-assayed in the National Cancer Institute (60 human tumour cell line panel), where moderate *in vitro* cytotoxicity (mean GI_{50} = 6.0μM) has been demonstrated. The first synthetic study towards pestalone obtained its synthetic analogues.[131] Total synthesis of pestalone was also successfully accomplished (scheme 4).[132]



scheme 4

Preliminary biological activities screening of the crude extract of the marine fungus *Halorosellinia oceanica* BCC 5149 led to the isolation of structurally unique isocoumarin glucosides, halorosellins A **176** and B **177**, together with other new minor metabolites including 4, 8-dihydroxy-6-methoxy-4, 5-dimethyl-3-methyleneisochroman-1-one **178**, 3-acetyl- 7-hydroxy-5-methoxy-3, 4-dimethyl-3*H*isobenzofuran-1-one **179** and an ophiobolane sesterterpene, 17-dehydroxyhalorosellinic acid **180**.[133] Compound **179** exhibited mild anti-mycobacterial activity (MIC

value of 200 μg mL^{-1}).



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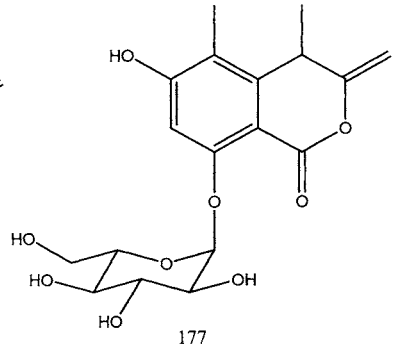

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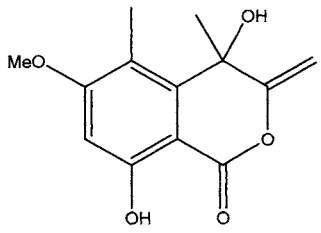

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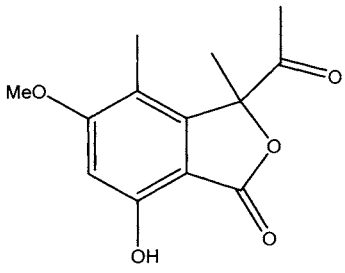

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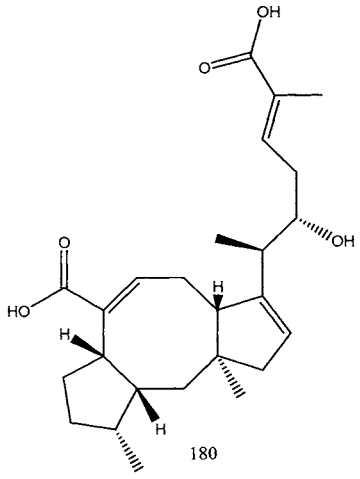
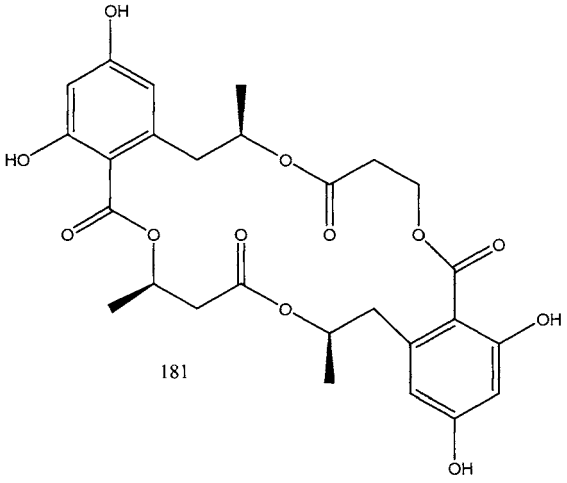


180

Cultures of the marine fungus Hypoxylon oceanicum (LL-15G256) were found to have potent anti-fungal activity in assays designed to detect

inhibitors of fungal cell wall biosynthesis. Bioactivity-guided isolation provided a series of unreported macrocyclic polyesters named as 15G256i **181**, 15G256v **182**, 15G256o **183**, 15G256a-2 **184**, 15G256b-2 **185**, 15G256n **186** and 15G256p **187**,[133] their biosynthetic sequences were proposed. 15G256i **181** and 15G256v **182** showed mild anti-fungal activity in tests using Neurospora crassa OS-1 in vitro.



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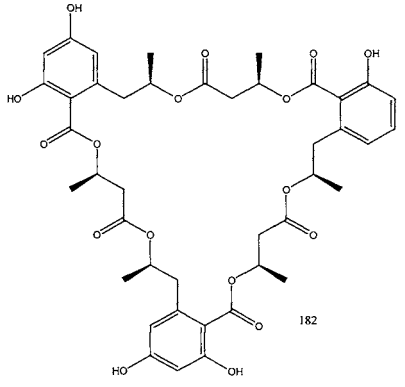

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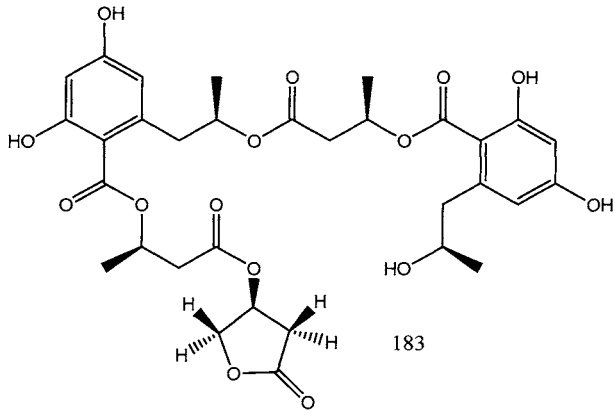

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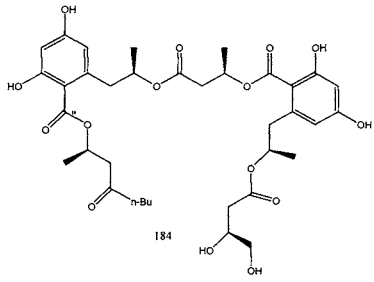

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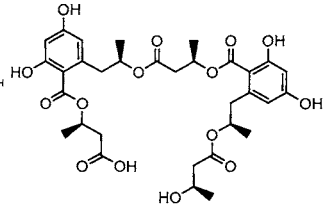

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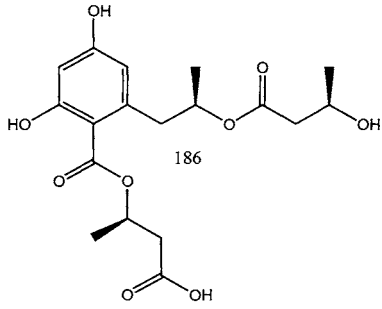

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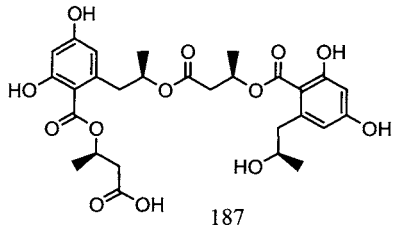
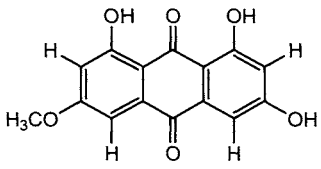


187

The fungus *Curvularia lunata*, isolated from the marine sponge *Niphates olemda*, yielded the new 1,3,8-trihydroxy-6-methoxyanthraquinone, named lunatin **188**.[134] Lunatin was found to be active against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli.*

Fungal isolates of culture filtrates of the fungi *Penicillium cf.*

montanense, which was obtained from the marine sponge *Xestospongia exigua* collected from the Bali Sea, Indonesia, yielded three novel decalactone metabolites, xestodecalactones A **189**, B **190**, and C **191**.[135] These compounds are structurally related to a number of biologically active metabolites found in terrestrial fungal strains. Due to the additional stereocenter at C-9, **190** and **191** are diastereomers. Compound **190** was found to be active against the yeast *Candida albicans*; and caused inhibition zones of 25, 12, and 7 mm at concentrations of 100, 50, and 20 μmol, respectively in the agar diffusion assay. Their synthetic derivatives were obtained.[136,137] The first synthesis of xestodecalactone A **189** was accomplished by the use of methyl 5-hydroxyhexanoate in its R- or S-configured form, or as its racemate as the precursors.[138] Both enantiomers of **189** as well as the racemic compound were obtained. Comparison of these synthetic products with the natural product by circular dichroism (CD) spectroscopy and by HPLC on a chiral phase revealed the natural product to have the (R)-configuration.



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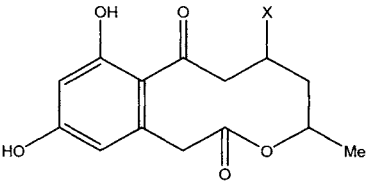
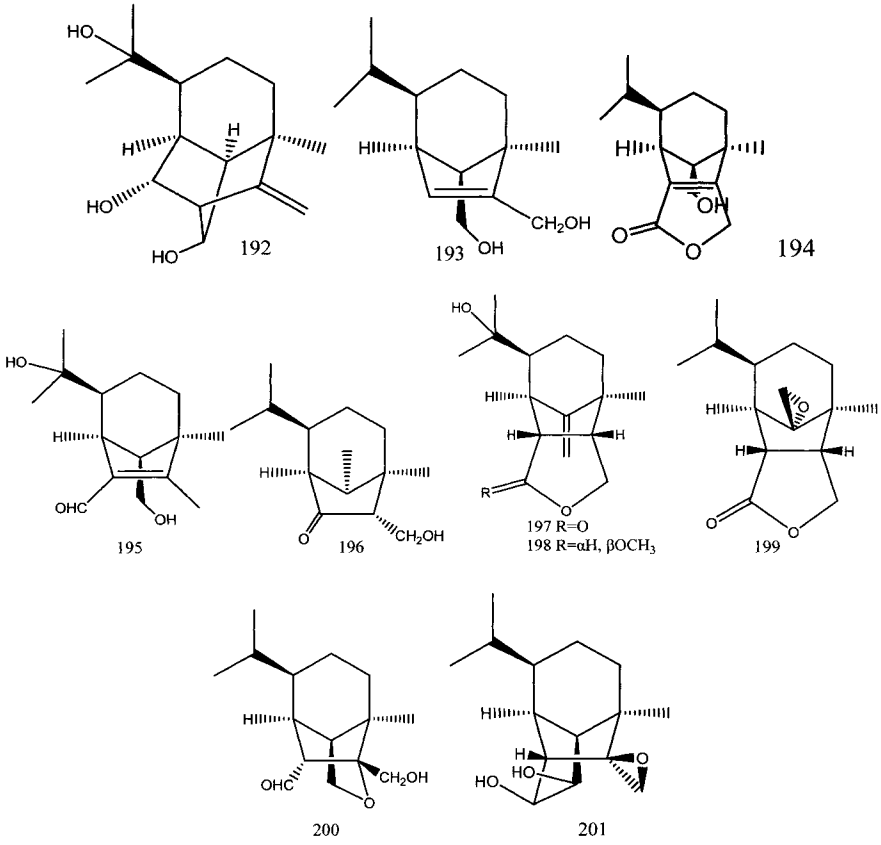
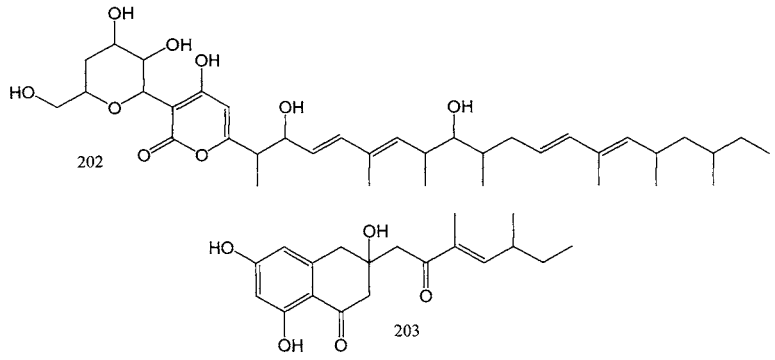


189 X=H
190 X=OH; 9,11-*cis*
191 X=OH; 9,11-*trans*

From the inner tissue of the marine red alga *Liagora viscida*, fungus *Drechslera dematioidea* was isolated. The fungus was mass cultivated to investigate the secondary metabolite content, and from which 10 new sesquiterpenoids: isosativenetriol **192**, drechslerines A **193** and B **194**, 9-hydroxyhelminthosporol **195**, drechslerines C-G **196-200**, and sativene epoxide **201** were isolated.[139] Most of these compounds demonstrated moderate anti-fungal activity. Compounds **198** and **200** also exhibited anti-plasmodial activity against *Plasmodium falciparum* strains K1 and NF54.

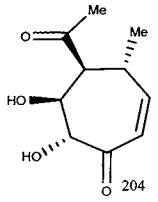


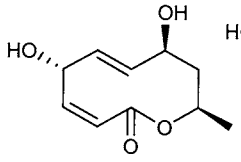
YM-202204 **202**, a new anti-fungal antibiotic, was found in the culture broth of marine fungus *Phoma* sp. Q60596.[140] This antibiotic exhibited potent anti-fungal activities against *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*; and also inhibited glycosyl- phosphatidyl-inositol (GPI)-anchoring in yeast cells. The IC_{80}s of **202** against *Candida albicans* ATCC10231, *Cryptococcus nesformans* TIMM0362, *Aspergillus fumigatus* TIMM1776, and *Saccharomyces cerevwasiae* YFC805 were 6.25 μg mL^{-1}, 1.56 μg mL^{-1}, 12.5 μg mL^{-1} and 1.56 μg mL^{-1}, respectively.



Bioactivity-guided fractionation of the culture of Keissleriella sp., a marine filamentous fungus (strain number: YS4108), afforded an anti-fungal metabolite with a new carbon skeleton. The molecule was identified as 3, 6, 8-trihydroxy-3-[3, 5-dimethyl-2-oxo-3(E)-heptenyl]-2, 3- dihydronaphthalen-1 (4H)-one **203**.[141] In vitro anti-fungal assay of **203**, inhibitory activity to the growth of the human pathogenic fungi *Candida albicans*, *Tricophyton rubrum* and *Aspergillus niger* (with MICs of 40, 20 and 80 μg mL^{-1}) has been evidenced. Keissleriella sp. However, after repeated cultivation of this species, a new anti-microbial metabolite, keisslone **204**, was found.[142] This compound **204** inhibited the human pathogenic fungi *Candida albicans*, *Trichophyton rubrum,* and *Aspergillus niger* at MICs of 50, 70, 40 μg mL^{-1}, respectively.

Two new 10-membered macrolides, modiolides A **205** and B **206**, and a new linear pentaketide, modiolin **207**, were isolated from the culture broth of fungus *Paraphaeosphaeria* sp. (N-119), which was separated from a marine horse mussel. [143] Both compounds **205** and **206** showed antibacterial activity against *Micrococcus luteus* (MIC value 16.7 μg mL^{-1}) and anti-fungal activity against *Neurospora crassa* (MIC value 33.3 μg mL^{-1}).





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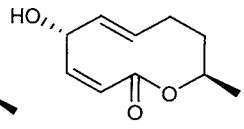

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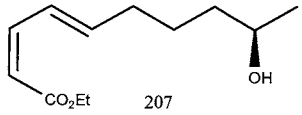
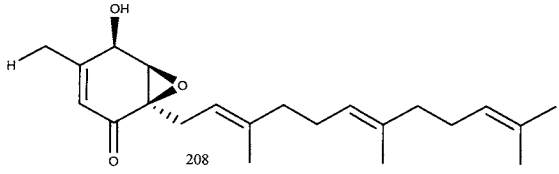


207

New polyoxygenated farnesylcyclohexenones, 7-deacetoxyyanuthone A **208** and its 2,3-hydro derivative **209**, were isolated from genus *Penicillium*.[144] Compounds **208** showed moderate in vitro cytotoxicity in a panel of five human tumor cell lines, and also exhibited mild in vitro antibacterial activity against methicillin-resistant and multidrug-resistant *Staphylococcus aureus* (MIC, 50 μg mL^{-1}).



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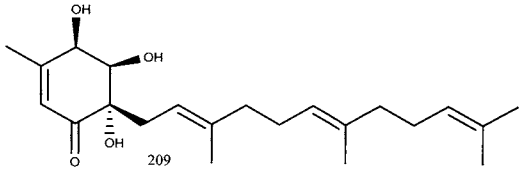
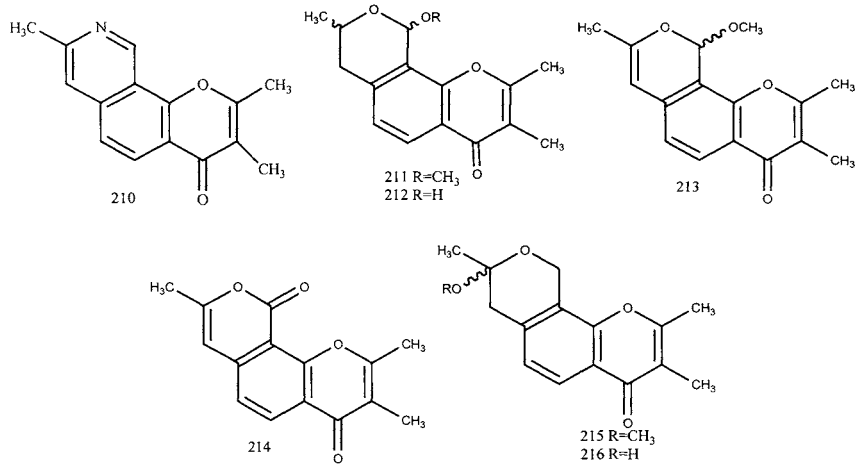
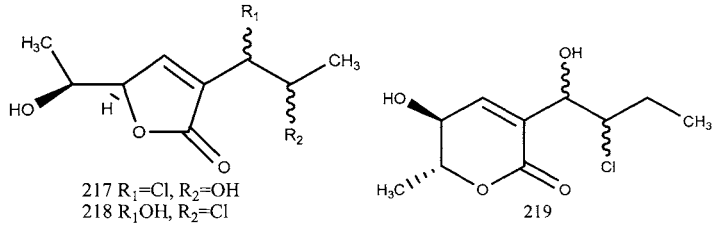


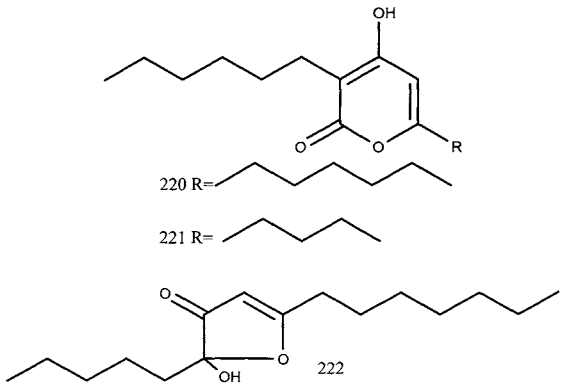
209

From the marine sponge *Xestospongia exigua* collected from Indonesia, fungus *Aspergillus versicolor* was isolated. Following the cultivation in a seawater-based medium seven new angular tricyclic chromone derivatives, aspergillitine **210** and aspergiones A-F **211-216**, were obtained from the mycelia and culture filtrate.[145] Aspergillitine **210** displayed only moderate antibacterial activity against *Bacillus subtilis*.



Three new chlorine containing compounds, 8-chloro-9-hydroxy-8, 9-deoxyasperlactone **217**, 9-chloro-8-hydroxy-8, 9-deoxyasperlactone **218**, and 9-chloro-8-hydroxy-8, 9-deoxyaspyrone **219** were obtained from a marine-derived fungus *Aspergillus ostianus strain* TUF 01F313 isolated from a marine sponge at Pohnpei.[146] Compound **217** inhibited the growth of marine bacterium *Ruegeria atlantica* found on a glass plate submerged in coastal water at 5 μg/disc (inhibition zone: 12.7 mm), while **218** and **219** were active at 25 μg/disc (10.1 and 10.5 mm, respectively).

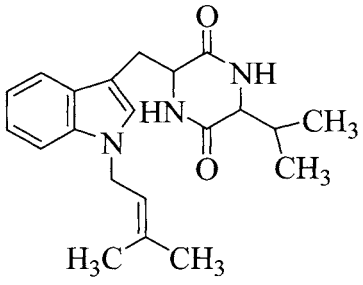




Inhibitors of the enzymes involved in fatty acid biosynthesis (FAB) have been reported as antibacterial agents. The search for new FAB inhibitors, using a lacZ reporter cell-based screening, led to several confirmed hits. Thereinto, a *Pseudomonas* sp. based on 16S profiling, was found to produce two α-pyrones **220** and **221**. The pyrones were unstable under acidic conditions, and they were rearranged into a furanone derivative **222**.[147] Of these compounds, pyrone **220**was the most active one with MICs (μg mL^{-1}) against *B. subtilis* (1-2), MRSA (2-4), *M. catarrhalis* (4), and VRE (2-64). Effects of **221** on macromolecular synthesis and membrane functions were tested using *B. subtilis*. Pyrone **220** nonspecifically inhibited the incorporation of radiolabeled precursors into DNA, RNA, and protein within 5 minutes of drug exposure, similar to that of triclosan. Both compounds also inhibited the cellular uptake of these radiolabeled precursors. Pyrone **220** and triclosan were membrane-active (BacLight test); however, pyrone **220** (at ≤128 μg mL^{-1} concentration) was not hemolytic to human RBCs in contrast to triclosan, which was hemolytic at 16 μg mL^{-1}. These data suggest that pyrone **220**, unlike triclosan, selectively affected bacterial membrane function.

Marine fungi, which produces anti-fungal compounds, were screened against *Pyricularia oryzae* (*P.oryzae*); and from the culture extracts of marine fungus M-3 isolated from laver (*Porphyra yezoensis*), a novel

metabolite, diketopiperazine **223**, was isolated.[148] This compound showed potent anti-fungal activity against *P.oryzae* at MIC of 0.36 μM.



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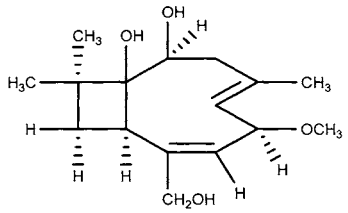
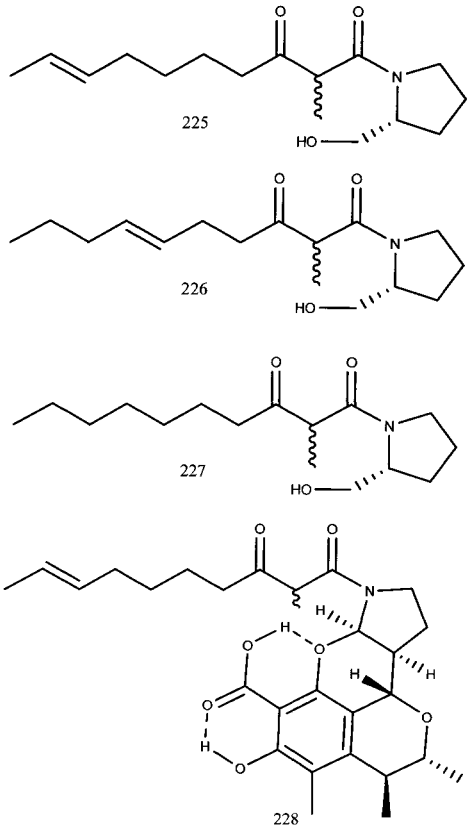


224

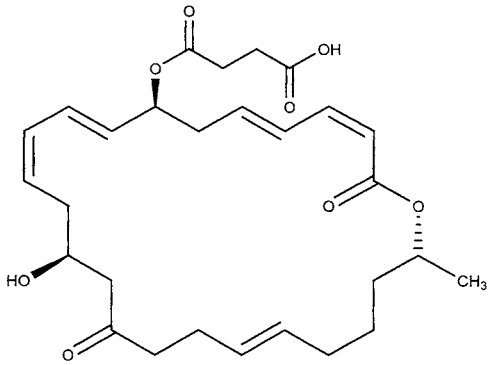
A new sesquiterpene of the caryophyllene series, fuscoatrol A **224** was isolated from the marine fungus *Humicola fuscoatra (Traaen)* KMM 4629 associated with the Kuril colonial ascidium.[149] Compound **224** possesses an antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* (MIC = 12.5 μg mL^{-1}) and exhibits a cytotoxic action on the developing eggs of sea urchin *Strongylocentrotus intermedius* (MIC_{50} = 40 μg mL^{-1}).

Three new pyrrolidine alkaloids, scalusamides A-C **225-227** were isolated from the cultured broth of the fungus *Penicillium citrinum*, which was separated from the gastrointestine of a marine fish.[150] Each of **225-227** was found to be a mixture of epimers at C-7. Scalusamide A **225** exhibited antifungal activity against *Cryptococcus neoformans* (MIC 16.7 μg/mL) and antibacterial activity against *Micrococcus luteus* (MIC 33.3 μg/mL). A novel tetracyclic alkaloid, perinadine A **228**, was isolated from the same fungus *Penicillium citrinum.* [151] Biogenetically, perinadine A **228** may be derived from citrinin, a well-known mycotoxin, and a scalusamide A-type pyrrolidine alkaloid. It showed weak cytotoxicity against murine leukemia L1210 cells (IC50, 20 μg/mL) and antibacterial activity against *Micrococcus luteus* (MIC, 33.3 μg/mL) and *Bacillus subtilis* (MIC, 66.7 μg/mL).



3. 2 From bacteria

EtOAc extract of a marine *Bacillus* sp. Sc026 culture broth, in bioactivity-guided fractionation, led to the isolation of two new compounds, 7-*O*-succinyl macrolactin F **229** and 7-*O*-succinyl macrolactin A **230**.[152] Both exhibited antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. (inhibition zones of 8-28 mm at 50-100 μg/disk).



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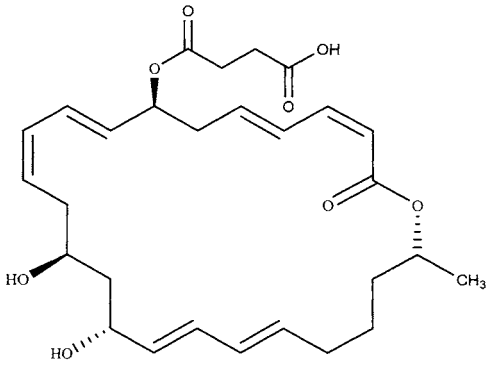
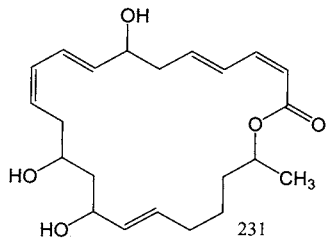


230

Seven new macrolactins G-M **231-237** were isolated from a culture broth of *bacillus* sp. PP19-H3.[153] The strain was isolated from macroalga *Schizyvenia dubyi* collected from the Omaezaki coast of Shizuoka prefecture in Japan. All compounds were examined and showed relatively weak antibacterial activities against bacteria *Staphylococcus aureus* (IFW 12732) and *Bacillus subtilis* (IFO 3134).



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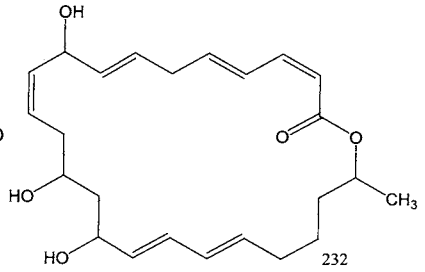

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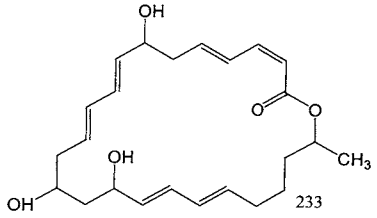

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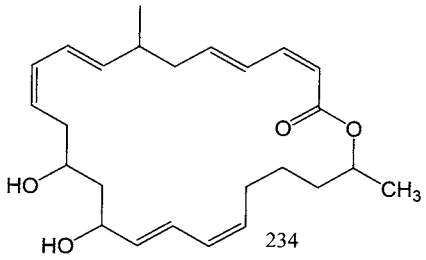

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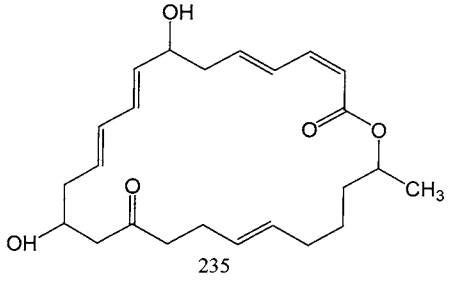

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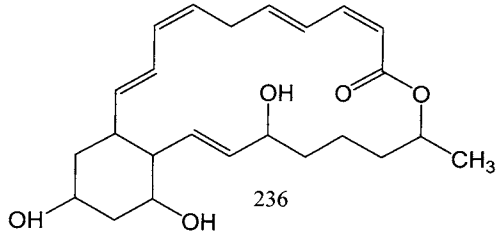

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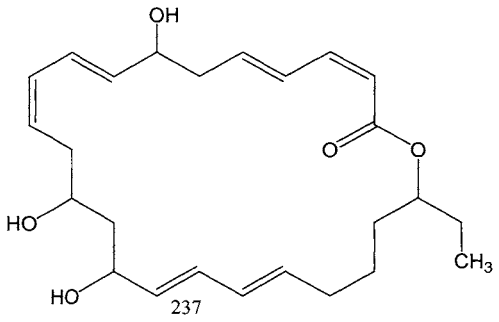
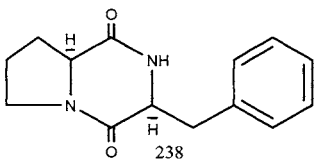


237

Bacterial strains CF-20 (CECT5719) and C-148 (CECT5718), isolated from cultures of larvae of molluscs, produced substances **238-242** with strong antibiotic activity against *Vibrio anguillarum* (MIC: 0.03-0.07 μg mL^{-1}).[154, 155] These substances were identified as the DD-diketopiperazines cyclo(D)-Pro-(D)-Phe **238** cyclo(D)-Pro-(D)-Leu **239**, cyclo(D)-Pro-(D)-Val **240**, cyclo(D)-Pro-(D)-Ile **241**, and cyclo-*trans*-4-OH-(D)-Pro-(D)-Phe **242**.



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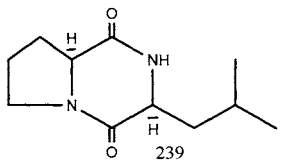

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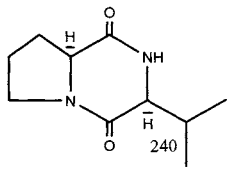

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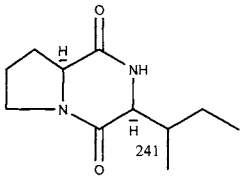

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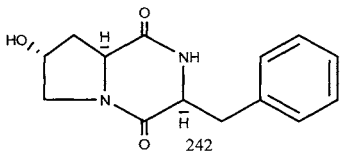

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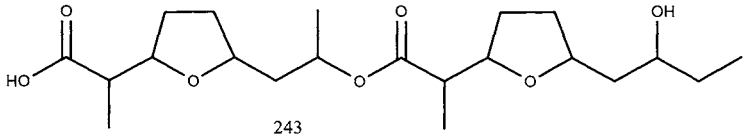
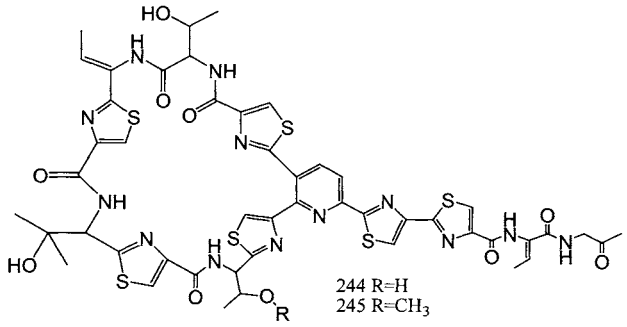


243

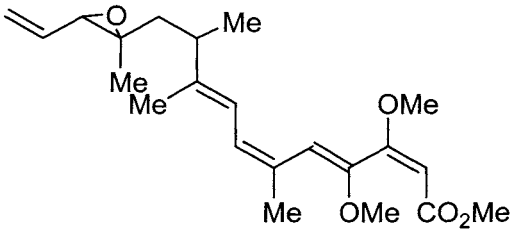
A new compound, bonactin **243**, was isolated from the liquid culture of a *Streptomyces* sp. BD21-2 obtained from a shallow-water sediment

sample from Kailua Beach, Oahu, Hawaii.[156] Compound **243** displayed anti-microbial activity against both Gram-positive and Gram-negative bacteria as well as anti-fungal activity.

Novel antibiotics, YM-266183 **244** and YM-266184 **245**, were found in the culture broth of *Bacillus cereus* QN03323 isolated from the marine sponge *Halichondria japonica*.[157] They both exhibited potent antibacterial activities against staphylococci and enterococci including multiple drug resistant strains with MICs between 0.02 and 1.56μg mL^{-1}. They were inactive against Gram-negative bacteria.

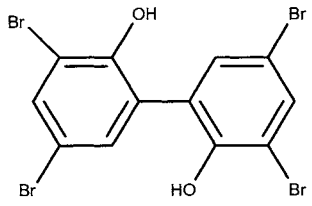


Geometrical isomers of haliangicin **346** were separated from the extract of the marine myxobacterium *Haliangium ochraceum.* The major component, *cis*-haliangicin **246**, was a cis-isomer of the epoxide portion of haliangicin **346**. Haliangicins B-D **247-249** were geometrical isomers of the polyene moiety, and present as an inseparable mixture of *cis* and *trans* epoxide isomers.[158] Their anti-fungal activities were also evaluated, and they showed lower activities than haliangicin.



Compound	Geometry			
	C2	C4	C6	Epoxide
cis-halinagicin (246)	*E*	*Z*	*Z*	*cis*
haliangicin B(247) (+*cis isomer*)	*Z*	*Z*	*Z*	*trans* (+*cis*)
haliangicin C(248) (+*cis isomer*)	*E*	*E*	*Z*	*trans* (+*cis*)
haliangicin D(249) (+*cis isomer*)	*E*	*E*	*E*	*trans* (+*cis*)

An anti-MRSA (methicillin-resistant Staphylococcus aureus) substance MC21-A **250** was purified from the methanol extract of the cells of *Pseudoalteromonas phenolica* O-BC30T.[159] Its anti-MRSA activity against 10 clinic isolates of MRSA was comparable to that of vancomycin (MC21-A MICs, 1 to 2 μg mL^{-1}; vancomycin MICs, <0.25 to 2 μg mL^{-1}). In a "time-kill" study, MC21-A demonstrated bactericidal activity, and its killing rate was much higher than that of vancomycin. Furthermore, **250** was not cytotoxic to human normal fibroblast, rat pheochromocytoma, and Vero cells at concentrations up to 50 μg mL^{-1}. All research results suggested that MC21-A **250** might be useful as a lead compound in the development of new types of anti-MRSA substances with modes of action different from those of vancomycin and teicoplanin.



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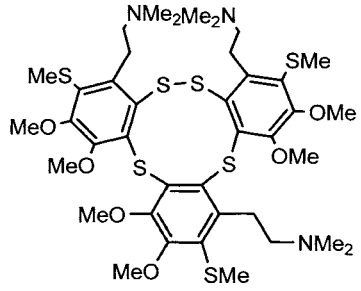


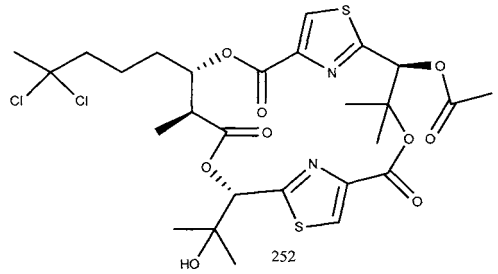
251

A novel tribenzotetrathiepin alkaloid, named lissoclibadin 1 **251**, was isolated from the ascidian Lissoclinum sp. (cf. L. badium Monniot and

Monniot, 1996).[160] Compound **251** inhibited the growth of the marine bacterium *Ruegeria atlantica* (15.2 mm at 20 μg/disk).

3. 3 From cyanobacteria

Hectochlorin **252** was isolated from *Lyngbya majuscula* collected from Hector Bay, Jamaica, and Boca del Drago Beach, Bocas del Toro, Panama.[161] Hectochlorin showed both potent inhibitory activity toward the fungus *Candida albicans* and a unique profile of cytotoxicity by the COMPARE algorithm. In addition, hectochlorin demonstrated its ability to promote actin polymerization with EC_{50} value at 20 μM, but was unable to displace a fluorescent phalloidin analogue from polymerized actin. Structurally, hectochlorin resembled dolabellin and compounds of the lyngbyabellin class. And its successful total synthesis was reported.[162, 163] The synthesis effort was initiated to develop a flexible route to hectochlorin which would allow access to analogues with potentially improved activity and/or attributes relative to the natural product.



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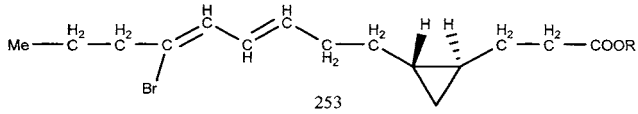
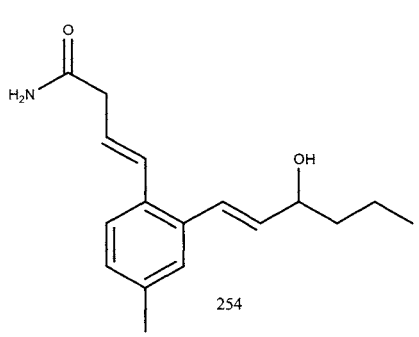


253

The novel antifungal compound majusculoic acid **253** was isolated from a marine cyanobacterial mat microbial community.[164] **253** exhibits antifungal activity against *Candida albicans* ATCC 14503 (MIC 8 μM).

3. 4 From actinomycete

A marine actinomycete (MST-MA190) from a beach sand sample collected near Lorne on the southwest coast of Victoria, Australia, yielded two new aromatic amides, lorneamide A **254** and lorneamide B **255**, both of which are novel tri-alkyl-substituted phenyl compounds.[165, 166] Lorneamide A was active against *Bacillus subtilitis*, exhibiting a LD_{99} of 50 μg mL^{-1}.



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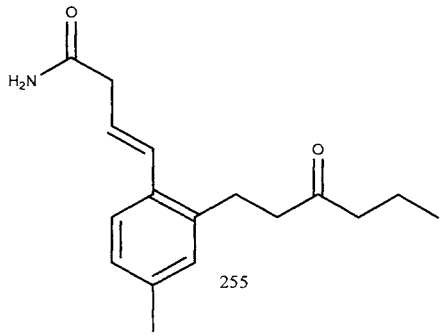
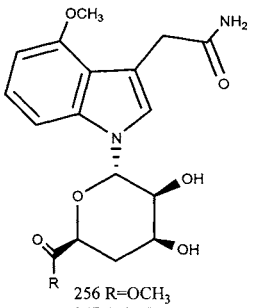


255

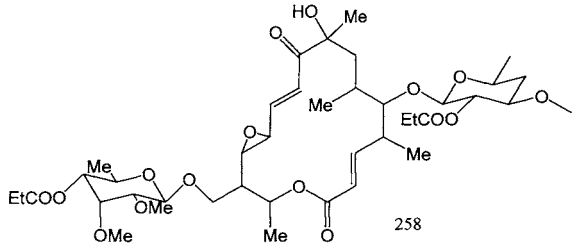


256 R=OCH_3
257 R=NH_2

Two new indole nucleosides kahakamides A **256** and B **257** were isolated from the actinomycete *Nocardiopsis dassonvillei* which is obtained from a shallow water sediment sample collected from the island of Kauai, Hawaii.[167] Compound **256** exhibited anti-microbial activity toward the Gram positive bacterium *Bacillus subtilis*.

In the screening of marine streptomycete isolates for bioactive components, a new macrolide antibiotic designated as chalcomycin B **258**

was isolated from the culture broth of a marine *Streptomycete* isolate B7064 as the active principal component.[168] Antibacterial and anti-fungal activities of **258** were qualitatively determined. And the preparations of chalcomycin derivatives were also reported.[169]



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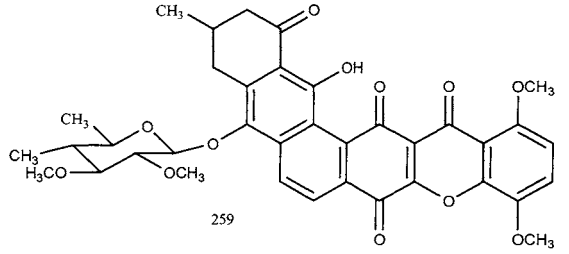
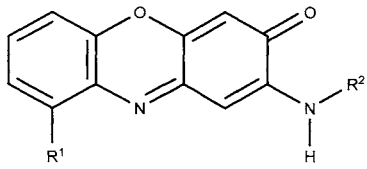


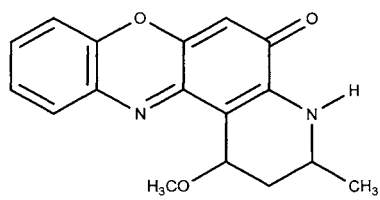
259

Strain BL-42-PO13-046 identified as *Astinomadura* sp. was isolated from the northern coast of Spain. A new compound, IB-00208 **259**, was isolated from the fermentation broth of the mycelial cake of this strain.[170, 171] IB-00208 exhibited good antibiotic activity against Gram-positive organisms, however, poor activity against the Gram-negative ones. Besides, this compound also showed a potent cytotoxic activity against several lines of tumor cells, both human and murine.

Three novel antibiotics, chandrananimycin A **260**, B **261**, and C **262**, were isolated from the culture broth of a marine *Actinomadura* sp.[172] isolate M048. Their anti-microbial activities ranged from moderate to strong, and they were also active against some human tumor cell lines.

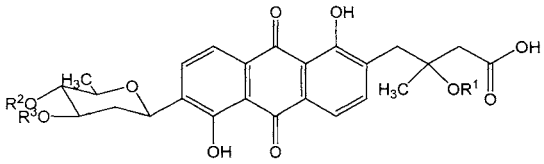


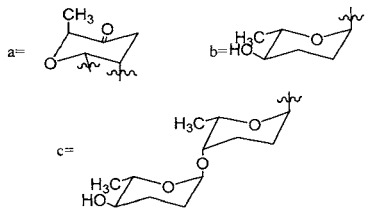
260 R^1=OH, R^2=$COCH_3$
261 R^1=H, R^2=$COCH_2CH_3$



262

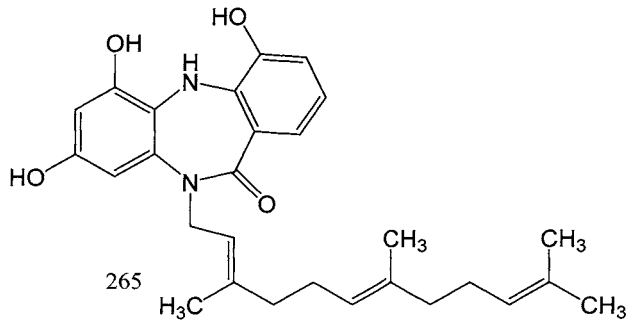
Himalomycin A **263**and B **264**, two new anthracycline antibiotics, were isolated from the culture broth of the marine *Streptomyces* sp.[173] isolate B6921, and both exhibited strong antibacterial activity but no anti-algal activity against the tested micro-algae.





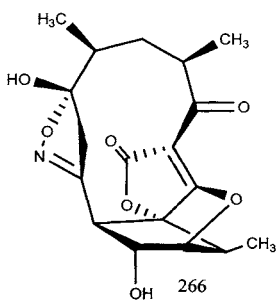
263 R^1=c,R^2=C-1",R^3=C-2" of a
264 R^1=c,R^2=b, R^3=H

A new dibenzodiazepine alkaloid, diazepinomicin **265**, was isolated from the culture of a marine actinomycete of the genus *Micromonospora*.[174] Compound **265** showed modest anti-microbial activity against selected Gram-positive bacteria with MICs of about 32 μg mL^{-1}. The method of expression the biosynthetic pathway genes in transformed host cells, and the novel polynucleotide sequences and their encoded proteins involved in the biosynthesis of **265** were discussed.[175] Derivatives of **268** were prepared.[176]

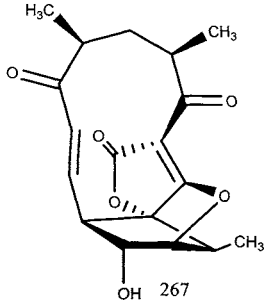


265

A screening method was established to detect inhibitors of the biosynthetic pathways of aromatic amino acids and *para*-aminobenzoic acid, the precursor of folic acid, using an agar plate diffusion assay modified as an antagonism test. By this screening method, a family of three novel polycyclic polyketides named as abyssomicins B **266**, C **267** and D **268**, were isolated from a marine *Verrucosispora* Strain AB-18-032.[177] The main component, abyssomicin C, inhibited the pathway between chorismate and *para*-aminobenzoic acid and was very active against Gram-positive bacteria, including multi-resistant clinical isolates of *Staphylococcus aureus*. The MIC value of abyssomicin C against strains *S. aureus* N315 and *S. aureus* Mu50 were in the range of 4μg mL^{-1} and 13μg mL^{-1}, respectively. The activity of **267** can be explained by the irreversible trapping of the targeted enzymes by a Michael addition.[178]



266



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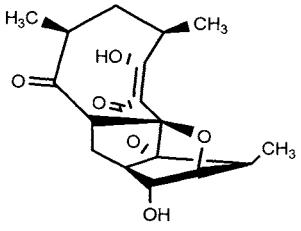
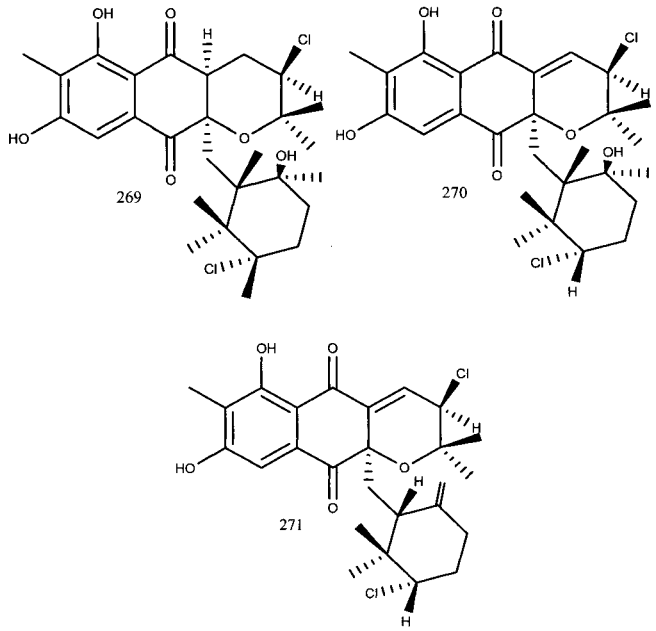


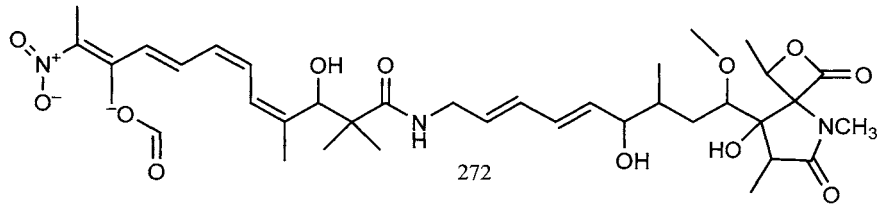
268

Three new chlorinated dihydroquinones **269-271** were obtained from a marine actinomycete CNQ-525, which was recognized as a member of a new genus (tentatively called MAR4) within the family Streptomycetaceae by 16S rDNA sequence based phylogenetic analysis.[179] The compounds are related to several previously reported metabolites of the napyradiomycin class. They show significant antibacterial properties against the drug-resistant pathogens methicillinresistant *Staphylococcus aureus* (MRSA) and vancomycinresistant *Enterococcus faecium* (VREF). In addition, compounds **269-270** were found to be cytotoxic toward HCT-116 human colon carcinoma with IC_{50} values of 2.40 and 0.97μg/mL, respectively.



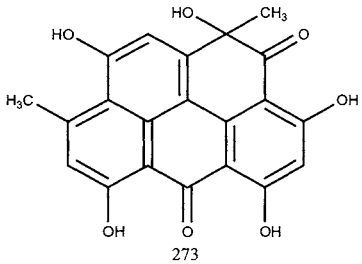
A strain of *Streptomyces nodosus* (NPS007994) isolated from Scripps Canyon, La Jolla, California, was found to produce lajollamycin **272**, a nitro-tetraene spiro-β-lactone-γ-lactam antibiotic.[180] Lajollamycin

showed antimicrobial activity against both drug-sensitive and -resistant Gram-positive bacteria with MICs ranging from 1.5-20μg/mL and inhibited the growth of B16-F10 tumor cells in vitro with an EC_{50} of 9.6 μM.

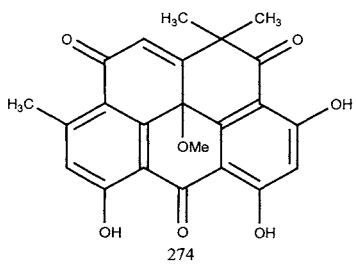


272

A streptomycete *Streptomyces chibaensis* AUBN1/7 which was isolated from marine sediment of the Bay of Bengal, India, yielded a new cytotoxic compound 1-hydroxy-1-norresistomycin **273**.[181] It exhibited antibacterial activities against Gram-positive and Gram-negative bacteria. It also showed a potent cytotoxic activity against cell lines HMO2 (gastric adenocarcinoma) and HePG2 (hepatic carcinoma) in vitro. It was also obtained from cultivation of the marine-derived streptomycete isolate B8005, a member of the rare resistomycin class.[182] The formation of **273** is of interest from a biosynthetic point of view. From a related marine strain B4842, resistoflavin methyl ether **274** was isolated.



273



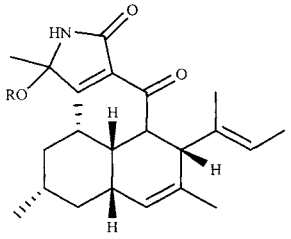
274

4 Enzyme inhibition agents

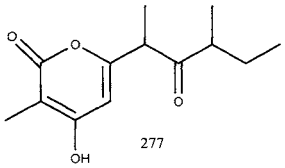
4. 1 From fungus

Two unusual tetramic acid containing compounds ascosalipyrrolidinones A **275** and B **276** and a new natural product

ascosalipyrone **277** were obtained from the endophytic marine fungus *Ascochyta salicornia*, which was isolated from the green alga *Ulva* sp.[183] Compound **275** inhibited tyrosine kinase p56[kk] to 70% of its activity at a concentration of 40 μg/mL and to 23% at a concentration of 200 μg/mL. It also exhibited cytotoxic activity against rat skeletal muscle myoblast cells and mouse peritoneal macrophages, and exhibited anti-plasmodial activity towards *Plasmodium falciparum* strains K1 and NF 54, antimicrobial activity in agar diffusion assays against *Bacillus megaterium* (5 mm), *Mycotypha microsporum* (4 mm) and *Microbotryum violaceum* (2 mm) at a concentration of 50μg/filter disk.



275 R=-$CH_2CH_2CH_2CH_3$
276 R=-CH_3



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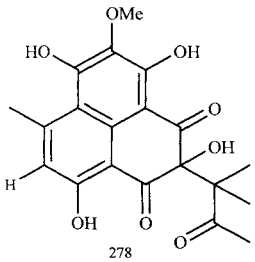
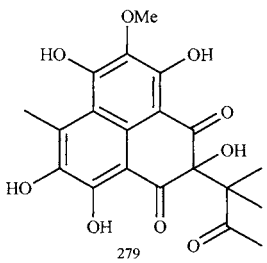


278

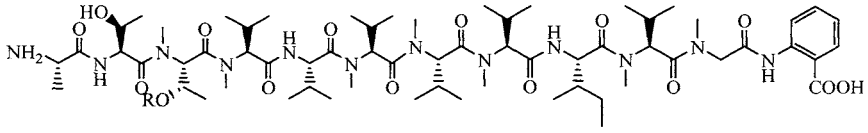


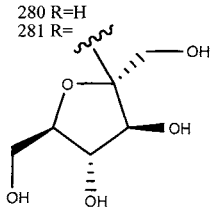
279

Sculezonones A **278** and B **279**, which contain phenalene-dione nucleus, were isolated from culture broth of the fungus *Penicillium* sp., which was separated from the Okinawan marine bivalve *Mytilus coruscus*.[184] Their effects upon DNA polymerase activity were studied.[185]

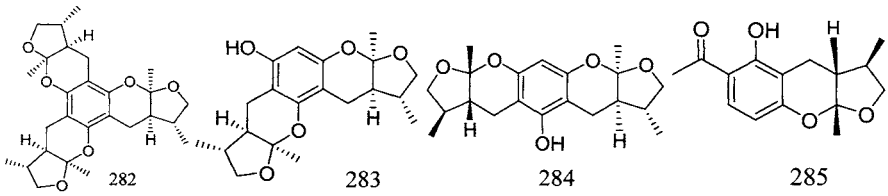
From extracts of the mycelium of the fungus (K063), which was separated from an Okinawan marine red alga, two new peptides, dictyonamides A **280** and B **281** were isolated.[186] Peptide **281** showed

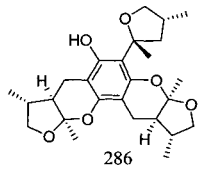
inhibitory activity against cyclin-dependent kinase 4 with IC_{50} value of 16.5 μg mL^{-1}.





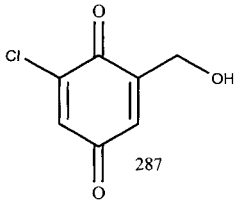
Five unique metabolites, ketal compound xyloketals A **282** and its analogues B **283**, C **284**, D **285**, and E **286** were isolated from mangrove fungus *Xylaria* sp. (no. 2508) obtained from the South China Sea.[187] Xytoketal C can be slowly transformed to xytoketal B in DMSO-*d6* solution at room temperature. Xyloketal A exhibited the activity of inhibiting acetylcholine esterase at 1.5×10^{-6} mol L^{-1} ($p < 0.01$). All xyloketals **282-286** and derivatives of xyloketal A **282** were successfully synthesized.[188-192] The xyloketal family of compounds was synthesized by condensation of phloroglucinol or 2,4-dihydroxyacetophenone with enones in multistep, one-pot, domino reactions, leading to the xyloketals and 5-demethylxyloketals, respectively.[190] In the case of condensation with phloroglucinol, the mono-, bis-, and tris- adducts are formed and their ratios depend on the ratio of starting materials and the reaction time.





286

Chlorogentisylquinone **287**, which was obtained from the culture broth of a marine fungal strain FOM-8108, inhibited neural sphingomyelinase activity of rat brain membranes (with IC_{50} value of 1.2 μM).[193,194] The strain FOM-8108 was considered to be a member of genus Phoma family.[195]



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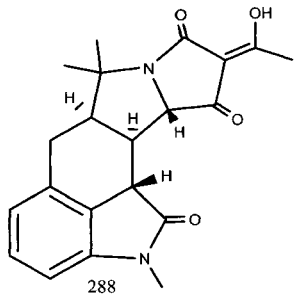
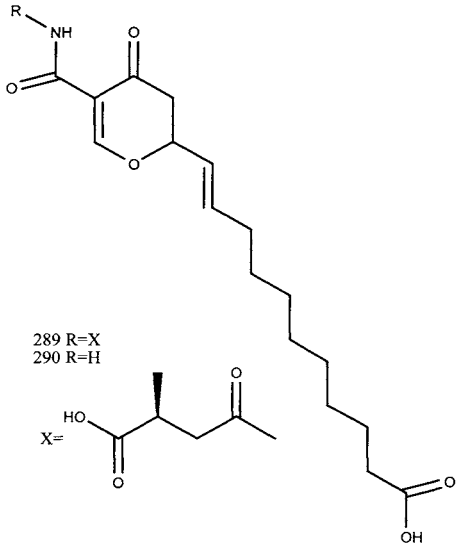


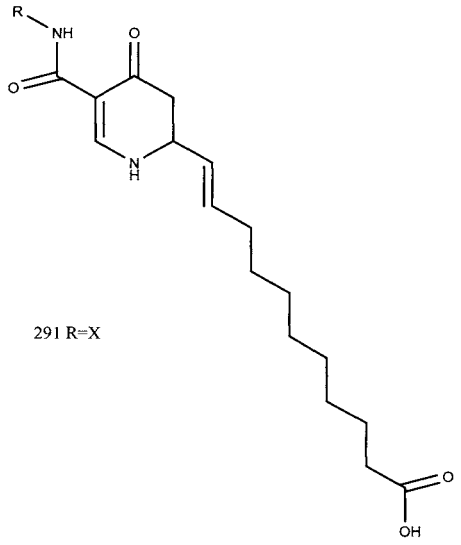
288

A new pentacyclic oxindole alkaloid, speradine A **288** was isolated from the culture broth of fungus *Aspergillus tamarii,* which was separated from driftwood obtained from the seashores of Okinawa.[196] Compound **288** exhibited inhibitory activity against Ca2t-ATPase (IC_{50} 8 μM) and histone deacetylase (IC_{50} 100 μg mL^{-1}), and antibacterial activity against *Mycrococcus luteus* (MIC 16.7 μg mL^{-1}).

A new ubiquitin-activating enzyme (E1) inhibitor himeic acid A **289** and two new related compounds, himeic acids B **290** and C **291**, were isolated from a marine-derived fungus, Aspergillus sp.[197] The formation of an E1-ubiquitin (Ub) intermediate was 65% inhibited by **289** at the concentration of 50μM, while **290** and **291**, showed little inhibitory activity even at 100μM.



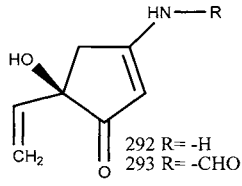
289 R=X
290 R=H



291 R=X

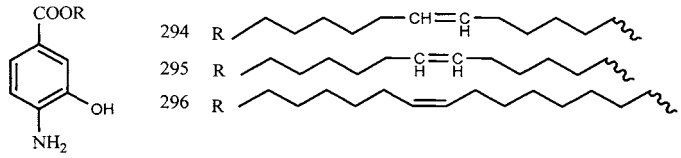
New 3-amino-5-ethenylcyclopentenones, myrothenones A **292** and B **293**, were isolated together from the marine algicolous fungus of genus of Myrothecium.[198] Compounds **292** exhibited a tyrosinase inhibitory activity with IC_{50} values of 6.6 μM, which is more active than kojic acid

(IC_{50}, 7.7 μM) currently being used as a functional personal-care compound.



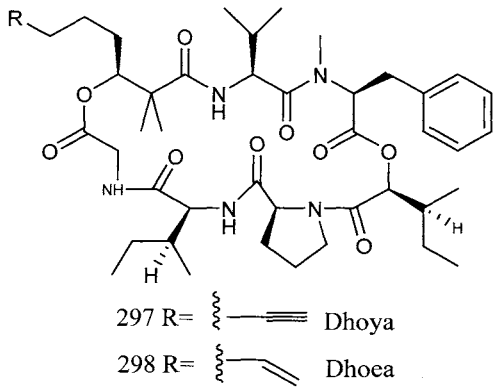
4. 2 From bacteria

A series of new esters of 4-amino-3-hydroxybenzoic acid with unsaturated long-chain, B-5354a, b and c **294** – **296,** were found in a culture broth of a novel marine bacterium, SANK71896.[199] They inhibited sphingosine kinase activity with IC_{50} values of 21, 58 and 38 μM, respectively. Kinetic studies revealed that B-5354c **296** inhibited sphingosine kinase with a *Ki* value of 12 μM. It also inhibited sphingosine-1-phosphate formation in human platelets. *Via* structure-activity relationship study, it indicated that all the three substituents on the benzene ring were necessary for the inhibitory activity against sphingosine kinase.[200] The inhibitory effect of B-5354c on recombinant human SPH kinase isoforms (hSPHK1 and hSPHK2) was evaluated and compared with N,N-dimethylsphingosine (DMS), a well-characterized SPH kinase inhibitor. The inhibitory potency of B-5354c for hSPHK1 was almost the same as that of DMS. However, those for hSPHK2 were about two to four times more potent than DMS.[201] B-5354c was also used to investigate the α-toxin-induced hemolysis of sheep erythrocytes.[202]

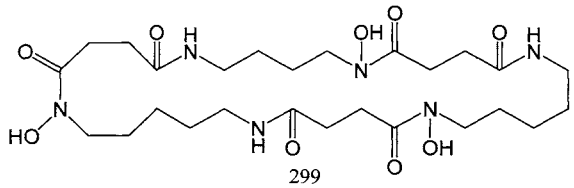


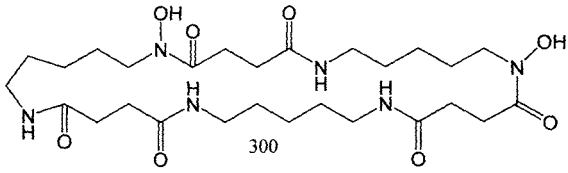
4. 3 From cyanobacteria

Two new cyclodepsipeptides, pitipeptolides A **297** and B **298**, were isolated from a population of marine cyanobacterium *Lyngbya majuscula* collected at Piti Bomb Holes, Guam.[203] Compounds **297** and **298** possessed stimulated elastase activity and moderate anti-mycobacterial activity; and both compounds exhibited weak cytotoxicity against LoVo cancer cells with IC_{50} values of 2.25 and 1.95μg mL^{-1} respectively.



Two new cyclic peptides **299** and **300** were isolated from the culture broth of an actinomycete of the genus Streptomyces isolated from an unidentified marine sponge, their structures were defined to be the dehydroxy and desmethylenyl derivatives of nocardamine, respectively.[204] They exhibited weak inhibition against the enzyme sortase.

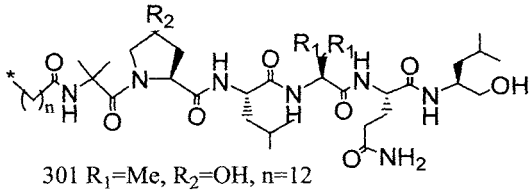




300

5 Antivirus agents

Halovirs A **301**, B **302**, C **303**, D 3**04** and E **305** were produced during the saline fermentation of a marine-derived fungus of the genus *Scytalidium*.[205, 206] These lipophilic, linear peptides were in vitro potent inhibitors of the herpes simplex viruses 1 and 2. Halovirs A, B, and C showed ED_{50} values of 1.1, 3.5, and 2.2 μM, respectively, when added to cells infected with HSV-1 for 1 h. Halovirs D and E had ED_{50} values of 2.0 and 3.1 μM, respectively, indicating that the length of the lipophilic chain also modulates the antiviral activity. Evidence indicated that halovirs directly inactivate herpes viruses through a mechanism of action that could be applicable in the prevention of HSV transmission. Halovirs A **301** was synthesized and structure-activity relationships defining key structural elements for optimal viral inhibition of halovirs were studied.[207, 208]



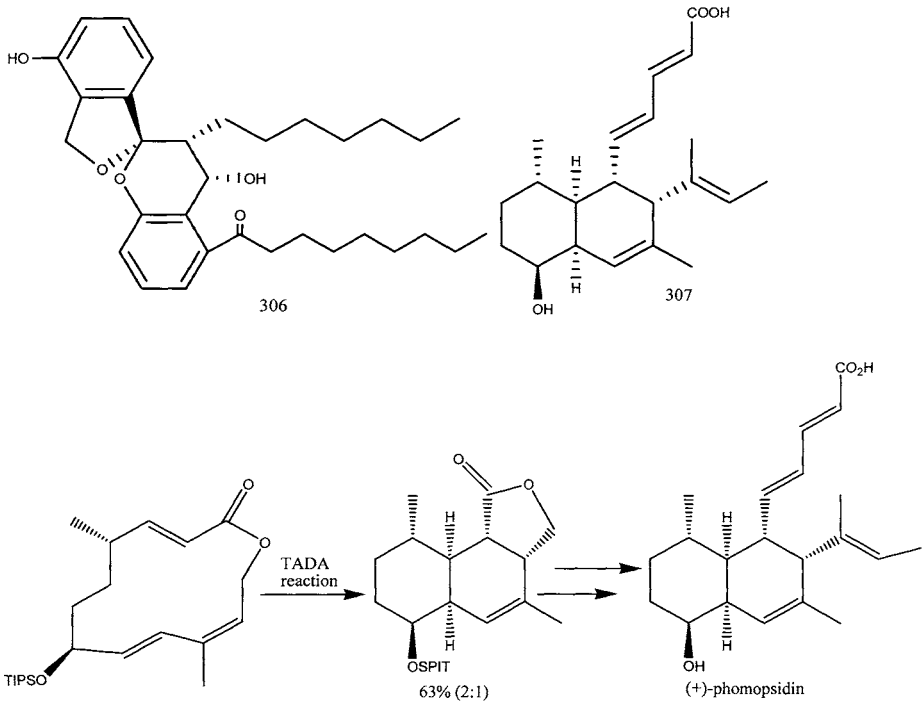
301 R_1=Me, R_2=OH, n=12
302 R_1=H, R_2=OH, n=12
303 R_1=Me, R_2=H, n=12
304 R_1=Me, R_2=OH, n=10
305 R_1=Me, R_2=H, n=10

6 Miscellaneous bioactive agents

6. 1 From fungus

A spiroacetal compound paecilospirone **306** was isolated from a marine fungus *Paecilomyces* sp. collected from Yap Island.[209,210]

Phomopsidin **307** was isolated from marine-derived fungi *Phomopsis* sp. which was separated from a fallen mangrove branch at the bottom of a coral reef in Pohnpei.[210] Both showed inhibitory activity, **306** (20% inhibition at 50 μM) was much weaker than **307** (IC_{50}, 5.7 μM), to purified porcine brain microtubule proteins. The first total synthesis of (+)-phomopsidin was achieved *via* a diastereoselective transannular Diels-Alder (TADA) reaction (scheme 5).[211, 212] The biosynthetic study using ^{13}C -labeled precursors revealed the origin of all carbon atoms in phomopsidi, which was built with nine acetates and three methyl groups from L-methionine.[213] A chemoenzymatic synthesis of the cis-decalin core associated **307** was also described.[214]

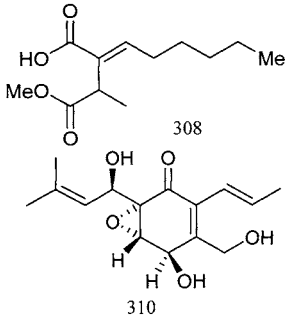


scheme 5

Two new metabolites, 2-hexylidene-3-methylsuccinic acid 4-methyl ester **308** and an ophiobolane sesterterpene named halorosellinic acid **309**, were

isolated from a culture broth of the marine fungus *Halorosellinia oceanica* BCC 5149.[215] Compound **309** showed anti-malarial activity with IC_{50} value of 13 μg mL^{-1} and possessed only weak anti-mycobacterial activity with the minimum inhibitory concentration of 200 μg mL^{-1}.

Rosnecatrone **310** is a phytotoxic metabolite isolated from cultures of a virulent strain of the fungus *Rosellinia necatrix*.[216] In the initial tests using apple and geranium seedlings, **310** showed higher phytotoxicity than cytochalasin E.



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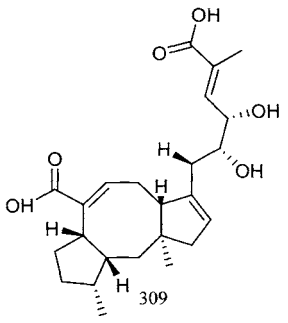
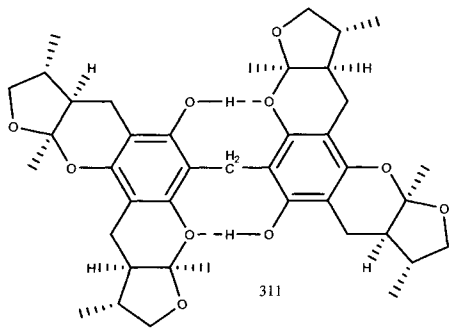
310



309

Xyloketal F **311**, an unusual metabolite with strong L-calcium channel blocking activity, was isolated from the mangrove endophytic fungus *Xylaria* sp. (#2508) collected at the South China Sea coast.[217] Compound **310** was synthesized by condensation of xyloketal B **283** with formaldehyde. The L-calcium channel blocking activities of xyloketals B **283**, A **282**, and F **311** were determined, and at the same concentration (0.03 μmol/L), the inhibiting rates were 12.05%, 21.47%, and 50.33% respectively.



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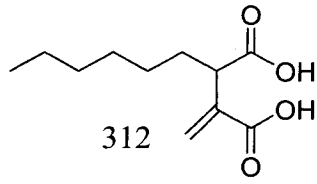
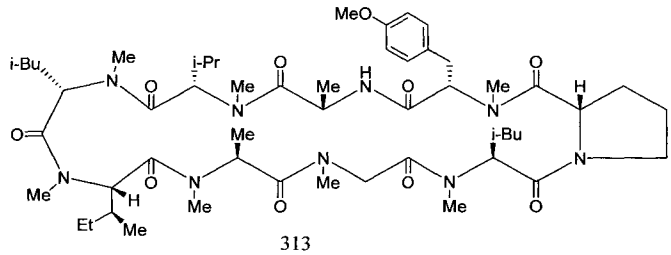


312

A new inhibitor of p53–HDM2 interaction, (-)-hexylitaconic acid **312**, was isolated from a culture of marine-derived fungus, *Arthrinium* sp.[218] The inhibition of p53–HDM2 binding was tested by the ELISA method, and **312** inhibited the binding with an IC_{50} value of 50 μg/mL. Although a number of synthetic inhibitors of p53–HDM2 interaction have been reported so far, **312** is the second inhibitor isolated from natural resources.

Two new anti-dinoflagellates, clonostachysins A **313** and B **314**, were obtained from a marine sponge derived fungus *Clonostachys rogersoniana* strain HJK9.[219] Both compounds exhibited a selectively inhibitory effect on a dinoflagellate *Prorocentrum micans* at 30 mM, but not any on other microalgae nor bacteria even at 100 mM.



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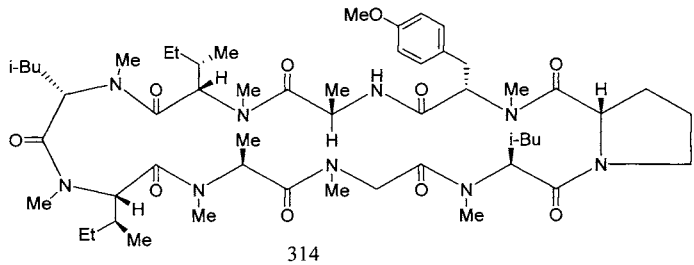
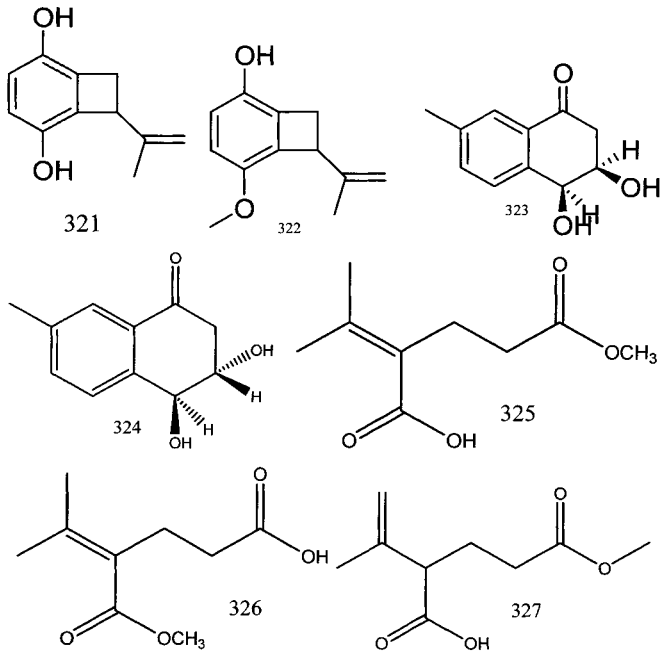
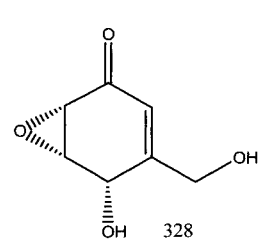


314

YCP **315**, a mitogenic polysaccharide with its molecular weight (MW) of 2.4×10^3 kDa, was isolated from the mycelium of the marine filamentous fungus *Phoma herbarum* YS4108.[220] It was found to be able to increase the phagocytic activity of mice in vitro and in vivo, indicating that it may be looked up as a potent immunomodulator that could activate macrophages. And the free radical-scavenging properties and antioxidant activity of YCP and its two chemically sulfated derivatives YCP-S1 **316** and YCP-S2 **317** were compared.[221] It was found that sulfated YCP was more potent than YCP at protecting erythrocytes against oxidative damage hemolysis. The data suggest for the first time that sulfation of polysaccharide significantly increases its antioxidant activity. And three sulfated derivatives of YCP **315**, YCP-SL **318**, YCP-SM **319** and YCP-SH **320** were synthesized, the degree of substitution DS of which were determined to be 0.13, 0.99 and 1.3, with the average molecular weight 0.64×10^3, 0.57×10^3 and 0.45×10^3 kDa, respectively[222] The YCP sulfates showed significant anticoagulant activity and antiplatelet aggregation activity. YCP-SH also possessed potent antiplatelet aggregation activity in vitro compared with aspirin. YCP sulfates specifically interfered with different stages of the coagulation cascade, and the anticoagulant activity improved with the increasing DS and decreased MW.



A marine fungal isolate, identified as *Acremonium* sp., was mass cultivated and found to produce two novel hydroquinone derivatives, 7-isopropenylbicyclo[4.2.0]octa-1, 3, 5-triene-2, 5-diol **321** and 7-isopropenylbicyclo-[4.2.0]octa-1, 3, 5-triene-2, 5-diol-5-*β*-D-glucopyranoside **322**.[223] The new natural products (3*R**, 4*S**)-3,4-dihydroxy-7-methyl-3,4- dihydro-1(2*H*)-naphthalenone **323** and (3*S**,4*S**)-3,4-dihydroxy-7-methyl-3,4- dihydro-1(2*H*)- naphthalenone **324** were very difficult to separate and were obtained as a 1:0.8 mixture. In addition to these compounds an inseparable mixture of three new isomeric compounds, pentanedioic acid 2-(1-methylethylidene)-5-methyl ester **325**, pentanedioic acid 2-(1-methylethylidene)-1-methyl ester **326**, and pentanedioic acid 2-(1- methylethenyl)-5-methyl ester **327**, were also obtained. Compounds **321** and **322** had significant DPPH radical scavenging effects (85.5, 17.5%, respectively, at 25.0 μg mL^{-1}), and with **322** being also able to inhibit peroxidation of linolenic acid (35.5%, at 37.0 μg mL^{-1}).



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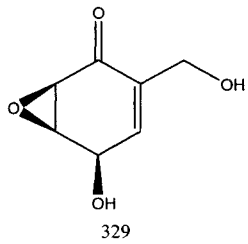

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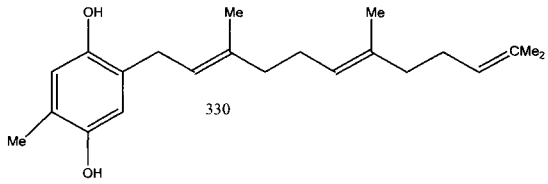
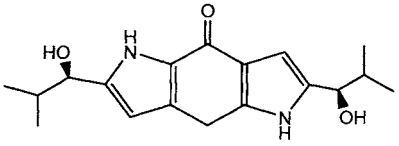


330

Bioactivity-guided fractionation of an organic extract of the broth from the marine-derived fungus culture of *Aspergillus parasiticus* gave a new gabosine derivative, parasitenone **328**.[224] Parasitenone showed moderate activity in the free radical scavenging assay. Short, simple and enantioselective synthesis the putative structure of **328** was accomplished from the readily available chiral building block. Parasitenone was later found to be the known natural product epoxydon **329**.[225]

Farnesylhydroquinone **330** was isolated from the mycelium of a marine-derived fungus of the genus Penicillium.[226] This compound exhibited potent free radical scavenging activity (IC_{50}, 12.5 μM) against the DPPH.

Terreusinone **331**, a novel chiral dipyrrolobenzoquinone compound, was isolated from the marine algicolous fungus *Aspergillus terreus*.[227] Compound **331** exhibited a UV-A absorbing activity with ED_{50} value of 70μg mL^{-1}. Microbial transformation of **331** resulted in the isolation of a new oxidized metabolite, terreusinol **332**.[228]



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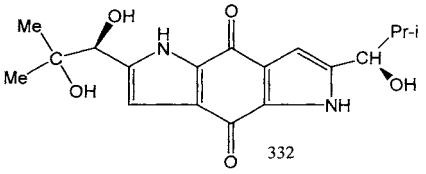

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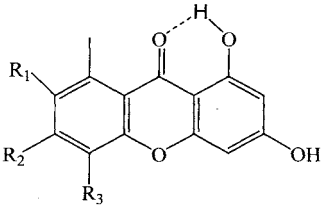
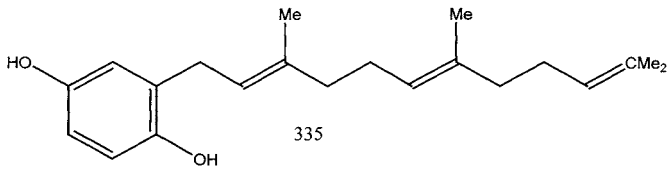


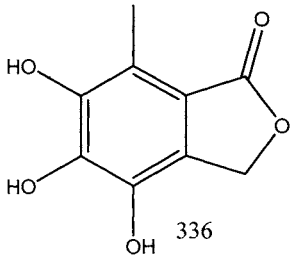
333 R_1=R_2=OH,R_3=H
334 R_1=R_2=R_3=OH

A marine fungal isolate, identified as *Wardomyces anomalus,* was cultivated and found to produce two new xanthone derivatives, 2, 3, 6, 8-tetrahydroxy-1-methylxanthone **333** and 2, 3, 4, 6, 8-pentahydroxy-1-methylxanthone **334**.[229] Compounds **333** showed significant antioxidant activity and significant DPPH radical scavenging effect (94.7% at 25.0 μg mL^{-1}), and inhibitory effect on peroxidation of linolenic acid (17.0% at 7.4 μg mL^{-1}) and on $p56^{lck}$ tyrosine kinase (100% enzyme inhibition at 200 μg mL^{-1}).

Farnesylhydroquinone **335**, which exhibited DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity, was separated from *Penicillium* sp. marine fungi;[230] and the method of the preparation of this compound was described.



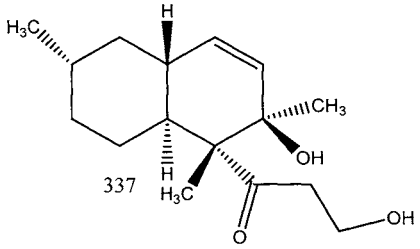
335



336

Fungus Epicoccum sp. was isolated from the marine brown alga *Fucus vesiculosus*. From the cultivated fungus, a new secondary metabolite 4,5,6-trihydroxy-7-methylphthalide **336** (epicoccone) was found.[231] **336** was found to be potently active, showing 95% DPPH radical scavenging effects at 25 μg mL^{-1}. It also inhibited the peroxidation of linolenic acid in the TBARS assay (62 % inhibition at 37 μg mL^{-1}).

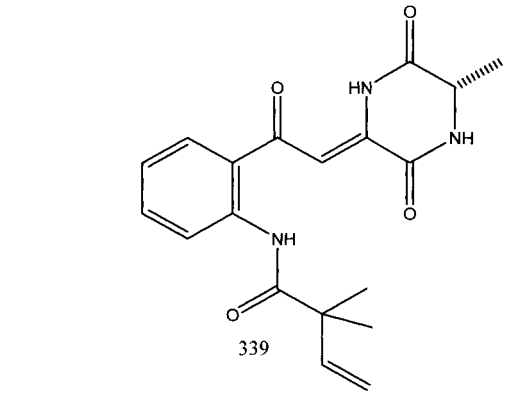
A new polyketide, aspermytin A **337**, was isolated from a marine fungus *Aspergillus* sp.[232] The fungus was separated from the mussel *Mytilus edulis*. Compound **337** induced neurite outgrowth in rat pheochromocytoma (PC-12) cells at a concentration of 50 μM.



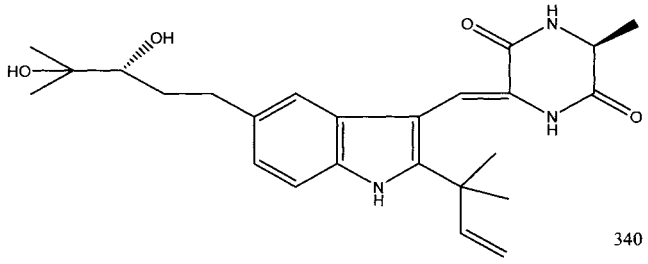
337

EPS2 **338** (mean molecular weight = 1.3×10^5) is an antioxidant exopolysaccharide separated from the broth of a marine filamentous fungus Keissleriella sp. YS 4108.[233] It is composed of galactose, glucose, rhamnose, mannose and glucuronic acid in approximate proportions of 50:8:1:1:0.4. The glycan was assessed in different in vitro systems for its antioxidant properties and showed that EPS2 exhibited profound scavenging activities in superoxide radical. In a more elaborate study,

radical scavenging effects of EPS2 with both site-specific and non site-specific hydroxyl radical were also discerned using the deoxyribose assay method. Furthermore, EPS2 also was found to be effective in blocking the non site-specific strandbreaking of DNA induced by the Fenton reaction at concentrations of 0.1 and 1 mg/mL and effective in inhibiting the copper-mediated oxidation of human low density lipoprotein (LDL) in a dose-dependent manner.



339



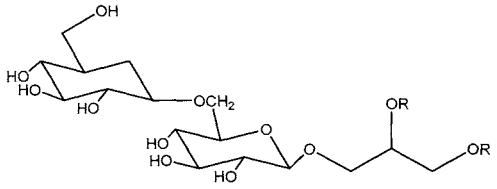
340

Two new diketopiperazine alkaloids, golmaenone **339** and dihydroxyisoechinulin A **340**, were isolated from the culture broth of the marine-derived fungus *Aspergillus* sp.[234, 235] Compounds **339** and **340** both exhibited significant radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC_{50} values of 20 μM; both responded similarly to the positive control, ascorbic acid (IC_{50}, 20 μM). **339** and **340** also showed activity in protection against ultraviolet-A

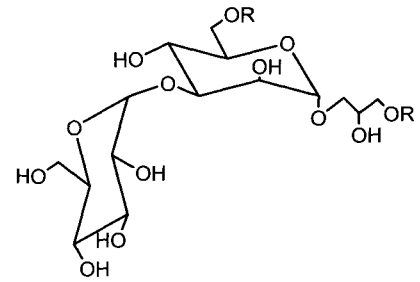
(UV-A)(320-390nm) with ED_{50} values of 90 μM and 130 μM, respectively. They are more active than oxybenzone (ED_{50}, 350 μM) which was currently used as sunscreen.

6. 2 From bacteria

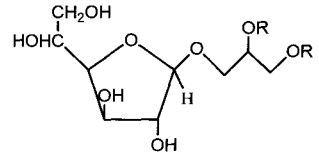
Bacterium *Microbacterium* sp., isolated from the sponge *Halichondria panicea,* produced four unusual cell-associated glycoglycerolipids GGL.1 **341**, GGL.2 **342**, GGL.3 **343**, and GGL. 4 **344**.[236] **342**, 1-*O*-acyl-3-[R-glucopyranosyl-(1-3)-(6-*O*-acyl-R-mannopyranosyl)]glycerol, with a 14-methyl-hexadecanoic acid and 12-methyl-tetradecanoic acid positioned at C-6 of the mannose unit and at the glycerol moiety, was the main component. Glycolipid production was correlated with growth and reached a maximum value of 200 mg/L when grown on artificial seawater medium with 20 g L^{-1} glucose. Compound **342** can decrease the surface tension of water down to 33 mN m^{-1} and the interfacial tension of the water/*n*-hexadecane system down to 5 mN m^{-1}. In addition to this good surface-active behavior, **342** also showed anti-tumor activities.



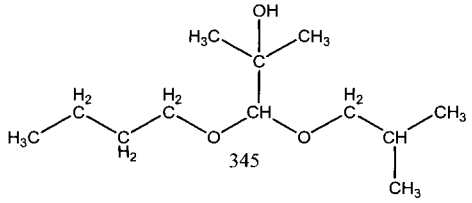
341 R=anteiso-C15:0 and -C17:0; but also iso-C16:0



R=anteiso-C15:0 and-C17:0; but also iso-C16:0
342 R=H, 343 R=$COCH_3$

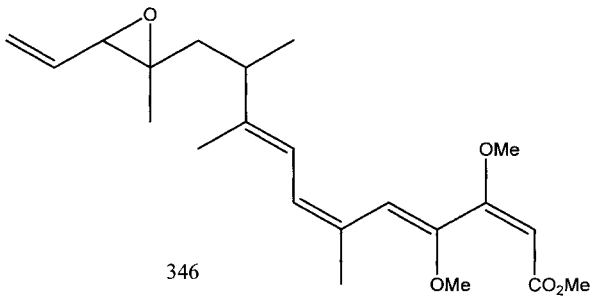


344 R=anteiso-C15:0 and -C17:0; but also iso -C16:0



345

The new metabolite [1-(2'-methylpropoxy)-2-hydroxy-2-methylpropoxy]butane **345** was isolated from the cell-free culture supernatant of the marine bacterium *Vibrio angustum* S14 as part of studies investigating the role of chemical signals in prokaryote-prokaryote and prokaryote-eukaryote interactions.[237] Compound **345** induced the acylated homoserine lactone (AHL) reporter system in *Agrobacterium tumefaciens* and bioluminescence in *Vibrio harveyi*.



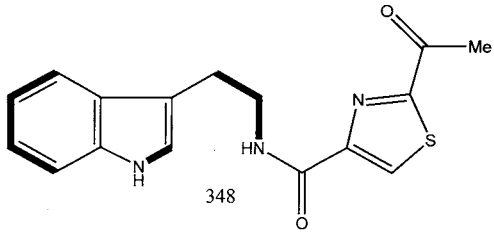
346

Haliangicin **346**, a novel β-methoxyacrylate antibiotic with a conjugated tetraene moiety, was isolated from the culture broth of marine myxobacterium, *Haliangium luteum*.[238] The strain was isolated from a seaweed sample collected at a sandy beach in the Miura Peninsula, Kanagawa, Japan. In studies, haliangicin inhibited the growth of a wide spectrum of fungi but was inactive against bacteria.[239] In mitochondrial respiratory chains, haliangicin interfered with the electron flow within the cytochrome *b-cl* segment. In the in vitro bioactivity, it inhibited the electron transfer with IC_{50} value at 2.5 nM. The configuration of the epoxide in **346** was determined.[240]

The aerobic, mesophilic, and heterotrophic marine bacterium HYD657 named *A.macleodii* subsp. *fijiensis* biovar deepsane, which was isolated from a East Rise deep-sea hydrothermal vent polychaete annelid, produced an unusual exopolysaccharide (EPS) **347** in a given specific medium.[241] EPS consists of glucose, galactose, rhamnose, fucose and mannose along with glucuronic and galacturonic acids and a diacidic hexose which was identified as a 3-0-(1 carboxyethyl)-D-glucuronic acid. Its average molecular mass was 1.6×10^6 Da. Due to interesting biological activities of EPS, its applications have been found in cosmetics. Its probable function to the filamentous microbial mat in the *Alvinella pompejana* micro-environment was also mentioned.

A new algicide, bacillamide **348**, was isolated from the marine bacterium *Bacillus* sp. SY-1.[242] This compound showed algicidal activity

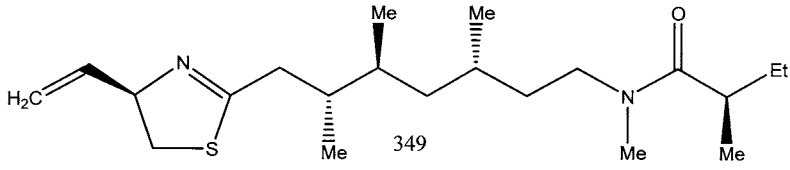
against the harmful dinoflagellate, *Cochlodinium polykrikoides* with LC_{50} of 3. 2 μg mL^{-1}. Synthesis of bacillamide was achieved by using tryptamine and the mixed anhydride derived from 2-acetylthiazole-4-carboxylic acid and pivaloyl chloride. [243]



348

6. 3 From cyanobacteria

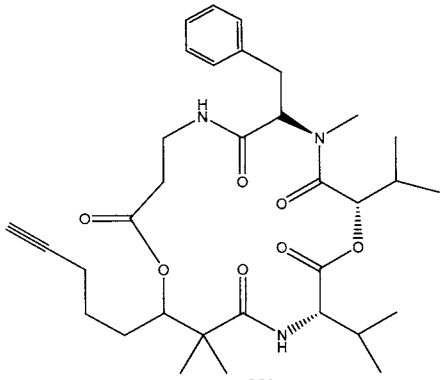
The biological properties, synthesis, and complete stereo-structure of kalkitoxin **349** a novel neurotoxic lipopeptide from a Caribbean collection of *Lyngbya majuscule*, were reported in detail.[244, 245] Compound **349** was synthesized through 17-step reactions, and five synthetic isomers of **349** were also synthesized. [244] Their spectroscopic data were discussed and biological properties were compared.[245] Several other synthetic methods of this compound, and another natural source, crude organic extract from a Puerto Rican collection of *Lyngbya majuscule*, that **349** can be obtained were reported.[246-250] Kalkitoxin was assayed and proved to interact with voltage-sensitive sodium channels in cerebellar granule neurons.[251]



349

Yanucamides A **350** and B **351** were isolated from the lipid extract of a *Lyngbya majuscula* and *Schizothrix* sp.[252] assemblage collected at Yanuca Island, Fiji. Both compounds contained a unique 2,2-dimethyl-3-hydroxy-7-octynoic acid moiety, they were previously described only as a component of kulolide-1 **352** and kulokainalide-1 **353**,

metabolites from the marine mollusk *Philinopsis speciosa*. Thus, the isolation of the yanucamides from this cyanobacterial assemblage supported the hypothesis that the kulolides and related metabolites were of cyanobacterial origin. Both yanucamides A and B exhibited strong brine shrimp toxicity (LD_{50}, 5 ppm). The first total synthesis of yanucamide A was reported *via* amide and ester couplings of the key components.[253,254] This synthesis has established the configuration at the previously ambiguous 3-position, and also revised the absolute configuration of **353** to **354**.



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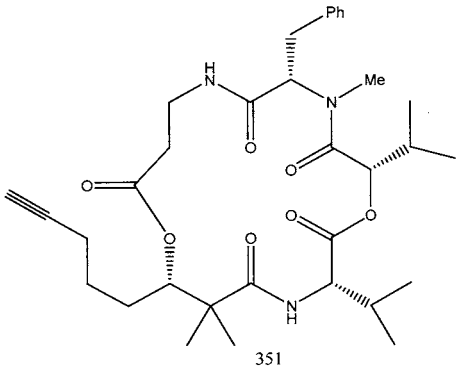

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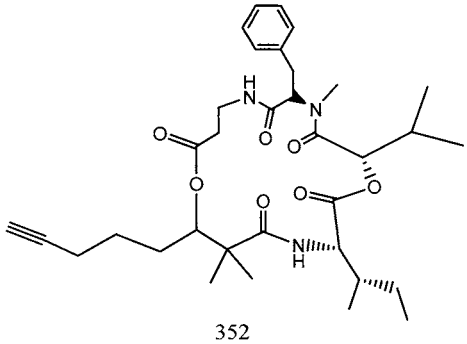

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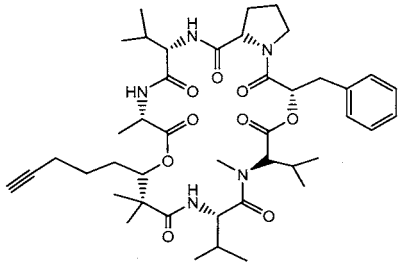

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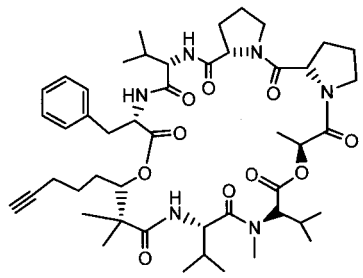

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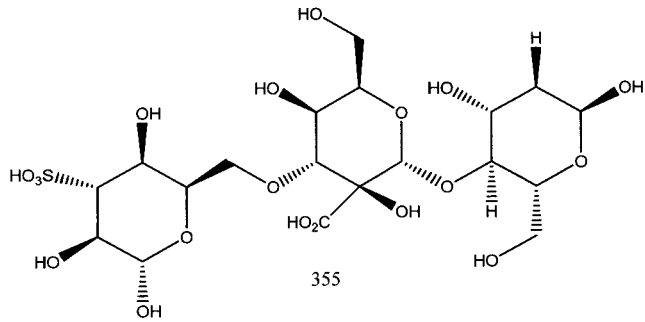
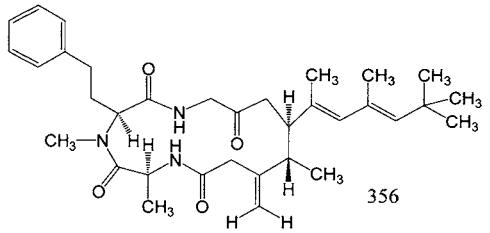


355

Cyanobacterium, *Cyanothece* sp. ATCC 51142, produced high concentration of gel-like exopolysaccharide **355** and the gel formation property of this compound was investigated for its potential application in removing metals from solution.[255]

Bioactivity-guided fractionation of organic extracts from two *Lyngbya majuscula* collections led to the isolation of a new secondary metabolite, antillatoxin B **356**, an unusual *N*-methyl homophenyl-alanine analogue of the potent neurotoxin antillatoxin **357**.[256,257] Compound **356** exhibited significant sodium channel-activating (EC_{50} = 1.77 μM) and ichthyotoxic (LC_{50} = 1 μM) properties.



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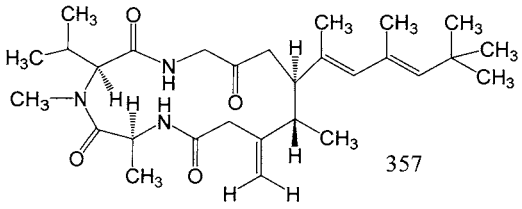
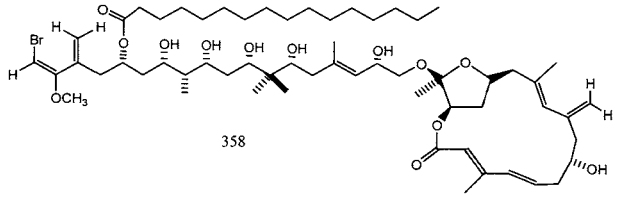


357

The extract from a laboratory culture of an Indonesian cyanobacterium *Phormidium* sp. displayed inhibitory activity in a Ras-Raf protein interaction assay. Assay-guided fractionation of the extract led to the isolation of the major inactive metabolite, phormidolide **358**; which was nevertheless highly toxic to brine shrimp (LC_{50} = 1.5 μM).[258] Compound **358** was one of the only few reported macrolide-type natural products, which were obtained from marine cyanobacteria.



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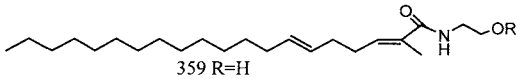
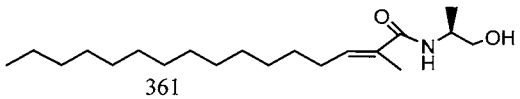


359 R=H
360 R=Ac



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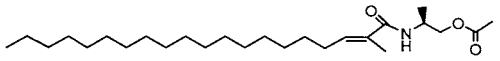

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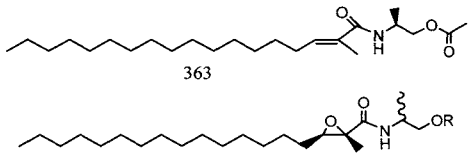
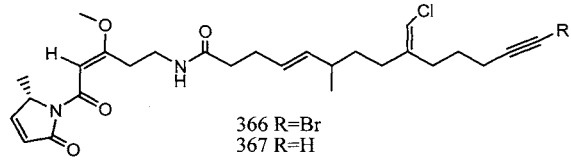


363

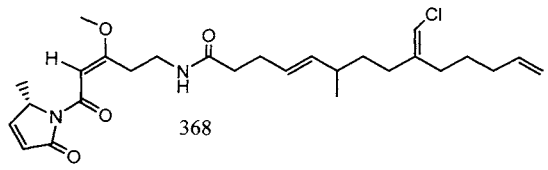
364 R=H
365 R=Ac

Semiplenamides A **359** to G **365**, a series of new anandamide-like fatty acid amides, were isolated from a 1997 Papua New Guinea collection of the marine cyanobacterium *Lyngbya semiplena*; and all these new metabolites displayed toxicity in the brine shrimp model system.[259] Only semiplenamides A **359**, B **360**, and G **365** showed weak affinity for the rat cannabinoid CB1 receptor. Semiplenamide A **364** was also a moderate inhibitor (IC_{50} = 18.1 μM) of the anandamide membrane transporter (AMT). Novel *syn*-aldol/dehydration methodology was developed for the stereoselective synthesis of the core (*E*)-α, β-unsaturated amide functionality of this class of natural product, and employed for the efficient synthesis of semiplenamide C.[260]

In a screening program for bioactive compounds from marine cyanobacteria led to the isolation of jamaicamides A–C **366-368**.[261] Jamaicamide A **366** is a novel and highly functionalized lipopeptide containing an alkynyl bromide, vinyl chloride, β-methoxy eneone system, and a pyrrolinone ring. The jamaicamides showed sodium channel blocking activity and fish toxicity. Precursor substrates were fed to jamaicamide-producing cultures and mapped out the series of acetate and amino acid residues helping to develop an effective cloning strategy for the biosynthetic gene cluster.

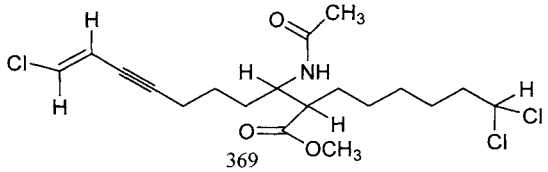


366 R=Br
367 R=H



368

Brine shrimp toxicity guided fractionation of the extracts from two mixed Fijian collections of the cyanobacteria *Lyngbya majuscula* and *Schizothrix* sp. led to the isolation of eleven novel chlorinated lipids, taveuniamides A–K **369-379**.[262] All of these metabolites showed an intriguing constellation of unsaturation (olefinic and acetylenic bonds) and chlorination at the two termini of a 15-carbon chain. The central carbon atom of the chain (C-8) was substituted in each case with an N-acetate function. Taveuniamides F **374**, G **375** and K **379** were the most potent brine shrimp toxins with LD_{50}s between 1.7 - 1.9 mg mL^{-1}, while taveuniamides A–E **373-377** showed moderate activity LD_{50} 3 - 5 mg mL^{-1}. Taveuniamides G **375**, I **377**, and J **378** were essentially inactive at a concentration of 10 mg mL^{-1}.



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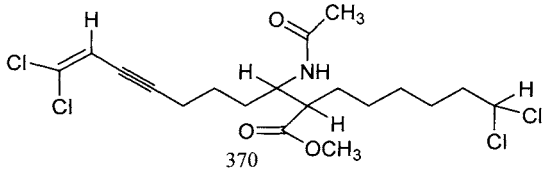

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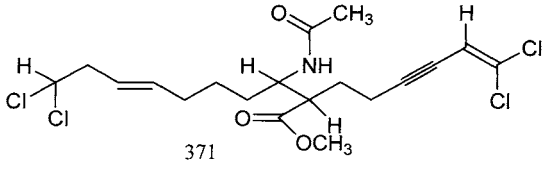

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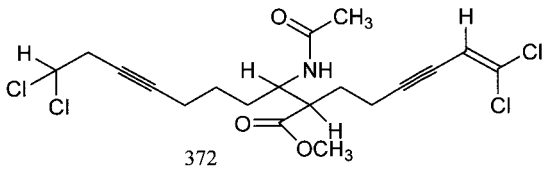

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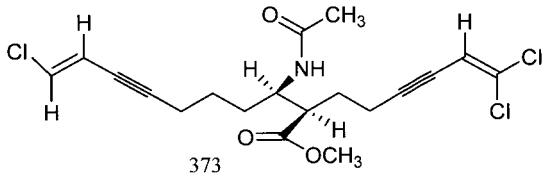

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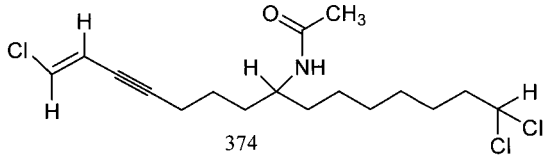

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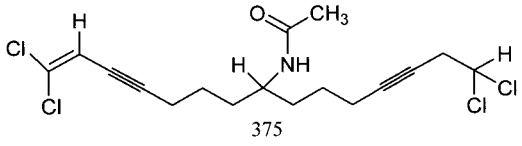

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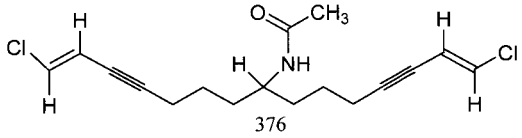

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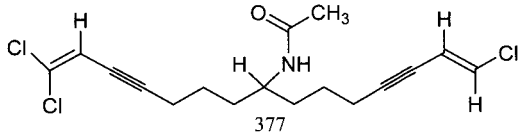

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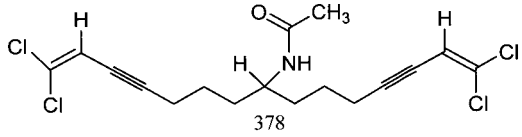

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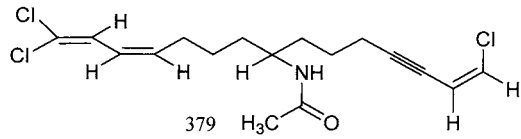
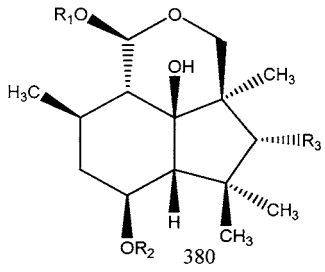
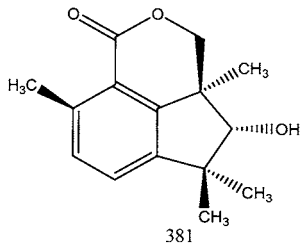
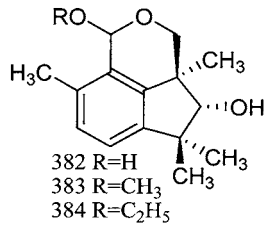
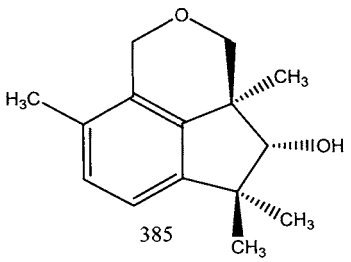


Eleven new botryane metabolites botrydial sesquiterpenoids, 7-hydroxy-10-methoxydeacetyldihydrobotrydial **380**, 7-hydroxy-10-oxodehydrodihydro- botrydial **381**, 7-10-dihydroxydehydrodihydrobotrydial **382**, 7-hydroxy-10-methoxydehydro- dihydrobotrydial **383**, 7-hydroxy-10-ethoxydehydrodihydrobotrydial **384**, 7-hydroxy-10-dehydroxydehydrodihydrobotrydial **385**, 7-hydroxydeacetylbotryenalol **386**, 7, 10-dihydroxy- deacetyldihydrobotrydial-110-ene **387**, 4, 10-didehydroxy-7-hydroxydeacetyldihydro- botrydial-110, 59-diene **388**, 7-hydroxy-10-dehydroxydeacetyldihydrobotrydial-110, 59-diene **389**, and 15α-hydroxy-14-aldehyde probotryan-45-ene **390** were isolated from the mitosporic fungus *Geniculosporium* sp., which was associated with the red alga *Polysiphonia* sp. [263] They have some inhibitory activity against *Chlorella fusca*, *Bacillus megaterium*, and *Microbotryum violaceum.*









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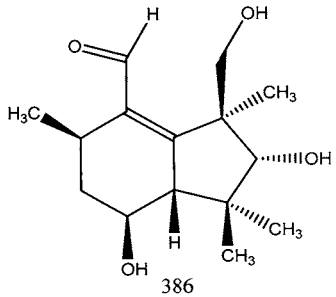

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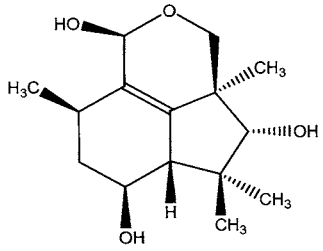

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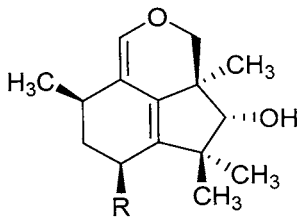
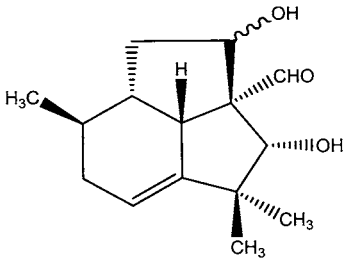


388 R=H
389 R=OH



390

7 Prospect

This review has used almost 263 citations and described nearly 390 new metabolites from marine microorganisms. The data implies that marine microorganisms are just the same as their terrestrial congeners on the diversity of metabolites. As an abundant source of bioactive compounds, they are not at an inferior position compared with other organisms. The unique advantages of the research in this area, the abundance, structure diversity, and biological activities, offers

opportunities to fruitful results and which can be easily industrialized; and this should result in greater interest shown by international science groups around research and development.

People still know very little about marine microorganisms at present and are still unaware of such basic concepts as how to differentiate the marine microorganisms and terrestrial ones. Of course, if we take the deep sea, geothermic ocean environment and the worldwide oceanic anoxic habitats into account, marine microorganisms should be distinctly different from the terrestrial corresponding ones to environment. As a result, it is no doubt that the microorganisms from the extreme environment will have unusual biosynthetic routes. However, the isolation and cultivation of these microorganisms are very difficult, and the livability won't exceed 10% if cultivated with classical methods. Unfortunately, we know little about the specific natural nourishments and growing factors that marine microorganisms need. The ingredient of common culture medium such as peptone, mono-sugars for land congeners are not the required essentials for oceanic ones. They are obviously substituted with the more complicate carbon source like chitin, sulfated polysaccharides and marine proteins. And we know very little about the influence of inorganic elements such as infrequent metals or silicone, while they are abundant in the seawater. These facts limit our abilities to isolate and cultivate the new marine microorganisms greatly. It is very evident that the basic biological study needs to be paid enough attention to and strengthened before there is effective exploitation of marine microorganisms.

Molecular biology has made significant progress in the field of microorganism in these several years. The new technology of molecular biology has given microorganism diversity a totally new concept, and will be applied in marine microorganism abroad very soon, exerting its tremendous function, especially in the microorganism taxonomy, the field of enzyme and the useful compounds synthesized by microorganisms.

Marine microorganisms have innumerable new genes and biochemical approaches, and available enzymes, antibiotics and other useful molecules. As to biological technology, they are really a huge gold mine. Thus, the combination of biological technology and marine microorganism research will create a mutual benefit prospect.

The main goal of marine microorganism research at present is to exploit new drugs. In the nearly 300 new compounds covered in this review, almost 80% of them refer to bioactivities, especially some important discoveries of pharmacology and toxicology. However, the whole subject is just underway and the applicable achievements are few. But with the rapid progress of molecular biology, study towards marine microorganisms and their metabolites will receive a breakthrough; and to the marine microorganisms living in extreme environments, the probability increases greatly. Though we described little about the big molecule from marine microorganisms in this review, the field is worthy of attention. Because of its "marine" environment and the newfound biological function of polysaccharide, marine microorganism derived polysaccharides contain enormous development potential.

But the microorganisms from mangrove haven't yet gained enough recognition until now. Mangrove has a special ecological status, for they locate at the transitions between marine-derived and terrene-derived plants. Some scientists evaluate that 80% of marine microorganisms can be found in mangroves. Mangrove is a big source of diverse microorganisms and their metabolites, and will attract more and more scientists to devote time to its research.

Along with the continual development of marine microorganism research, the species library, metabolites library and genes library of marine microorganisms can be brought into the agenda. The foundation of the three libraries certainly will promote the progress of this field and the relative subjects. It is beyond any doubt that the marine microorganism research will become a new growth point in gaining greater interest and

recognition in the future.

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BIOLOGICAL ACTIVITIES OF SULFATED GLYCOSIDES FROM ECHINODERMS

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ABSTRACT: The classes Asteroidea (starfishes) and Holothuroidea (sea cucumbers) belonging to the phylum Echinodermata are characterized by their content in toxic saponins. Asterosaponins from starfishes are sulfated steroidal glycosides whereas holothurins from sea cucumbers are triterpenoid glycosides with sulfate groups attached to the monosaccharide residues in sixty percent of the saponins isolated so far. Starfishes also contain steroidal mono- and diglycosides which occur as complex mixtures with asterosaponins. Due to their toxicity and membranotropic action, these polar compounds have attracted the attention of chemists and pharmacologists and a wide spectrum of biological activities has been found for these saponins. The purpose of this communication is to review the structural characteristics and biological properties of the saponins isolated from starfishes and sea cucumbers in the last five years, focusing on the structural elucidation and evaluation of antifungal, cytotoxic and antiviral properties of some examples of the author's laboratory isolated from starfishes and sea cucumbers collected near the Patagonian shore and Antarctica.

INTRODUCTION

Marine organisms are sources of new natural products with unusual structural features, many of which have no counterpart among secondary metabolites of terrestrial origin [1]. The interest in marine chemicals lies in the pharmacological and toxicological properties of many of these secondary metabolites [2] and in their biological role in the natural environment [3]. Echinoderms belonging to the classes Asteroidea (starfishes or sea stars) and Holothuroidea (sea cucumbers) produce complex mixtures of saponins that are responsible for their general toxicity and that may play a defensive role due to their membranotropic action [4, 5]. These secondary metabolites are very common in plants but their presence in the animal kingdom is very rare. While the occurrence of saponins is constant in starfishes and sea cucumbers, they have been

found only rarely in some sponges, gorgonians, alcyonarians, green alga and in fishes of the genus *Pardachirus* [6].

Starfishes and sea cucumbers also contain glycosphingolipids (cerebrosides and gangliosides) that have a wide range of biological activities, presumably related to the amphipathic nature of these molecules [7, 8].

Several structural features of the aglycones and the oligosaccharide chains differentiate starfish saponins (asterosaponins) from those isolated from sea cucumbers (holothurins). Asterosaponins contain a steroidal nucleus while holothurins are characterized by a triterpenoid aglycone. All asterosaponins contain a sulfate group attached at C-3 of the aglycone while approximately sixty percent of the holothurins isolated so far have sulfate groups linked to the monosaccharide units of the oligosaccharide chain. It is suggested that sulfated compounds could have a role as stabilizers for hydroxyl or amino groups or as detoxifiers for non sulfated toxic compounds [9].

In addition to Asteroidea and Holothuroidea, the phylum Echinodermata (Greek *echinos*, spiny; *derma*, skin) comprises the classes Ophiuroidea (brittle stars), Crinoidea (sea lilies and feather stars) and Echinoidea (sea urchins). There is no report of occurrence of steroid or triterpenoid glycosides in sea lilies, feather stars or sea urchins. Brittle stars contain sulfated polyhydroxylated steroids [10-12] and only two sulfated steroidal monoglycosides have been isolated from the brittle star *Ophioderma longicaudum* [13].

Starfish and sea cucumber saponins have shown a wide spectrum of biological effects: antifungal, cytotoxic, hemolythic, antiviral, antibacterial and immunomodulatory activities [14, 15]. These biological activities are a consequence of their membranotropic action against Δ^5 sterols in cellular membranes. Saponins form complexes with these sterols, developing single ion channels and larger pores which cause an alteration of the physico-chemical properties of membranes [16]. Starfish and sea cucumber cell membranes are resistant to their own saponins due to the presence of Δ^7- and $\Delta^{9,11}$ sterols, sulfated Δ^5 sterols and β-xylosides of sterols instead of the free Δ^5 sterols [17].

Initially, most of the work on biological activities of asterosaponins has been performed on purified extracts of starfish and only few pharmacological studies have been carried out on the pure glycosides. This trend has changed and most of the new structures published

nowadays in the literature include biological studies that may contribute to a better understanding of the role of these secondary metabolites in starfish and to establish structure-activity correlations.

Several monographs concerning the structures and biological activities of asterosaponins and holothurins have been published [5, 14, 15, 18-20]. The aim of the present communication is to discuss the most recent findings in the field, focusing on the structural characteristics and biological activities of starfish steroidal glycosides and sea cucumber triterpene glycosides. Structural elucidation of some examples of the author's laboratory will be presented.

STEROIDAL GLYCOSIDES FROM ASTEROIDEA

Asterosaponins

Starfishes are rich in sulfated steroidal oligoglycosides which occur as complex mixtures of highly oxygenated compounds, such as steroidal mono- and diglyclosides and free and sulfated polyhydroxysteroids [18]. The term asterosaponin has been applied to designate the oligoglycosides containing a $\Delta^{9,11}$-3β,6α-dihydroxy steroidal aglycone with a sulfate group at C-3 and an oligosaccharide chain, usually made up of five or six units, glycosidically linked to C-6. The most common steroidal side chain in asterosaponins contains 20α-hydroxy and 23-oxo functionalities, as in thornasterol A sulfate (**1**), the most common aglycone in asterosaponins, Fig. (**1**).

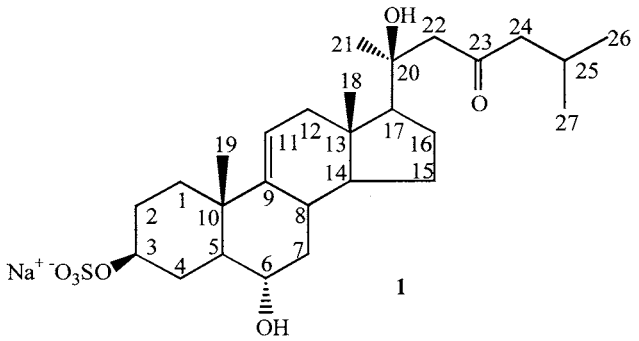
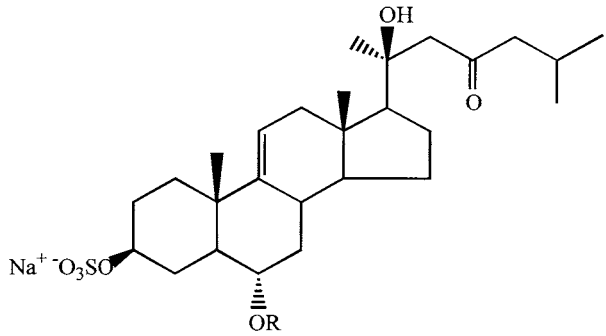


Fig. (1). Structure of Thornasterol A sulfate

Steroid **1** has been isolated from the starfish *Aphelasterias japonica* and showed hemolytic activity to mouse erythrocytes with an ED_{50} value

of 1.1 x 10^{-4} M [21]. Thornasterol A has also been isolated as the 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolinium (salsinol) salt from the starfish *Lethasterias nanimensis chelifera* [22]. Comparison of the action of the sodium and salsinol salts of thornasterol A sulfate on the development of the sea urchin *Strongylocentrotus nudus* showed that the sodium salt was toxic with an EC_{50} value of 40 μg/ml in contrast with the salsinol salt which was non-toxic up to a concentration of 100 μg/ml.

The most common monosaccharide units in asterosaponins are D-xylose, D-quinovose, D-fucose and D-galactose, always in their pyranose form with β-anomeric configuration (α for L-arabinose). The large majority of asterosaponins are pentaglycosides and the first monosaccharide unit (usually D-glucosyl or D-quinovosyl) linked to the aglycone is always glycosylated at C-3 by a branching glycosyl group (usually D-xylosyl or D-quinovosyl) with a terminal quinovose unit linked to C-2 [19, 20]. The branching sugar is glycosylated at C-4 by the remaining part of the oligosaccharide chain. An example of these characteristic features is thornasteroside A (**2**), the asterosaponin isolated from the common Pacific starfish *Achantaster planci* and the most widely distributed oligoglycoside in starfishes, Fig. (**2**) [19].



2 Thornasteroside A R = [Fuc-(1→2)-Gal-(1→4)]-[Qui-(1→2)]-Xyl-(1→3)-Qui
3 Labidiasteroside A R = [Fuc-(1→2)-Qui-(1→4)]-[Qui-(1→2)]-Glc-(1→2)-Glc
4 Luidiaquinovoside A R = [Fuc-(1→2)-Qui-(1→4)]-[Qui-(1→2)]-Glc-(1→3)-Qui
5 Psilasteroside R = [Ara-(1→3)-Fuc-(1→2)-Gal-(1→4)] [Qui-(1→2)]-Xyl-(1→3)-Qui

Fig. (2). Structure of asterosaponins from the starfishes *Acanthaster planci*, *Labidiaster annulatus*, *Luidia quinaria* and *Psilaster cassiope*

Several reviews on steroidal glycosides from starfishes with descriptions of their structures, distribution and spectroscopic characteristics have been published [10, 18, 19].

Recently, we have isolated from the polar extracts of the Antarctic starfish *Labidiaster annulatus* the pentaglycoside labidisteroside A (**3**), the first asterosaponin with a 2,4-disubstituted glucose unit as a branching point in the oligosaccharide chain, Fig. (**2**) [23]. Asterosaponin **3** and the recently isolated luidiaquinovoside A (**4**) are the only two examples reported so far of asterosaponins containing a branched glucose in the oligosaccharide chain, Fig. (**2**) [24].

Luidiaquinovoside (**4**) and psilasteroside (**5**), isolated from *Psilaster cassiope*, Fig. (**2**) showed marginal in vitro cytotoxicity against RBL2H3 (rat basophilic leukemia) cell lines (IC_{50} 31.3 and 5.4 μg/ml, respectively) [25].

A few asterosaponins containing hexasaccharide chains have been described. Versicoside A (**6**) was the first example of a group of hexaglycosides containing a single branching sugar unit [26] while pectinioside E was the first asterosaponin of a second group of hexasaccharides with two branching points in the sugar chain [27]. Anasterosides A (**7**) and B (**8**) are further examples of new hexasaccharides isolated together with versicoside A from the Patagonian starfish *Anasterias minuta*, Fig. (**3**) [28].

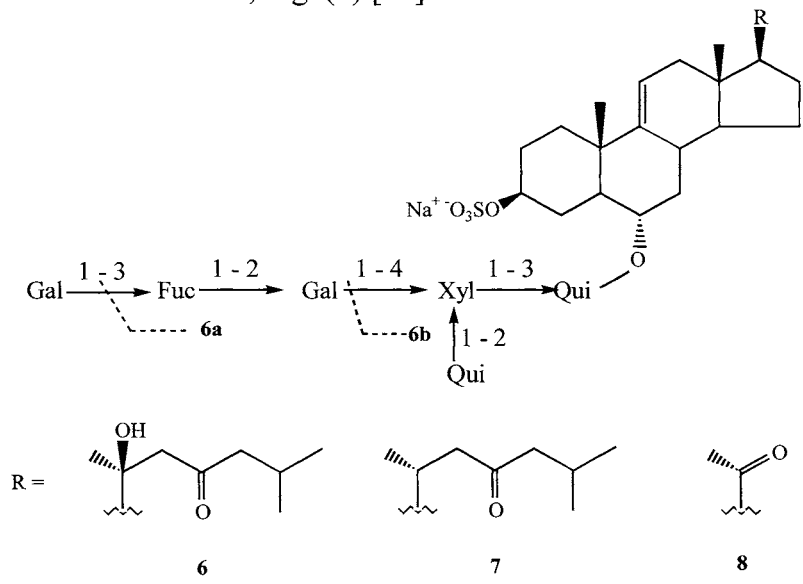


Fig. (3). Structures of asterosaponins from the starfish *Anasterias minuta*

The saponins were isolated by bioactivity-guided fractionation of the ethanolic extract of the starfish using the brine shrimp (*Artemia salina* L.)

larvae mortality assay. The three hexaglycosides differed in the aglycone side chain, but contained the same hexasaccharide chain, composed of galactose, fucose, xylose and quinovose in the ratio 2:1:1:2. Anasteroside A (**7**) contained 23-oxo-5α-cholest-9(11)-en-3β,6α-diol 3-sulfate, an aglycone present in asterosaponins isolated from the starfishes *Marthasterias glacialis*, *Coscinasterias tenuispina*, *Luidia maculata* and *Neosmilaster georgianus* [19].

Anasteroside B (**8**) contained 5α-pregn-9(11)-en-3β,6α-diol 3-sulfate (3-*O*-sulfoasterone), which has been isolated for the first time as a natural product from the starfish *Aphelasterias japonica* [21]. Asterone has been reported as an artifact easily obtained by retroaldol cleavage of glycosides containing steroidal nucleus with 20-hydroxy-23-oxo side chains. The mild extraction and processing conditions employed in the isolation of the asterosaponins of *Anasterias minuta*, suggested that anasteroside B is a naturally occurring hexaglycoside. Two further examples of hemolytic monoglycosides containing 3-*O*-sulfoasterone were recently isolated from the starfish *Aphelasterias japonica* [21]. Recently, the 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolinium (salsinol) salt of 3-*O*-sulfoasterone has been isolated from the starfish *Lethasterias nanimensis chelifera* [22].

The structural elucidation of the hexaglycosides of *A. minuta* required a combination of spectroscopic analysis (NMR and FABMS), chemical transformations and enzymatic degradations. Characteristic signals in the highfield region of the ^{1}H-NMR spectra gave information on methyl groups belonging to the aglycones and the 6-desoxy sugars, fucose and quinovose. ^{1}H- and ^{13}C-NMR data were consistent with the presence of a steroidal aglycone with a 9(11)-double bond [δ_C 145.3 (s, C-9) and 116.2 (d, C-11); δ_H 5.19 (1H, m, H-11)], one sulfated oxomethine [δ_C 78.0 (d, C-3); δ_H 4.84 (1H, m, H-3)] and one oxomethine [δ_C 79.5 (d, C-6); δ_H 3.82 (1H, m, H-6)]. The methyl singlet at δ 2.17 ppm in the ^{1}H-NMR spectrum of **8** and the signals at δ 210.0 (C-20) and 31.0 ppm (C-21) in the ^{13}C-NMR spectrum confirmed the presence of asterone as the aglycone. Six doublets at δ 4.82, 4.86, 4.90, 5.06, 5.09 and 5.18 ppm were assigned to the anomeric protons. The β-stereochemistries at the anomeric carbons were deduced from the coupling constant values (J = 7.3-7.7 Hz). The position of the interglycosidic attachments was determined using a combination of ^{1}H-^{1}H COSY, relayed COSY, ^{13}C-NMR and HETCOR experiments. These data were further confirmed by methylation of the

saponin **6** followed by acid hydrolysis and GC-MS analysis of the partially methylated alditol acetates derived of each sugar unit.

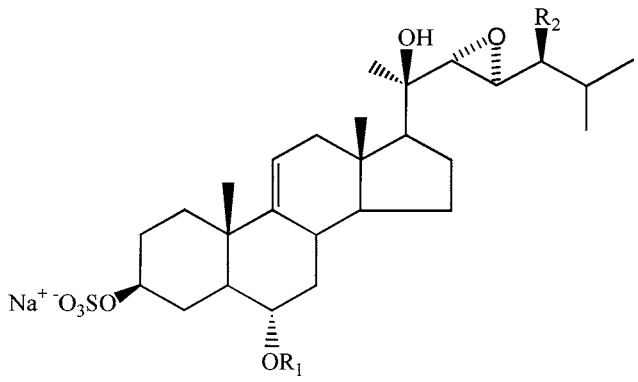
Some saponins have been reported to show antifungal properties [14]. Therefore, the hexaglycosides **6-8** were evaluated by a bioautographic technique [29] for their antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*. In order to evaluate the influence of the oligosaccharide chain moiety on the antifungal activity, we hydrolyzed versicoside A (**6**) with *Charonia lampas* glycosidase mixture to obtain, after separation by HPLC, the pentaglycoside thornasteroside A (**6a**) and the triglycoside forbeside H (**6b**). The hexaglycosides versicoside A (**6**) and anasteroside A (**7**) as well as the pentaglycoside thornasteroside A (**6a**) were active in a concentration-dependent manner. Anasteroside A (**7**), lacking the hydroxyl group at C-20 was the most active saponin, while anasteroside B (**8**), with a pregnane side chain was inactive in all the concentrations range. On the other hand, the triglycoside forbeside H containing the same aglycone as thornasteroside A and versicoside A showed no activity at all the tested concentrations. Desulfation of versicoside A by solvolysis in dioxane/pyridine (1:1) rendered a totally inactive saponin. These results suggest that the sugar chain, together with the side chain in the steroidal aglycone moiety and the presence of a sulfate group at C-3 play an important role in the antifungal activity of these saponins.

Culcita novaeguineae, an abundant starfish distributed in the South China Sea, is used as a folk medicine for the treatment of rheumatism and as a tonic in China. The *n*-BuOH extract of this starfish showed significant deforming effect against the plant pathogenic fungus *Pyricularia oryzae* P-2b. Bioassay-guided fractionation led to the isolation of three new asterosaponins (**9-11**), together with four known pentaglycosides, regularosides A and B, thornasteroside A and marthasteroside A_1, Fig. (**4**) [30, 31].

Asterosaponins **9**, **10** and **11** were tested for in vitro cytotoxicity against human leukemia K-562 cells (IC_{50}'s 8.60, 4.90 and 4.95 μg/ml, respectively) and human hepatoma BEL-7402 cells (IC_{50}'s 9.2, 4.1 and 3.4 μg/ml, respectively).

Further analysis of the *n*-BuOH extract of *Culcita novaeguineae* resulted in the isolation of three new bioactive asterosaponins, Fig. (**5**) [32]. Compounds **12-14** possessed the same pentasaccharide moiety

linked to C-6 of the steroidal aglycone and differed from each other in the side chains.



9 R_1 = [Fuc-(1→2)-Fuc-(1→4)]-[Fuc-(1→2)]-Qui-(1→3)-Glc, $R_2 = CH_3$
10 R_1 = [Fuc-(1→2)-Ara-(1→4)]-[Fuc-(1→2)]-Qui-(1→3)-Glc, $R_2 = CH_3$
11 R_1 = [Fuc-(1→2)-Qui-(1→4)]-[Fuc-(1→2)]-Qui-(1→3)-Glc, R_2 = H

Fig. (4). Structures of asterosaponins with a 22,23-epoxide in the side chain isolated from the starfish *Culcita novaeguineae*

Asterosaponins **12** and **13** showed significant cytotoxicity against two cancer cell lines, K-562 (IC_{50}'s 3.57 and 3.75 μg/ml, respectively) and BEL-7402 (IC_{50}'s 2.55 and 1.89 μg/ml, respectively).

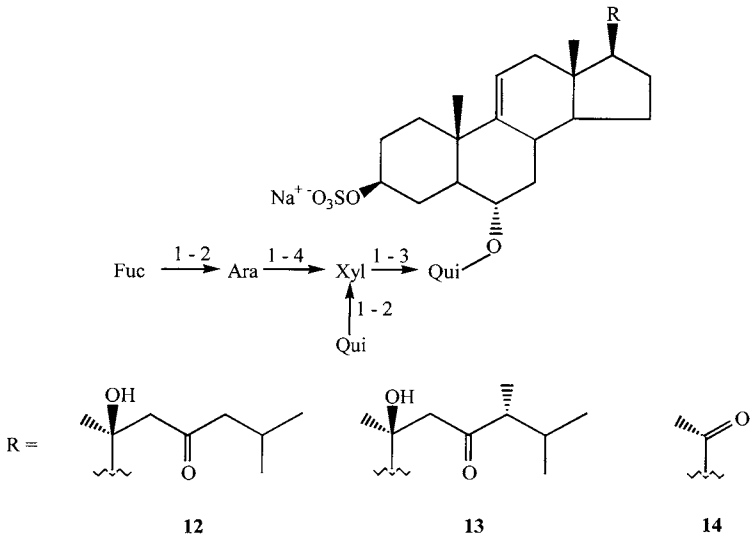


Fig. (5). Structures of asterosaponins from the starfish *Culcita novaeguineae*

On the contrary, asterosaponin **14** possessed no cytotoxicity. Compounds **12** and **13** showed hemolytic activity to rabbit erythrocytes with ED_{50} values of 16 and 31 μg/ml, respectively while **14** was inactive. These results are coincident with the lack of antifungal activity of anasteroside B (**8**), possessing the same 20-cetopregnane chain as asterosaponin **14**.

Glycosides of polyhydroxylated steroids

Besides asterosaponins, glycosides of polyhydroxysteroids are widespread in starfishes. These compounds usually occur as complex mixtures present in minor amounts. They are composed of a polyhydroxylated steroidal aglycone and a carbohydrate portion usually made up from one or two monosaccharide units, often linked to each other. Although the most common glycosylation position is C-24 of the steroidal aglycone, several glycosides with the sugar unit attached at C-3, C-26, C-28 or C-29 have been reported. The most common monosaccharide units are D-xylopyranose, often methylated at positions 2 and/or 4 and L-arabinose in its furanose form. Polyhydroxysteroid glycosides show a much larger structural variability than asterosaponins due to the hydroxylation pattern of the steroidal nucleus, the identity and location of the sugar residue and the presence of sulfate groups [18]. Usually, steroidal aglycones contain hydroxyl groups at positions 3β, 6 (α or β), 8, 15 (α or β), 16 (α or β) and 24 of the aglycone with additional hydroxyl groups at 4β, 5α and 7α. Hydroxylation at C-3 (β) and C-8 is a characteristic feature of the large majority of these compounds. Several examples of polyhydroxysteroid glycosides contain sulfate groups attached to the aglycone, as in the diglycoside antarcticoside P [33] or at the sugar unit, as in luridoside A, isolated from the Patagonian starfish *Cosmasterias lurida* [34]. The most common positions are C-3, C-6, C-15 and C-24 of the aglycone and C-5 of the pentose unit. Only three sulfated compounds with additional fosfate residues at C-6 (α) have been isolated from the deep-water starfish *Tremaster novaecaledoniae* [35].

More than one hundred polyhydroxylated steroidal glycosides have been characterized but only few reports on their biological activity have been published. Recently, several examples of new bioactive mono- and diglycosides of polyhydroxysteroids as well as biological studies on known compounds have been reported.

Two new 24-*O*-xylosides, rathbuniosides R_1 (**15**) and R_2 (**16**) have been isolated from the starfish *Asterias rathbuni*, Fig. (**6**) [36]. The 3β,6α,15β,24-tetrahydroxy substitution pattern in **16** has been found for the first time in polyhydroxysteroids from starfish.

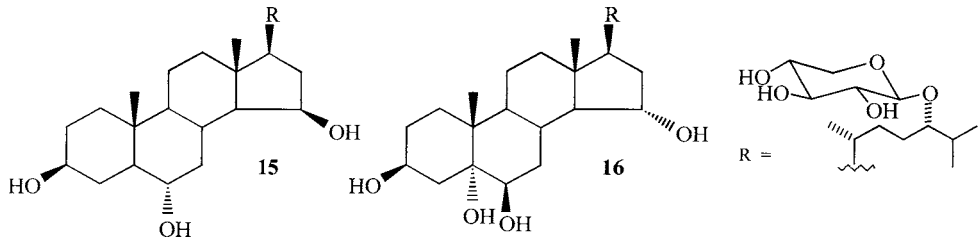
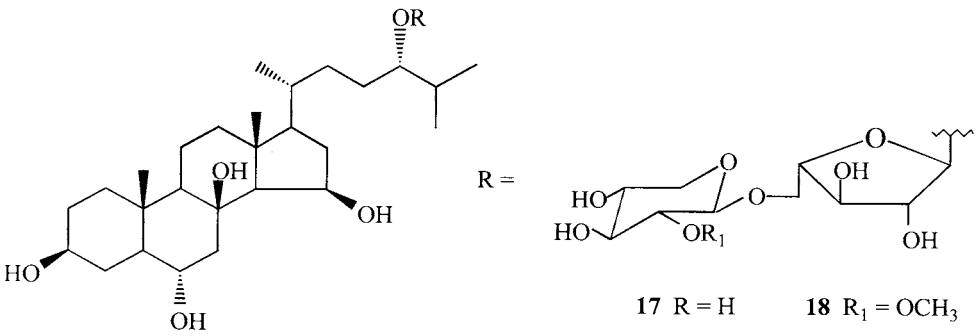


Fig. (6). Monoglycosides isolated from the starfish *Asterias rathbuni*

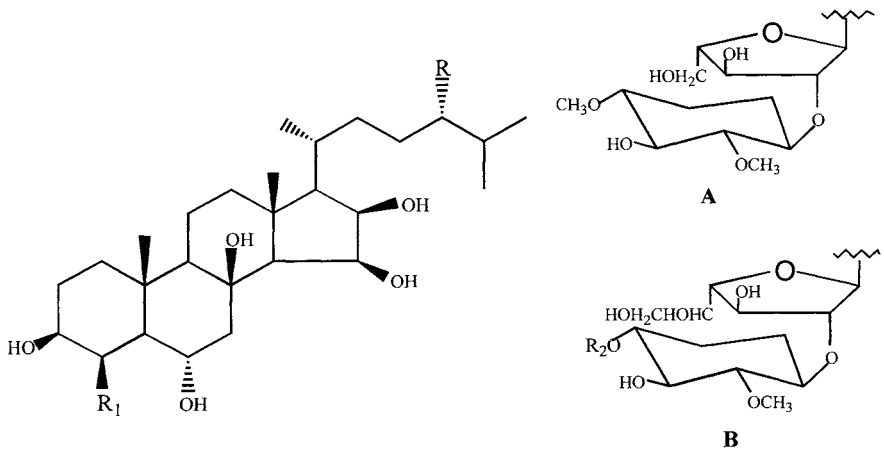
Rathbuniosides R_1 (**15**) and R_2 (**16**) as well as six related diglycosides (Structures **17-22**, Fig. (**7**) and (**8**)) isolated from the starfishes *Mediaster murrayi* [37], *Ceramaster patagonicus* [38] and *Culcita novaeguineae* [39] showed inhibition of cell division of the fertilized eggs of the sea urchin *Strongylocentrotus intermedius* as well as hemolytic activity [40].



17 R = H **18** $R_1 = OCH_3$

Fig. (7). Diglycosides isolated from the starfish *Mediaster murrayi*

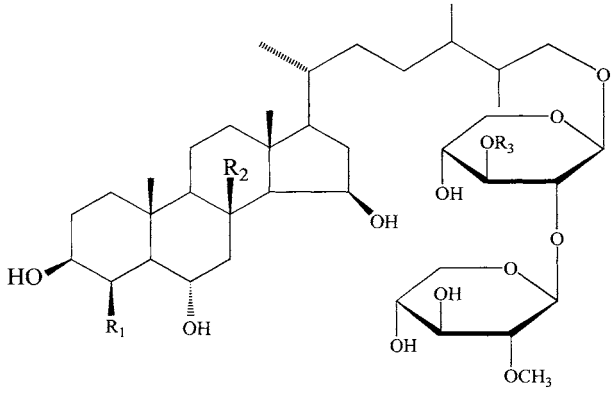
Differences in the activity of the eight compounds were correlated with the number of hydroxyl groups in the aglycones. Cytotoxic effects of the compounds were the result of cell membrane damage due to their ability to bind to membrane cholesterol.



19 Culcitoside C_2 R = A, R_1 = OH
20 Culcitoside C_3 R = A, R_1 = H
21 Ceramasteroside C_2 R = B, R_2 = CH_3, Δ^{22}
22 Ceramasteroside C_3 R = B, R_2 = H, Δ^{22}

Fig. (8). Culcitosides C_2 and C_3 from *Culcita novaeguineae* and Ceramasterosides C_2 and C_3 from *Ceramaster patagonicus*

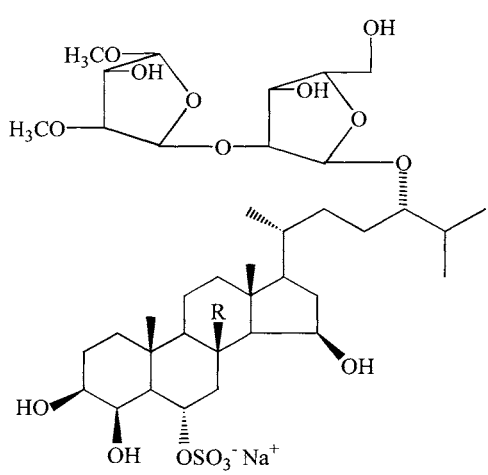
The starfish *Certonardoa semiregularis* has shown to be a rich source of bioactive sterols and glycosides of polyhydroxylated steroids. Purification of the brine shrimp active fraction of the methanolic extract of *C. semiregularis* led to the isolation of nine new sulfated polyhydroxylated steroidal diglycosides [41].



23 Certonardoside A R_1 = OH, R_2 = OH, R_3 = $SO_3^-Na^+$, $\Delta^{24,28}$
24 Certonardoside B R_1 R_2 = H, R_3 = $SO_3^-Na^+$, $\Delta^{24,28}$
25 Certonardoside C R_1 = H, R_2 = OH, R_3 = $SO_3^-Na^+$, $\Delta^{24,28}$
26 Certonardoside D R_1 = OH, R_2 = OH, R_3 = $SO_3^-Na^+$, Δ^{22}
27 Certonardoside E R_1 = H, R_2 = OH, R_3 = $SO_3^-Na^+$, Δ^{22}

Fig. (9). Structures of Certonardosides A – E

Certonardosides A-E (**23-27**), Fig. (**9**) are sulfated at C-3' of the xylopyranose unit attached to C-26 of the steroidal side chain, while certonardosides F-I (**28a**, **28b**, **29a**, **29b**), Fig. (**10**) contain a sulfate group at C-6 (α) of the steroidal aglycone. The isolated compounds were evaluated for their antiviral activity against HIV, HSV, CoxB, EMCV and VSV viruses. Certonardosides A-J were inactive within the range of non cytotoxic concentrations. Only weak antiviral activity against HSV was observed in compounds **29a** and **29b** and the desulfated analog of **28b**.



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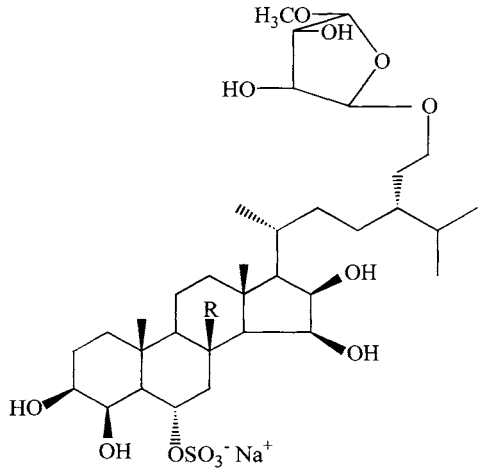


29

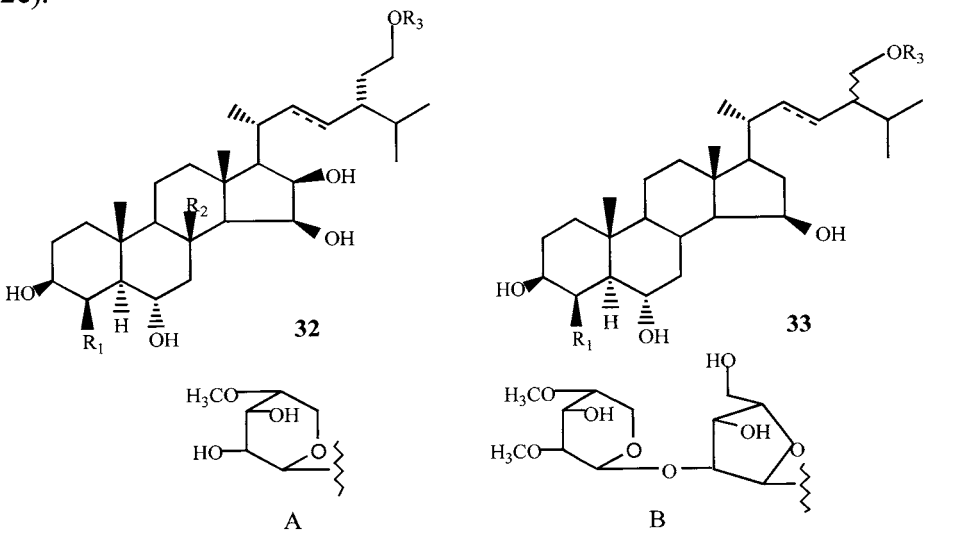
28a Certonardoside F R = OH
28b Certonardoside G R = H
29a Certonardoside H R = OH
29b Certonardoside I R =H

Fig. (10). Structures of Certonardosides F – I

Eight new polyhydroxysteroid glycosides (**32a-32e, 33a-33c**), Fig. (**11**), glycosylated at C-28 and C-29, and the C-24 glycosides Certonardosides H_3 and H_4 (**34** and **35**), Fig. (**12**), together with twenty two new polyhydroxysteroids were further isolated from the brine shrimp active fraction of the starfish *Certonardoa semiregularis* [42, 43].

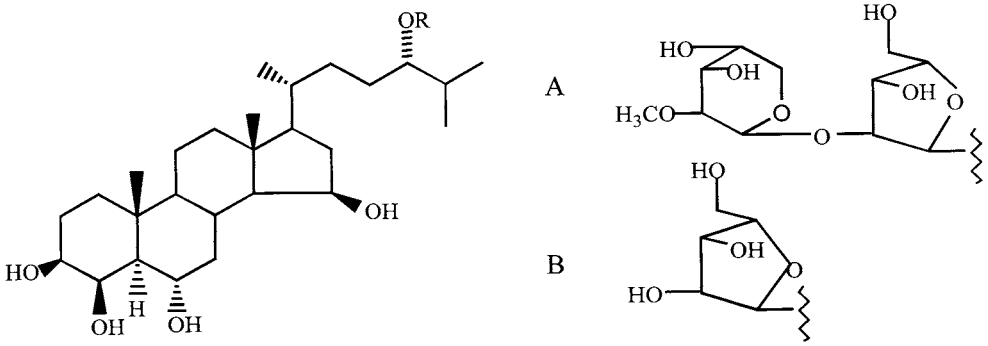
The isolated compounds were tested for cytotoxicity against a small panel of human solid tumor cell lines (A549, SK-OV-3, SK-MEL-2, XF498, CNS, HCT 15). The polyhydroxysteroids were more potent than

the corresponding glycosides except certonardosides P_1 (**32a**) and J_3 (**32c**).



32a Certonardoside O_1 R_1 = H, R_2 = H, R_3 = A
32b Certonardoside P_1 R_1 = H, R_2 = OH, R_3 = A
32c Certonardoside J_2 R_1 = OH, R_2 = H, R_3 = A
32d Certonardoside J_3 R_1 = OH, R_2 = H, R_3 = A, Δ^{22}
32e Certonardoside I_2 R_1 = OH, R_2 = OH, R_3 = A
33a Certonardoside H_2 R_1 = OH, R_3 = A, 24*R*
33b Certonardoside B_2 R_1 = H, R_3 = B, 24*S*
33c Certonardoside B_3 R_1 = H, R_3 = B, Δ^{22}, 24*R*

Fig. (11). Certonardosides O_1 – B_3 from *Certonardoa semiregularis*

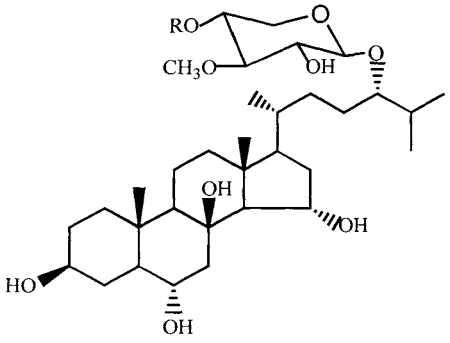


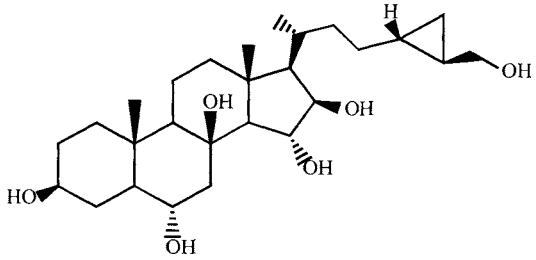
34 Certonardoside H_3 R = A
35 Certonardoside H_4 R = B

Fig. (12). Certonardosides H_3 and H_4 from *Certonardoa semiregularis*

Asterosaponins are usually considered to be responsible for the general toxicity of starfish. However, this investigation showed that sterols and another class of minor chemical components, such as glycosides of polyhydroxysteroids also play an important role in the general toxicity.

Recently, a new sulfated steroidal monoglycoside phrygioside B (**36**) together with its desulfated analog borealoside C (**36a**) [44] and the cyclopropane-containing steroid phrygiasterol (**37**), Fig. (**13**) have been isolated from the Pacific starfish *Hippasteria phrygiana* [45]. Compound **37**, the first cyclopropane-containing steroid isolated from echinoderms, inhibited the growth of Ehrlich carcinoma cells with an IC_{50} of 50 μg/ml, whereas borealoside C induced apoptosis of the same cells (EC_{50} = 70 μg/ml) and inhibited Ca^{2+} influx into mouse spleenocytes (EC_{50} = 20 μg/ml).





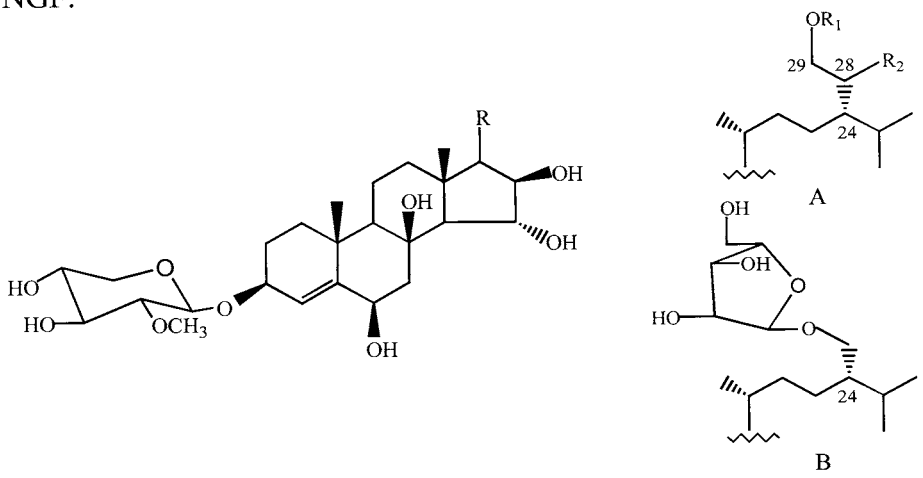
37

36 Phrygioside B R = $SO_3^-Na^+$
36a Borealoside C R = H

Fig. (13). Structures of phrygiasterol and related compounds from *Hippasteria phrygiana*

Bioactivity-guided fractionation of the alcoholic extract of the Okinawan starfish *Linckia laevigata* led to the isolation of five new neuritogenic polyhydroxysteroid diglycosides, Fig. (**14**) [46, 47]. These compounds are further examples of a group of rare steroidal glycosides in which the monosaccharide residues are located in two different positions of the steroidal nucleus [48]. Each of the linckosides possesses one 2-*O*-methylxylopyranose unit attached to C-3 of a 3β,6β,8β,16β-tetrahydroxy substituted steroidal aglycone and an additional monosaccharide unit at the aglycone side chain (C-24, C-28 or C-29).

Linckosides A (**38**) and B (**39**) differ in the sugar unit attached to C-29, an α-arabinofuranosyl in **38** and a xylose unit in **39**. Linckosides C (**40**) and D (**41**) are the first steroids that possess a hydroxyisopropyl substituent at C-24 of the side chain. The neuritogenic activity of diglycosides **38-42** was evaluated using rat pheochromocytoma (PC12) cells in comparison with NGF (nerve growth factor). Linckosides B, C and D with a xylose unit in the side chain showed significant neuritogenic activities of 62%, 56% and 73%, respectively, comparable with that of NGF.



38 Linckoside A R = A, R_1 = L-arabinose, R_2 = H
39 Linckoside B R = A, R_1 = D-xylose, R_2 = H
40 Linckoside C R = A, R_1 = D-xylose, R_2 = CH_3
41 Linckoside D R = A, R_1 = D-xylose, R_2 = CH_3, Δ^{22}
42 Linckoside E R = B

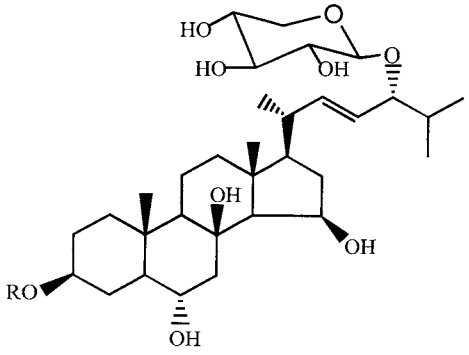
Fig. (14). Structures of Linckosides A-E from the starfish *Linckia laevigata*

On the other hand, linckosides A and B with an arabinose unit at the side chain exhibited lower activities (33% and 29%, respectively) than the others. This result suggests that the nature of the sugar moiety at the side chain plays an important role for the neuritogenic activity and that other structural factors, such as the presence or absence of a methyl group at C-28 and the double bond at C-22, seemed not to be important. All the linckosides showed significant synergistic effects on the NGF-induced neuronal differentiation of PC12 cells.

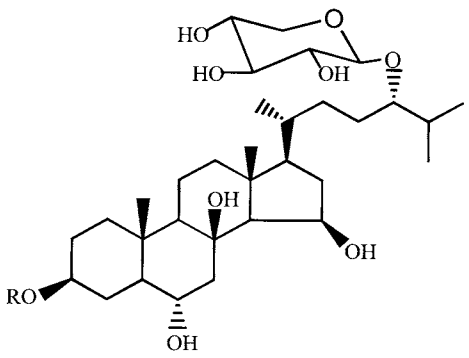
Bioactivity-guided fractionation of the ethanolic extract of the Patagonian starfish *Anasterias minuta* using the brine shrimp (*Artemia salina* L.) larvae mortality assay led us to the isolation of three sulfated

polyhydroxylated steroidal xylosides, the new minutosides A (**43**) and B (**44**), together with the known pycnopodioside B (**45**), Fig. (**15**) [49]. The structures were elucidated by extensive 1D and 2D NMR experiments as well as FABMS analysis and chemical methods.

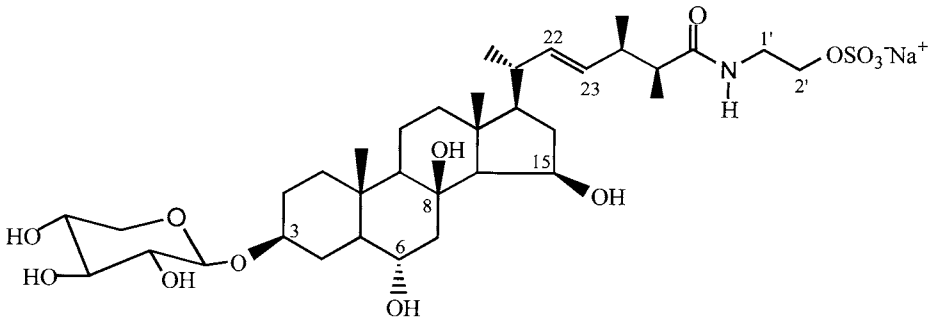
The molecular formula of compound **44** was established as $C_{35}H_{58}O_{12}NSNa$ on the basis of the pseudomolecular ion peak at *m/z* 762.3481 $[M + Na]^+$ in the HRFABMS. Examination of the ^1H- and ^{13}C-NMR spectra indicated that **44** possessed the same 3β,6α,8,15β-tetrahydroxy steroidal nucleus as glycosides **43** and **45** bonded to a monosaccharide unit.



43 R = $SO_3^-Na^+$
43a R = H



45 R = $SO_3^-Na^+$
45a R = H



44

Fig. (15). Bioactive monoglycosides from the starfish *Anasterias minuta*

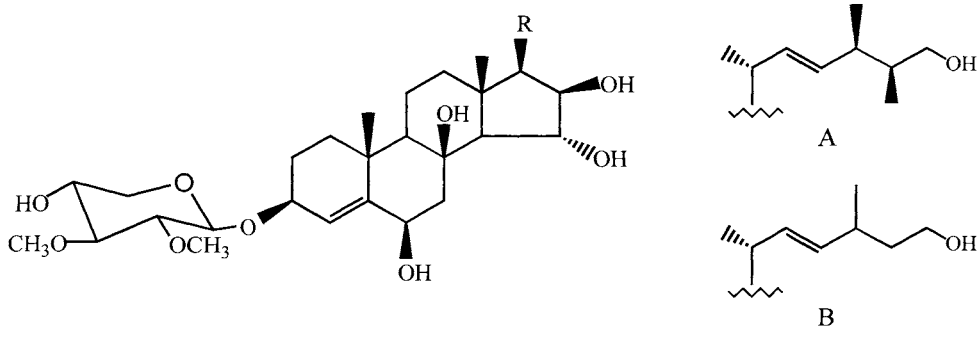
The relative stereochemistry of all chiral centers was established by analysis of selected NOE correlations. The hydroxyl groups at C-3 and C-6 were assigned to 3β and 6α on the basis of NOEs between H-3 and H-

5α and between H_3-19 and H-6. Correlations between H-14/H-15 and H-15/H-16α revealed the β-configuration of the hydroxyl group at C-15. The ^1H NMR spectrum contained three methyl doublets at δ 0.95, 0.96 and 1.07 ppm which were assigned to CH_3-28, CH_3-21 and CH_3-27, respectively by analysis of the ^1H-^1H COSY, HMQC and HMBC spectra. The ^1H-NMR spectrum of **44** also contained two methylene signals at δ 2.96 and 3.53 ppm coupled to each other in the ^1H-^1H COSY spectrum. Correlation of these protons in the HMQC spectrum to the signals at δ 51.5 (C-1') and 36.6 ppm (C-2'), together with the correlation of the triplet at δ 2.96 (H_2-1') to the Me-27 (δ_H 1.07) in the NOESY spectrum as well as the chemical shift of the carbonyl group (δ_C 178.5) suggested the presence of a $\Delta^{(22E)}$, 26-amide ergostane side chain [50]. The strong bands at 1212 and 1050 cm^{-1} in the FTIR spectrum, characteristic of a sulfonic acid salt, together with the fragment ion at *m/z* 455 [M - SO_3Na - Xyl + Na]$^+$ in the positive FABMS and the NMR data of **44** suggested the presence of a taurine residue in the side chain. This side chain has been previously identified in a polyhydroxysteroid isolated from the starfish *Myxoderma platyacanthum* [50]. Recently, the aglycone of minutoside B (**44**), triseramide, has been isolated from the starfish *Astropecten triseriatus* [51].

Analysis of the monosaccharide signals in the ^{13}C-NMR spectrum (103.1, 77.9, 75.0, 71.3 and 66.9) and further assignment of. all proton and carbon chemical shifts using ^1H-^1H COSY and HMQC experiments indicated the presence of a xylose residue. The presence of xylose was confirmed by acid hydrolysis of **44** with aqueous 2N trifluoroacetic acid followed by GC analysis of the corresponding peracetylated alditol. The D-configuration was determined by GC analysis of the 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivative as for minutoside A (**43**).The chemical shift of the anomeric carbon (δ_C 103.1) and the coupling constant of the anomeric proton (δ_H 4.36, $J_{1',2'}$ = 7.7 Hz) suggested that the sugar had a β-configuration. The location of the sugar at C-3 of the aglycon was established on the basis of the correlation between the anomeric proton and H-3α in the NOESY spectrum of **44**. Minutoside B (**44**) is the first example of a steroidal xyloside containing an amide function in the aglycone obtained from a natural source. Only a few examples of polyhydroxylated sterols with an amide function in their side chains have been reported previously from the starfishes *Myxoderma platyacanthum* [50] and *Styracaster caroli* [52].

Since some polyhydroxylated steroid glycosides have shown interesting biological activities, compounds **43-45** and the desulfated analogs **43a** and **45a** were examined against the pathogenic fungi *Cladosporium cucumerinum* and *Aspergillus flavus* by a bioautographic technique [29]. Minutoside A (**43**) and pycnopodioside B (**45**) were moderately active (inhibition zones of 7–10 mm) against *C. cucumerinum* at the tested concentrations (10–60 μg/spot), while minutoside B (**44**) was inactive at the lowest concentration (10 μg/spot) and weakly active (inhibition zones of 3–4 mm) at the highest concentrations (20–60 μg/spot). The three glycosides (**1-3**) were moderately active against *A. flavus* showing inhibition zones of 5–10 mm at the highest tested concentrations (20–60 μg/spot). The compounds were found to be less active than benomyl, a commercially available fungicide, which showed inhibition zones of 15 and 14 mm at a concentration of 5 μg/spot for *C. cucumerinum* and *A. flavus*, respectively. The three xylosides were also less active against *C. cucumerinum* than the asterosaponins versicoside A (**6**) and anasteroside A (**8**) isolated from *A. minuta*. As for the asterosaponins, the desulfated analogs **43a** and **45a** were inactive against *C. cucumerinum* and *A. flavus* at all the tested concentrations. These results suggest that the presence of a sulfate group in the aglycon moiety may play an important role in the antifungal activity of these monoglycosides.

Recently, two related monoglycosides, the known henricioside H_2 (**46**) and the new leviusculoside J (**47**) have been isolated from the Far Eastern starfish *Henricia leviuscula*, Fig. (**16**) [53].



46 Henricioside H_2 R = A
47 Leviusculoside J R = B

Fig. (16). Bioactive monoglycosides from the starfish *Henricia leviuscula*

Both 3,6-di-*O*-methylxylanosides differ in the aglycone side chain. Compounds **46** and **47** showed 70% and 100% hemolysis of mouse erythrocytes, respectively, at a concentration of 8.0 x 10^{-5} M.

TRITERPENOID GLYCOSIDES FROM HOLOTHUROIDEA

Holothurins isolated from sea cucumbers are triterpenoid oligoglycosides that contain an aglycone based on a "holostanol" skeleton [3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone] (**48**), Fig. (**17**) and a sugar chain of two to six monosaccharide units linked to the C-3 of the aglycone [54]. Quinovose, glucose, 3-*O*-methylglucose, xylose and, rarely 3-*O*-methylxylose are present in the carbohydrate moieties of these glycosides. The first monosaccharide unit is always xylose, while 3-*O*-methylglucose and 3-*O*-methylxylose are always terminal.

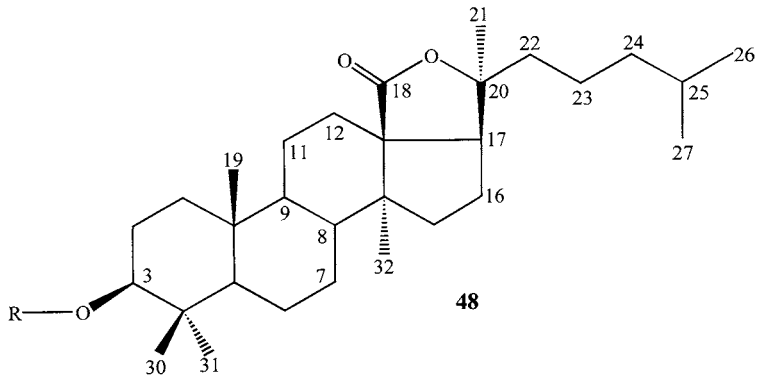


Fig. (17). Structure of hypothetical holostanol

Most of sea cucumber triterpene glycosides are tetra- or pentaglycosides. The majority of tetrasaccharides show a linear chain with the most common 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)-Xyl structure. The few disaccharides that have been isolated show a Qui-(1→2)-4-OSO_3Na-Xyl chain attached to C-3 of the triterpenoid aglycone. Some hexasaccharides have been isolated from sea cucumbers of the order Aspidochirota: *Stichopus japonica*, *Stichopus chloronotus*, *Parastichopus californius* and *Bohadschia bivittata* [55]. They are non-sulfated glycosides with a linear 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Xyl chain and a branching of a linear trisaccharide at C-2 of the xylose unit.

Sixty percent of the triterpene glycosides isolated so far from sea cucumbers have sulfate groups linked to the monosaccharide units of the oligosaccharide chain. Most of them are monosulfated oligoglycosides, but several di- and trisulfated glycosides have been isolated, mainly from the order Dendrochirotida. Most tetrasaccharides are sulfated at C-4 of the xylose unit. Additional sulfate groups at C-6 of the 3-*O*-Me-glucose unit and at C-6 of the glucose unit have been found in trisulfated tetraglycosides. Most of the pentasaccharide chains are monosulfated at C-4 of the xylose unit linked to the aglycone. Only a few disulfated or trisulfated pentaglycosides with additional sulfate groups at C-6 of the 3-*O*-Me-glucose and glucose units have been isolated [55].

More than 100 different holothurins have been isolated from sea cucumbers in the last 25 years. These saponins differ in the composition and number of the sugar units, the number and positions of the sulfate groups and the structural characteristics of the aglycone. Some examples of holothurins having non-holostane aglycones have been found in seven species of sea cucumbers belonging to the order Dendrochirotida. The majority are monosulfated at the glucose or xylose units [55]. Triterpene glycosides are produced in the skin and in the Cuvier's tubules of sea cucumbers and are ejected when the animals are disturbed. This behavior may be associated to a defensive function due to the ability of holothurins to form complexes with cholesterol and other Δ^5-sterols from cell membranes. This membranotropic action determines the wide spectrum of their biological activities [15]. Sea cucumbers are resistant to their own toxins due to the presence of Δ^7-, 14α-methyl- and 14α-dimethyl- $\Delta^{9,11}$-sterols as well as their conjugated forms such as steryl sulfates and steryl xylosides [56].

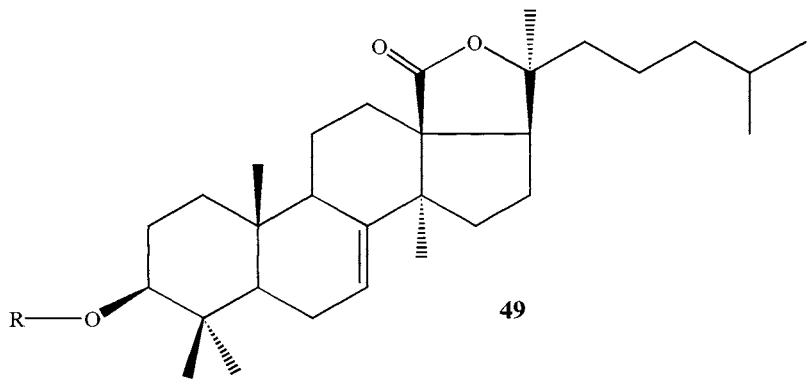
Several holothurins are specific for different taxonomic groups of sea cucumbers and structural characteristics of triterpene glycosides have been used to resolve taxonomic problems in the class Holothuroidea [57, 58]. For example, the triterpenoid glycosides distribution has been successfully applied in the reclassification of *Stichopus mollis* into the genus *Australostichopus* [59] and in the taxonomy of sea cucumbers belonging to the genus *Cucumaria* [60].

Recently, we have reviewed for the first time the chemical structures of sea cucumber glycosides and the principal spectral features of the aglycones and oligosaccharide chains in their ^{1}H- and ^{13}C-NMR spectra [55]. Most of these triterpenoid oligoglycosides contain an aglycone

based on a "holostanol" skeleton and two main series can be distinguished: glycosides based on a 3β-hydroxyholost-9(11)-ene aglycone and those containing a 3β-hydroxyholost-7-ene skeleton. Only a few examples of holothurins containing a non holostane aglycone have been isolated from sea cucumbers of the order Dendrocirotida. Usually aglycones that have a $\Delta^{9,11}$ double bond are characteristic of sea cucumbers belonging to the order Aspidochirota, while those with a Δ^{7} unsaturation were generally isolated from animals of the order Dendrochirotida.

3β-Hydroxyholost-7-ene aglycones

Several triterpene glycosides isolated from the sea cucumbers *Cucumaria frondosa, Cucumaria japonica, Stichopus chloronotus* and *Thelenota ananas* contain the simple 3β-hydroxyholost-7-ene as the aglycone (**49**) [55]. Recently, three new monosulfated pentaglycosides, frondosides A_2-2 (**49a**) A_2-3 (**49b**) and A_2-4 (**49c**), Fig. (**18**) were isolated from the sea cucumber *Cucumaria frondosa* [61, 62].



49

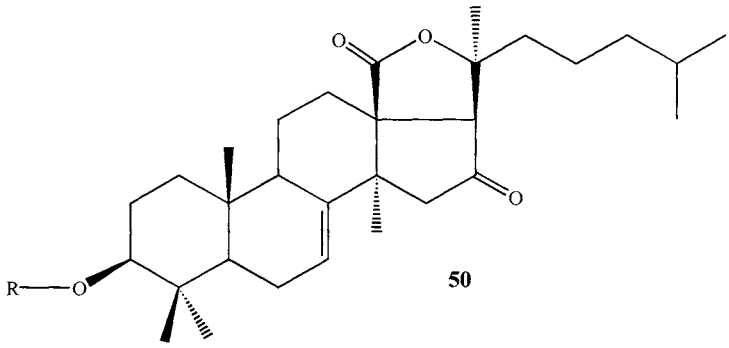
49a Frondoside A_2-2 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; 24-ceto, Δ^{25}
49b Frondoside A_2-3 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; 25-OH, Δ^{23} (*E*)
49c Frondoside A_2-4 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{24}

Fig. (18). Sulfated glycosides isolated from the sea cucumber *Cucumaria frondosa*

The three holothurins possess the same pentasaccharide moiety and differ from each other in the aglycone side chain. Frondoside A_2-3 (**49b**) contains a Δ^{23} double bond and a hydroxy group at C-25. This side chain has been previously identified in the aglycones of cucumarioside G_4 from

Eupentacta fraudatrix [63] and Eximisoside A from *Psolus eximius* [64]. In frondoside A_2-2 (**49a**), the simultaneous presence of a 24-ketone conjugated with a Δ^{25} double bond in the side chain is a unique characteristic for holothurins.

Dendrochirotida sea cucumbers belonging to the family Cucumariidae were extensively studied during the last years. Recently, the cytotoxic activities of the known pentaglycosides cucumariosides A_2-2 (**50a**) and A_7-1 (**50b**), Fig. (**19**) isolated from the sea cucumber *Cucumaria japonica* [65] were investigated using embryos of the sea urchin *Strongylocentrotus nudus* [66]. Cucumariosides A_2-2 and A_7-1 were highly toxic and induced morphological abnormalities and delay of development. EC_{50} values of glycosides **50a** and **50b** were determined as 0.3 and 1.98 μg/ml, respectively, whereas the aglycone was completely inactive at 100 μg/ml. Both cucumariosides differ only in the number of sulfate groups in the oligosaccharide chain. Cucumarioside A_2-2 is a monosulfated glycoside while A_7-1 is a trisulfated holothurin.



50

50a Cucumarioside A_2-2 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4 OSO_3Na-Xyl; Δ^{25}
50b Cucumarioside A_7-1 R = [6-OSO_3Na-3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
50c Cucumarioside A_3 R = [3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
50d Cucumarioside A_6-2 R = [6-OSO_3Na-3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
50e Frondoside A_2-1 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{24}
50f Mollisoside B_2 R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)- 4-OSO_3Na-Xyl; Δ^{25}

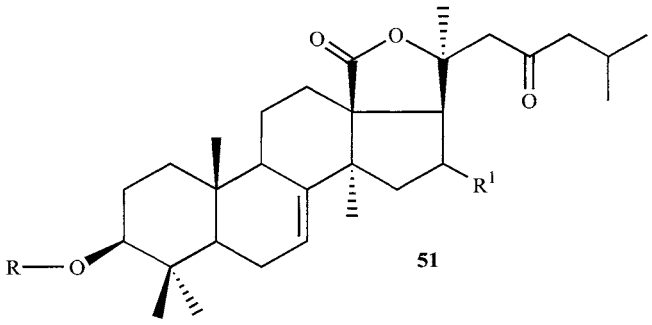
Fig. (19). Glycosides with a 3β-hydroxyholost-7-en-16-one aglycone

Disulfated cucumariosides A_3 (**50c**) and A_6-2 (**50d**), Fig. (**19**) differ from the monosulfated cucumarioside A_2-2 (**50a**) in the presence of an additional sulfate group at C-6 of the glucose unit in **50c** and at C-6 of the

3-*O*-methyl glucose unit in **50d**. Both holothurins showed cytotoxicity in vitro at IC_{50} = 1μg/ml against a selection of five human and mice tumoral cell lines [67].

Recently, a new monosulfated holothurin has been isolated from the sea cucumber *Cucumaria frondosa* [61]. The structure of frondoside A_2-1 (**50e**) is very similar with that of cucumarioside A_2-2 (**50a**), the only difference being the presence of a Δ^{24} double bond instead of a Δ^{25} one. Another new sulfated tetraglycoside, mollisoside B_2 (**50f**) was isolated from the sea cucumber *Australostichopus mollis*, collected at New Zealand and sotuhern Australia [68]. This holothurin has the same aglycone as Cucumariosides A_2-2, A_7-1, A_3 and A_6-2 and differs from them in the oligosaccharide chain. Mollisoside B_2 (**50f**) has a linear terasaccharide chain monosulfated at C-4 of the xylose unit.

Several triterpene glycosides isoalted from the sea cucumbers *Cucumaria echinata* and *Pentamera clacigera* contain aglycones with a carbonyl group at C-23 in the side chain. This structural feature is absent in 3β-hydroxyholost-9(11)-ene aglycones [55]. Two new glycosides containing this aglycone have been isolated from the sea cucumber *Pentamera calcigera*. Calcigerosides C_2 (**51a**) [69] and D_2 (**51b**) [70], Fig. (**20**) have a branched pentasaccharide chain with 3-*O*-methyl xylose as a terminal residue. Glycoside **51a** contains a terminal glucose attached to the quinovose residue. This feature has not been found before in sea cucumbers.



51

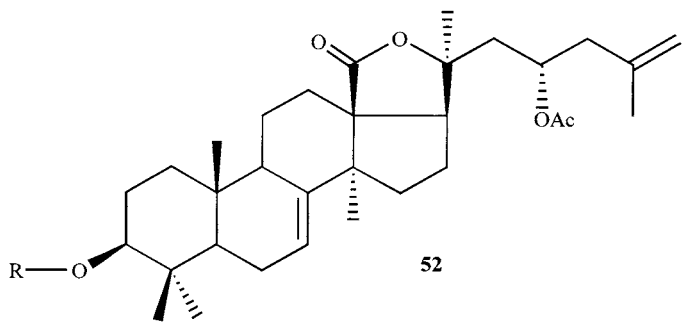
51a Calcigeroside C_2 R = [3-OMe-Xyl-(1→3)-Glc-(1→4)]-[Glc-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; R^1 = H
51b Calcigeroside D_2 R = [3-OMe-Xyl-(1→3)- 6-OSO_3Na-Glc-(1→4)]-[Glc-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; R^1 = H
51c Cucumarioside A_2-5 R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = OAc (β)

Fig. (20). Bioactive glycosides isolated from the sea cucumber *Pentamera calcigera*

Calcigeroside C_2 and its desulfated derivative were individually tested in vitro against four standard human and mouse tumoral cell lines (P-388, A-549, HT-29 and Mel-98). Only the desulfated derivative showed moderate cytotoxicity (IC_{50} = 5 μg/ml).

Another example of an holothurin containing aglycon **51** with a β-acetoxy group at C-16, Cucumarioside A_2-5 (**51c**), was isolated from the sea cucumber *Cucumaria conicospermium*, collected in the Sea of Japan [71]. The same aglycone has been found earlier only in Cucumarioside A_0-1, isolated from *Cucumaria japonica* [72].

Five non-sulfated triterpene glycosides, synallactosides A_1 (**52a**), A_2 (**52b**), B_1 (**52c**), B_2 (**52d**) and C (**52e**), Fig. (**21**) were isolated from the deep-water North Pacific sea cucumber *Synallactes nozawai* [73]. Synallactoside A_1 is the 25,26-dehydro derivative of thelenotoside A, isolated from *Thelenota ananas* as a mixture with thelenotoside A [74]. Compounds **52b-52e** are new compounds with interesting features in the carbohydrate chains of synallactosides A_2, B_1, and B_2. Synallactoside A_2 (**52b**) is the first glycoside isolated from sea cucumbers with two terminal 3-*O*-methylxylose residues in the oligosaccharide chain. On the other hand, the carbohydrate chains of synallactosides B_1 (**52c**) and B_2 (**52d**) have unprecedent structures among holothurins.



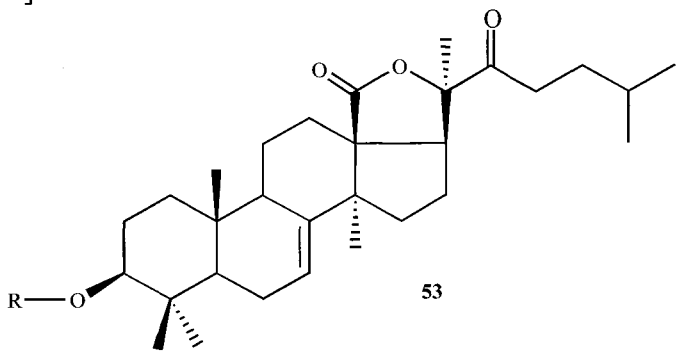
52

52a Synallactoside A_1 R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Quin-(1→2)]-Xyl
52b Synallactoside A_2 R = 3-*O*-Me-Xyl-(1→3)-Xyl-(1→4)-Quin-(1→2) -[3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-Xyl
52c Synallactoside B_1 R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Quin-(1→2) -[3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-Xyl
52d Synallactoside B_2 R = 3-*O*-Me-Xyl-(1→3)-Xyl-(1→4)-Quin-(1→2) -[Glc-(1→4)]-Xyl
52e Synallactoside C R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Quin-(1→2) -[Glc-(1→4)]-Xyl

Fig. (21). Glycosides isolated from the sea cucumber *Synallactes nozawai*

Recently, two new sulfated holothurins, pseudostichoposide B (**53a**) and mollisoside A (**53b**), Fig. (**22**) were isolated from two sea cucumbers belonging to the order Aspidochirotida, *Pseudostichopus trachus* [75] and

Australostichopus mollis [68]. Both glycosides contain an aglycone with a 22-keto group in the side chain, a novel feature for sea cucumber glycosides. Pseudostichoposide B (**53a**) contains a sulfate group at C-4 of the first xylose residue and an additional sulfate group at C-3 of the quinovose residue, a feature not earlier found in holothurins. The isolation of mollisosides A (**53b**) and B_2 (**50f**) together with the known neothyonidioside reported earlier from the sea cucumber *Neothyonidium magnum* [76] as well as morphological features justified the reclassification of *Stichopus mollis* in the new genus *Australostichopus* Levin [59].

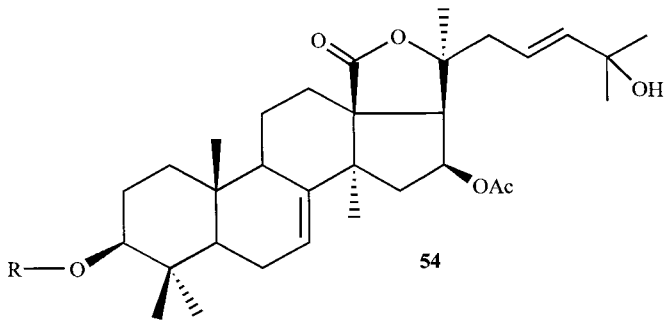


53

53a Pseudostichoposide B R = 3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-3-OSO_3Na-Quin-(1→2)-4-OSO_3Na-(1→4)-Xyl
53b Mollisoside A R = 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}

Fig. (22). Glycosides with a 22-keto-holost-7-en-3β-ol aglycone

Calcigeroside E (**54**), Fig. (**23**) is a new disulfated pentaglycoside isolated from the sea cucumber *Calcigera pentamera* [70].



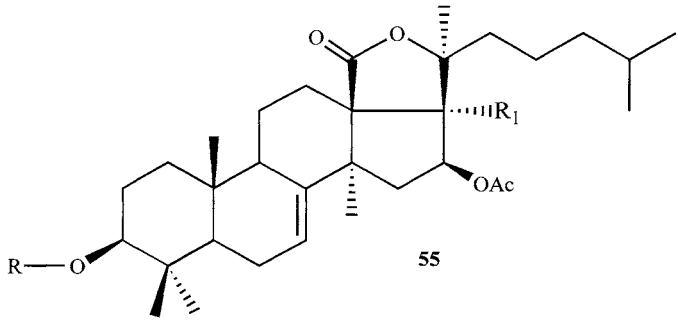
54

Calcigeroside E R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-Glc-(1→4)]-[Glc-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (23). Structure of Calcigeroside E isolated from *Calcigera pentamera*

The aglycone of this glycoside has been previously found in only two holothurins, cucumarioside G_4 [63] and eximisoside A [64].

One structural feature that has been found only in the 3β-hydroxyholost-9(11)-ene aglycones is the presence of an acetoxyl group at C-16 (α or β). Recently, nine new bioactive sulfated tetraglycosides (compounds **55a-55i**, (Fig. (**24**)) containing a 6β-acetoxy group at C-16 have been isolated from the sea cucumber *Mensamaria intercedens* [77, 78]. Intercedensides A (**55a**), C (**55c**), D (**55d**), E (**55e**), G (**55g**) and H (**55h**) contain a conjugated double bond (22,24-diene) in the aglycone side chain. This feature is very rare among sea cucumber glycosides. Analogous side chains were found only in cucumariosides C_1, C_2 [79] H [80] and G_3 [63]. Intercedensides C (**55c**), D (**55d**), E (**55e**), F (**55f**), H (**55h**) and I (**55i**) have a 17α-hydroxy group that is not characteristic for sea cucumbers of the Dendrochirotida order with exception of patagonicoside A, isolated previously from *Psolus patagonicus* [81].



55

55a Intercedenside A R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; 22*Z*; Δ^{24}; R_1 = H
55b Intercedenside B R = 6-OSO_3Na-3-OMe-Glc-(1→3)-Xyl-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{24}; R_1 = H
55c Intercedenside C R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-4-OSO_3Na-Xyl; 22*E*; Δ^{24}; R_1 = OH
55d Intercedenside D R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-4-OSO_3Na-Xyl; 22*Z*; Δ^{24}; R_1 = OH
55e Intercedenside E R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Xyl-(1→2)-4-OSO_3Na-Xyl; 22*Z*; Δ^{24}; R_1 = OH
55f Intercedenside F R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-4-OSO_3Na-Xyl; Δ^{24}; R_1 = OH
55g Intercedenside G R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Xyl-(1→2)-4-OSO_3Na-Xyl; 22*Z*; Δ^{24}; R_1 = H
55h Intercedenside H R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; 22*Z*; Δ^{24}; R_1 = OH
55i Intercedenside I R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{24}; R_1 = OH
55j Liouvilloside A R = 6-OSO_3Na-3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{24}; R_1 = H
55k Liouvilloside B R = 6-OSO_3Na-3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; R_1 = H
55l ThyonosideA R = 3-*O*-Me-Xyl-(1→3)-6-OSO_3Na-Glc-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}, R_1 = H
55m ThyonosideB R = 3-*O*-Me-Xyl-(1→4)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}; R_1 = H

Fig. (24). Sulfated glycosides from *Mensamaria intercedens*, *Staurocucumis liouvillei* and *Thyone aurea*

Intercedensides A-H showed significant in vitro cytotoxicity against 10 human tumor cell lines (A549, MCF-7, IA9, CAKI-1, U-87-MG, PC-3,

KB, KB-VIN, SK-MEL-2, HCT-8) with ED_{50} values in the range 0.6-4.0 μg/ml. Intercedenside A exhibited significant in vivo antineoplastic activity against mouse Lewis lung cancer and mouse S180 sarcoma.

Two new trisulfated tetraglycosides, Liouvillosides A (**55j**) and B (**55k**) were isolated from the Antarctic sea cucumber *Staurocucumis liouvillei* [83]. Both glycosides differ only in the presence of a Δ^{24} double bond in compound **55j**. The terminal isoproprenyl group in the side chain of liouvilloside A showed characteristic signals in the ^{13}C-NMR spectrum for the olefinic cabons at δ 123.9 ppm (C-24) and 131.3 ppm (C-25) as well as for the methyl groups attached to C-25 at δ 25.6 ppm (C-26) and 17.8 ppm (C-27). The presence of these two methyl vinyl groups was easily confirmed by the downfield shift of the methyl singlets in the ^{1}H-NMR spectrum at δ 1.54 ppm (H-26) and 1.64 ppm (H-27), while liouvilloside B (**55k**), the saturated analog, showed two nearly overlapped doublets (J = 6.6 Hz) at δ 0.83 and 0.84 ppm. Glycosides **55j** and **55k** showed a characteristic singlet at δ 2.0 ppm ($C\underline{H}_3CO_2$) as well as signals at δ 169.4 and 21.1 ppm for the carbonyl and methyl groups of the acetate moiety. The position of the acetoxyl group at C-16 was deduced from the chemical shift of the H-16 signal (δ 5.63 ppm) and its correlation with H-17, H-15α and H-15β in the ^{1}H-^{1}H COSY spectrum. The 16β-configuration was assigned by a NOESY experiment and by coupling constant analysis for the C-16 proton with the C-17α and C-15 protons. Calculated coupling constant values of 8.9 ($J_{15\alpha,16\alpha}$), 7.4 ($J_{15\beta,16\alpha}$) and 8.9 Hz ($J_{16\alpha,17\alpha}$) for the most stable conformation of 16β-acetoxyholosta-7,24-dien-3β-ol obtained by molecular mechanics were coincident with experimental and reported values [83] and differed considerably from those calculated for the 16α-isomer (4.1, 6.9 and 1.2 Hz, respectively).

In addition to the aglycone signals, the ^{1}H-NMR spectra of **55j** and **55k** showed four anomeric protons at δ 4.32 (xylose), 4.40 (glucose), 4.47 3-*O*-methylglucose) and 4.49 ppm (quinovose) that correlated with the anomeric carbon signals at δ 104.2, 103.1, 103.8 ppm. The β-stereochemistries at the anomeric carbons were deduced from the coupling constant values (J = 7.1–7.9 Hz). The position of the interglycosidic attachments was determined by a combination of ^{1}H-^{1}H COSY, relayed COSY and HETCOR experiments. The four carbohydrate units were determined to belong to the D-series by GC analysis of the mixture of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol

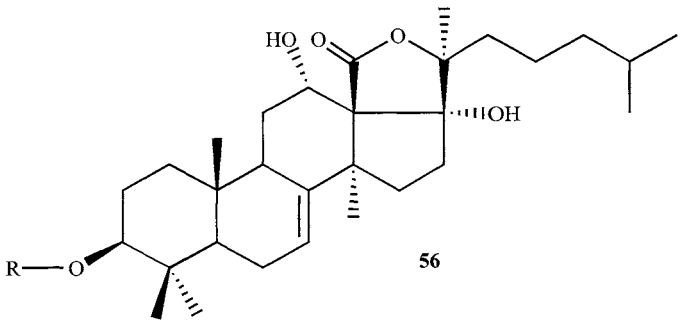
acetate derivatives. The position of the sulfate groups at C-6 of both glucose units and at C-4 of the xylose unit in the sugar chain was determined by comparison of ^{13}C-NMR data of both glycosides with those of the corresponding desulfated derivatives obtained by hydrolysis in anhydrous 0.15% HCl-MeOH at room temperature. This work is the first study of the glycosidic content of an Antarctic sea cucumber belonging to the genus *Staurocucumis* (family Cucumariidae, order Dendrochirotida).

Evaluation of the cytotoxicity of liouvillosides A and B at concentrations ranging from 6.25 to 50 μg/ml showed little or no cytotoxicity within eight hours of cell exposure to the compounds, but both saponins were cytotoxic following prolonged incubation periods. According to these results, the virucidal activity of both glycosides was then evaluated by incubation of a suspension of herpes simplex virus 1 (HSV-1) with the holothurins at concentrations below 10 μg/ml. Both saponins exerted an irreversible virucidal effect on HSV-1, but with different effectiveness: liouvilloside A produced a weak inactivation of HSV-1 since at the maximum concentration tested the residual infectivity was 24% with respect to the control virus sample, whereas after treatment with liouvilloside B in the same experimental conditions the remaining infectivity was 10-fold lower (2.5%). These results are in agreement with previous reports on the activity of triterpenoid compounds against several viruses, including important pathogens such as HSV and human immunodeficiency virus. In particular, naturally occurring saponins with different structures were found to be inhibitory of HSV through either a direct virucidal effect or by interference with an early step of the viral replicative cycle.

Two further examples of new sulfated holothurins containing aglycone **55** with a Δ^{25} double bond were isolated from the sea cucumber *Thyone aurea*, collected in Namibia [84]. Thyonosides A (**55l**) and B (**55m**) differ from each other in one glycosidic residue, xylose in glycoside **55m** instead of glucose-6-OSO_3Na in **55l** and in the β-(1→4) linkage between 3-*O*-methylxylose and xylose in Thyonoside B. Both glycosides contain the rare terminal 3-*O*-methylxylose.

Purification of the triterpenoid glycosides mixture of the Patagonian sea cucumber *Psolus patagonicus* led us to isolate patagonicoside A (**56a**), Fig. (**24**), the main holothurin in the mixture [81]. This disulfated tetraglycoside has an uncommon aglycone with two hydroxyl groups at

C-12α and C-17α, a structural feature characteristic for aspidochirotid sea cucumbers. This is the first report of an aglycone with simultaneous presence of 12α- and 17α-hydroxy groups and a Δ^7 double bond. The presence of two hydroxy groups attached to C-12 and C-17 was evidenced by two signals in the ^{13}C-NMR spectrum at δ 73.6 and 90.7 ppm, respectively. The C-7 signal at δ 90.7 ppm could be readily distinguished from the C-3 signal (δ 90.8 ppm) by a DEPT experiment.



56

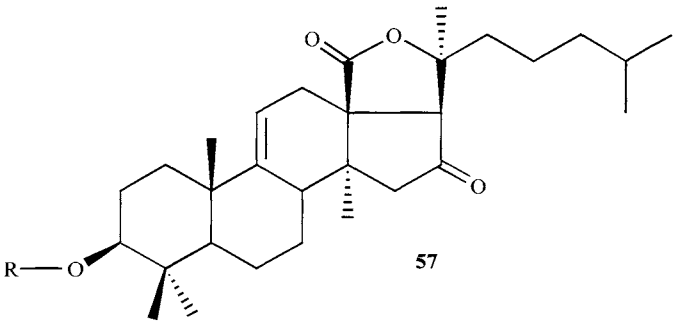
56a Patagonicoside A R = 3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl
56b Ds-Patagonicoside A R = 3-OMe-Glc-(1→3)-Glc-(1→4)-Quin-(1→2)-Xyl

Fig. (24). Structure of Patagonicoside A, an antifungal holothurin isolated from the sea cucumber *Psolus patagonicus* and its desulfated derivative

Since some holothurins with at least one oxygen function in positions 12, 16 or 17 of the aglycone moiety (e.g. holotoxins A and B, holothurins A and B, echinosides A and B, pervicosides A-C and Bivittosides A-D [55]) exhibit antifungal activities, patagonicoside A (**56a**) and its desulfated derivative, ds-patagonicoside A (**56b**) were examined against the phytopathogenic fungus *Cladosporium cucumerinum* by a bioautographic technique. Both saponins showed a marked difference in their antifungal properties. Patagonicoside A (**56a**) resulted to be considerable active, in a concentration dependent manner, showing inhibition zones of 8-19 mm at the tested concentrations (1.5-50 μg/spot). On the other hand, ds-patagonicoside A (**56b**) was inactive at the lowest concentrations (1.5 and 3 μg/spot) and weakly active (inhibition zones of 5-9 mm) at the highest tested concentrations (6-50 μg/spot). These results suggest that the presence of sulfate groups in the oligosaccharide chain may play an important role in the antifungal activity of these glycosides.

3β-Hydroxyholost-9(11)-ene aglycones

In the last four years, five new sulfated and two new non sulfated triterpene glycosides containing the aglycone 3β-hydroxyholst-9(11)-en-16-one (Structure **57**), Fig. (**25**) have been isolated from sea cucumbers.



57

57a Mollisoside B_1 R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Glc-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
57b Neothyonidioside C R = 3-OMe-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
57c Frondoside A_2-6 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
57d Hemoiedemoside A R = 3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
57e Hemoiedemoside B R = 6-OSO_3Na-3-OMe-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-Quin-(1→2)-4-OSO_3Na-Xyl; Δ^{25}
57g Parvimoside A R = [Glc-(1→3)-Glc-(1→4)]-[3-OMe-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl
57h Parvimoside B R = [Glc-(1→3)-Glc-(1→4)]-[3-OMe-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]-Xyl
57i Holotoxin B_1 R = [Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Xyl-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}
57j Holotoxin B R = [Glc-(1→3)-Glc-(1→4)]-[3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Qui-(1→2)]-Xyl; Δ^{25}

Fig. (25). Structures of 3β-hydroxyholost-9(11)-en-16-one aglycone based glycosides

The monosulfated tetraglicoside mollisoside B_1 (**57a**) was isolated from the sea cucumber *Stichopus mollis* [68] together with the major component of its glycosidic fraction, the known neothyonidioside C (**57b**) [59]. Glycosides **57a** and **57b** have holotoxinogenin as aglycone and a carbohydrate chain with a sulfate group at C-4 of the xylose group attached to C-3 of the aglycone, an uncommon feature for glycosides from sea cucumbers belonging to the family Stichopodidae.

The sea cucumber *Cucumaria frondosa* contains a complex mixture of Δ^7- and $\Delta^{9,11}$-holothurins. Frondoside A_2-6 (**57c**) [61] as well as the minoritary monosulfated pentaglycosides frondosides A_2-1 (**50e**), A_2-2 (**49a**), A_2-3 (**49b**) and A_2-4 (**49c**) [61, 62] have a glucose residue as the third monosaccharide unit in the carbohydrate chain in contrast to frondoside A, the major component of the glycosidic fraction [85].

Recently, we have isolated two new sulfated tetraglycosides, Hemoiedemoside A (**57d**) and B (**57e**), Fig. (**25**) from the sea cucumber *Hemoiedema spectabilis*, collected in the South Atlantic near the

Patagonian shore [86]. Both glycosides have the same aglycone, holotoxinogenin and a linear tetrasaccharide chain and differ in the number of sulfate groups attached to the monosaccharide residues. Glycoside **57d** is a disulfated compound while **57e** has three sulfate groups attached to C-6 of the two glucose units and C-4 of the xylose residue.

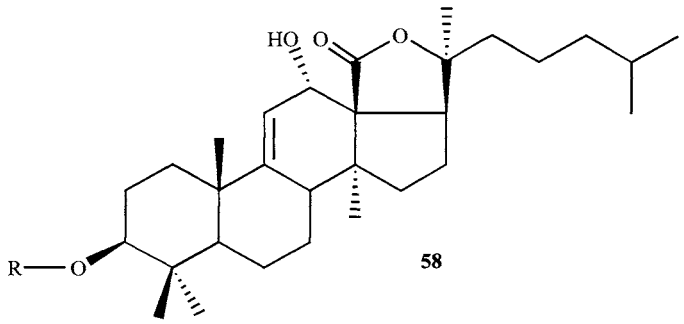
The assignment of the NMR signals associated with the aglycone moiety of **57d** and **57e** was derived from ^{1}H-^{1}H COSY, HETCOR and NOESY experiments. The ^{1}H-NMR signals of 3β-hydroxyholosta-9(11),25-dien-16-one were unambiguously assigned for the first time by application of the standard 2D NMR methods mentioned above. Analysis of NMR data indicated the presence of xylose, quinovose, glucose and 3-*O*-methylglucose in a ratio 1:1:1:1 in the oligosaccharide chain of both holothurins. This was confirmed by acid hydrolysis with aqueous 2N trifluoroacetic acid followed by GC analysis of the corresponding alditol peracetates. Analysis of the fragment ion peaks of **57d** in the FABMS (positive ion mode) at *m/z* 1209 $[M - SO_3Na + H + Na]^+$ and *m/z* 1083 $[M - 2SO_3Na + H]^+$ indicated the presence of two sulfate groups. This was confirmed by solvolytic desulfation of **57d** and comparison of its ^{13}C-NMR spectrum with that of the desulfated derivative. The pseudomolecular ion at *m/z* 1413 $[M + Na]^+$ of **57e** in the FABMS (positive ion mode) and fragment ions at *m/z* 1311 $[M - SO_3Na + H + Na]^+$, 1209 $[M - 2SO_3Na + 2H + Na]^+$ and *m/z* 1107 $[M - 3SO_3Na + 3H + Na]^+$ confirmed the presence of three sulfate groups in the saponin.

Hemoiedemosides A (**57d**) and B (**57e**) and their semisynthetic desulfated analog were evaluated for antifungal activity against the phytopathogenic fungus *Cladosporium cucumerinum*. Benomyl, a commercially available fungicide was used as reference compound. The three saponins were active in a concentration-dependent manner and the natural sulfated glycosides were more active than their desulfated analog which was inactive at the lowest concentrations (1.5–5 μg/spot) and weakly active at the higher ones (7.5–50 μg/spot). The disulfated holothurin was more active than benomyl at the higher concentrations (20–50 μg/spot), while trisulfated glycoside **57e** was slightly more active than the reference compound at 40 and 50 μg/spot. On comparing the antifungal activities against *C. cucumerinum* of hemoiedemoside A (**57d**) and patagonicoside A (**56a**), containing the same disulfated tetrasaccharide chain, glycoside **57d** showed inhibition zones of 8–33 mm

at the tested concentrations (1.5–50 μg/spot), while patagonicoside A was less active (8–19 mm) at the same concentrations. On the other hand, hemoiedemoside B (**57e**) was less active that **57d**. These results suggest that both the structure of the triterpenoidal aglycone and the presence and number of the sulfate groups at the oligosaccharide chain may play a significant role in the antifungal activity of these saponins. Further evaluation of the zootoxicities of the holothurins **57d** and **57e** and their desulfated analog using the brine shrimp (*Artemia salina* L.) larvae mortality bioassay showed that hemoiedemoside A (**57d**) had a noteworthy toxicity (LC_{50} 18.7 ppm). Hemoiedemoside B (**57e**) was 2 times less active (LC_{50} 47.5 ppm) than **57d** and nearly 10 times more active than the desulfated derivative (LC_{50} 424.5 ppm). These results correlated with the data on antifungal activity.

Most of the members of the Stichopodidae family (*Stichopus japonica* [87], *Stichopus chloronotus* [88], *Parastichopus californius* [89] and *Bohadschia bivittata* [90]) have been shown to produce hexosides as major components of the glycosidic fraction. They are non-sulfated glycosides with a linear 3-*O*-Me-Glc-(1→3)-Glc-(1→4)-Xyl chain and a branching of a linear trisaccharide at C-2 of the xylose unit. The only examples with a glucose unit instead of the terminal 3-*O*-Me-glucose are holotoxin B_1 (**57h**) and B (**57i**) [87, 89]. Recently, two new non-sulfated hexoglycosides, parvimosides A (**57f**) and B (**57g**), Fig. (**25**) were isolated from the sea cucumber *Stichopus parvimensis* [91]. Holothurin **57f** is related to holotoxin B, the difference being the absence of a double bond at position 25 in the aglycone of parvimoside A (**57f**). On the other hand, parvimoside B (**57g**) is the 25-dihydroderivative of holotoxin B_1 (**57h**).

Another structural feature in 3β-hydroxyholost-9(11)-ene aglycones is the presence of a 12α-hydroxyl group, such as in the hexaglycoside bivittoside D (Structure **58**), Fig. (**26**) [90]. Bivittoside D, widespread in sea cucumbers belonging to the genus *Bohadschia* (Holothuriidae) was described by Hedge *et al.* [92] as a new compound in the sea cucumber identified as *Telenata ananas*. Kalinin *et al.* [58] considered that this taxonomic classification relates to *Thelenota ananas* since the genus *Telenata* has never been described for sea cucumbers.

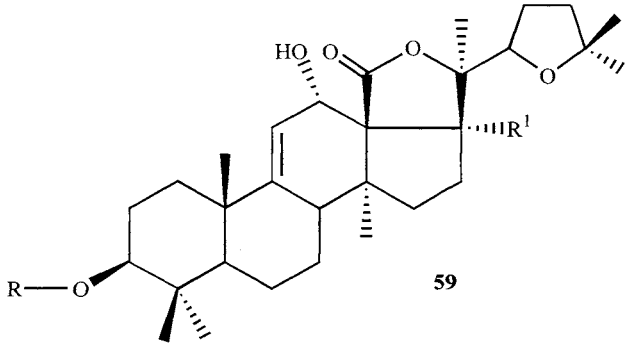


58

Bivittoside D R = [3-OMe-Glc-(1→3)-Gcl-(1→4)]-[3-OMe-Glc-(1→3)-Gcl-(1→4)-Quin-(1→2)]-Xyl

Fig. (26). Structure of Bivittoside D

T. ananas also contained a new sulfated triglycoside (**59a**), Fig. (**27**) with a 12α,17α-dihydroxyl and 22(25)-epoxy group in the aglycone, a very common for sea cucumbers from the family Holothuriidae [93, 94]. Holothurin **59a** contains a very uncommon trisaccharide carbohydrate chain.



59

59a R = [Glc-(1→4)]-[Quin-(1→2)]-Xyl; R^1 = OH
59b Holothurin B_3 R = Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H

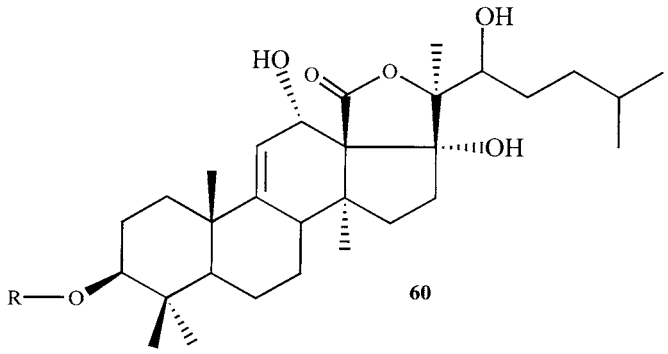
Fig. (27). Structures of glycosides from the sea cucumbers *T. ananas* and *Holothuria polii*

Bivittoside D (**58**) and triglycoside **59a** exhibited inhibitory activity in a chemokine receptor subtype 5 (CCR5) assay at concentrations of 30 and 5 μM, respectively [92]. Holothurin **59a** having only three monosaccharide residues is significantly lesser active than the tetraglycoside bivittoside D (**58**).

Holothurin B_3 (**59b**), Fig. (**27**), a new sulfated diglycoside, was isolated from the sea cucumber *Holothuria polii* [95]. This saponin contains the

same monosulfated disaccharide chain as holothurin B [94] and differs from it the absence of a 17α-hydroxyl group.

The sea cucumber *H. polii* also contained the monosulfated disaccharides holothurin B_2 (**60**), Fig. (**28**) and holothurin B_4 (**61**), Fig. (**29**) [95]. Both saponins have the same disaccharide chain as holothurin B_3 (**59b**).

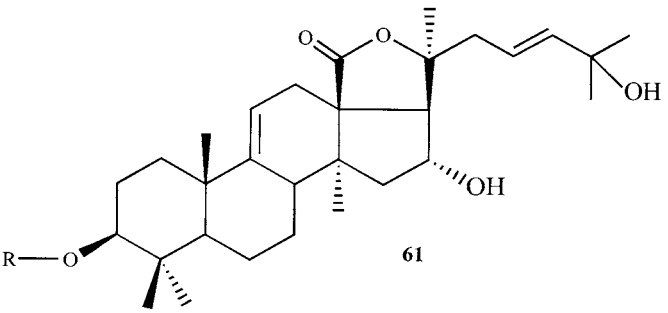


60

Holothurin B_2 R = Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (28). Structure of Holothurin B_3 from the sea cucumber *Holothuria polii*

Saponin **60** has the aglycone of holothurin A_1 [96] whereas holothurin B_3 has the same aglycon side chain as the 3β-hydroxyholost-7-ene aglycones in cucumarioside G_4 [63], eximisoside A [64] and calcigeroside E [69].



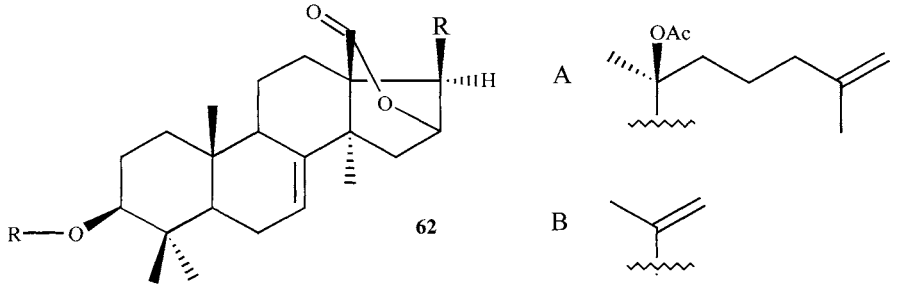
61

Holothurin B_4 R = Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (29). Structure of Holothurin B_4 from the sea cucumber *Holothuria polii*

Non-holostane aglycones

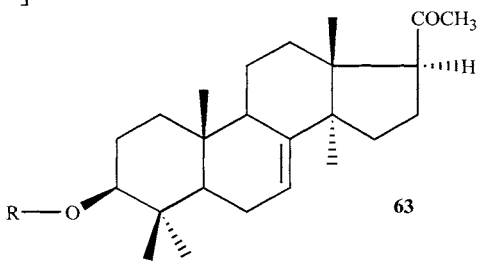
Some examples of sulfated holothurins with uncommon non-holostane aglycones have been isolated from eight species of sea cucumbers belonging to the order Dendrochirotida. Glycosides **62a-62e**, Fig. (**30**) contain aglycones with a 18(16)-lactone [69, 70, 97, 98].



62a Psolusoside B R = [6-OSO_3Na-Glc-(1→4)]-[Glc-(1→4)-Glc-(1→2)]-Xyl, R = A
62b Calcigeroside B R = [3-OMe-Xyl-(1→3)- 4-OSO_3Na Glc(1→4)]-[Quin(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl, R = B
62c Calcigeroside C_1 R = [3-OMe-Xyl-(1→3)-Glc(1→4)]-[Glc-(1→2)]-Quin(1→2)-4-OSO_3Na-Xyl, R = B
62d Calcigeroside D_1 R = 3-OMe-Xyl-(1→3)- 6-OSO_3Na-Glc-(1→4)]-[Glc-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl, R = B
62e Cucumarioside G_2 R = 3-*O*-Me-Xyl-(1→3)-Glc(1→4)-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (30). Structures of non-holostane glycosides with a 18(16)-lactone.

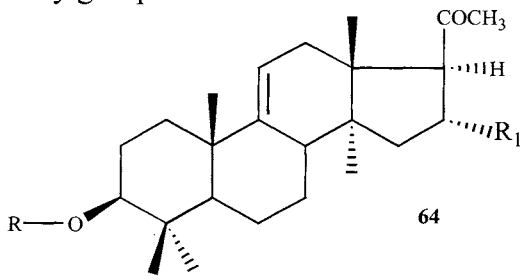
Calcigeroside B (**62b**) has a quinovose terminal residue attached to another quinovose unit. This structural feature is unique for sea cucumber glycosides [69]. Psolusoside B (**62a**) showed inhibition of rat brain Na^+, K^+-ATPase with an I_{50} value of 3 x 10^{-4} M [99]. Calcigerosides B (**62b**), C_1 (**62c**) and C_2 (**62e**) were individually tested in vitro against four standard human and mouse tumoral cell lines (P-388, A-549, HT-29 and Mel-98). Only their desulfated derivatives showed moderate cytotoxicity (IC_{50} = 5 μg/ml) [70].



63a Cucumarioside A_3-2 R = 3-*O*-Me-Glc-(1→3)-4-OSO_3Na-Glc-(1→4)-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl
63b Koreoside A R = [6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl

Fig. (31). Structures of non-holostane glycosides from the sea cucumbers *Cucumaria koraiensis* and *Cucumaria conicospermium*

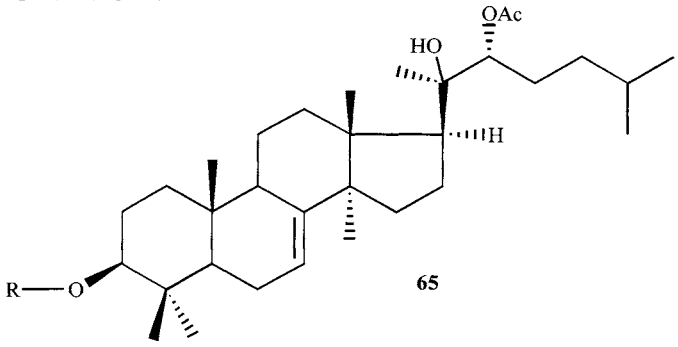
Six holothurins that lack a lactone function and have a shortened side chain have been isolated from sea cucumbers *Cucumaria koraiensis*, *Cucumaria conicospermium* and *Duasmodactyla kurilensis*. Cucumarioside A_3-2 (**63a**) [71] and Koreoside A (**63b**) [100], Fig. (**31**) contain a Δ^7 double bond while Cucumarioside A_3-3 (**64a**) [71], Isokoreoside A (**64b**) [71] and Kurilosides A (**64c**) and C (**64d**) [101], Fig. (**32**) contain a Δ^9 double bond. Kurilosides A (**64c**) and C (**64d**) contain a 16α-acetoxy group.



64a Cucumarioside A_3-3 R = 3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl, R_1 = H
64b Isokorioside A R = 6-OSO_3Na-3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)-[Xyl-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl, R_1 = H
64c Kuriloside A R = [3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Glc-(1→4)-Qui-(1→2)]-Xyl, R_1 = OAc
64d Kuriloside C R = [3-*O*-Me-Glc-(1→3)-6-OSO_3Na-Glc-(1→4)]-[Qui-(1→2)]-Xyl, R_1 = OAc

Fig. (32). Structures of non-holostane glycosides from the sea cucumbers *Cucumaria conicospermium* and *Duasmodactyla kurilensis*.

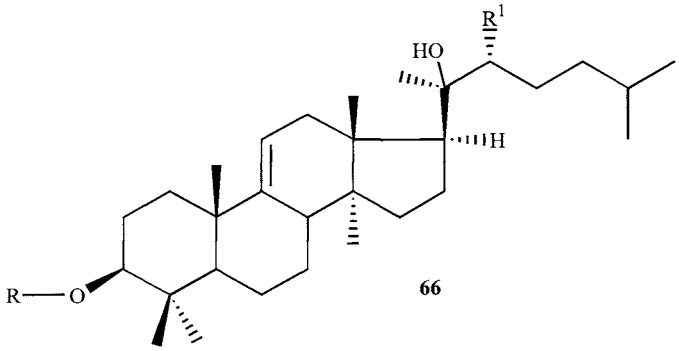
Recently, frondoside A_2-8 (**65**), a new saponin with a non-holostane aglycone containing a Δ^7 double bond, a hydroxyl group at C-20 and an acetoxy group at C-22 was isolated from the sea cucumber *Cucumaria frondosa*, Fig. (**33**) [62].



Frondoside A_2-8 R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; R^1 = OAc; Δ^{24}

Fig. (33). Structure of Frondoside A_2-8 from the sea cucumber *Cucumaria frondosa*

C. frondosa also contained frondosides C (**66b**) [58] and A_2-7 (**66c**) [62], two holothurins related to frondoside A_2-8 (**65**), Fig. (34) that differed from this holothurin in the presence of a $\Delta^{9,11}$ double bond and in the oligosaccharide chain. Previously, two saponins, Ds-penaustrosides A (**66c**) and B (**66d**), Fig. (**34**) lacking the acetoxy group at C-22 were isolated from the sea cucumber *Pentacta australis* [103].



66

66a Frondoside C R = [3-OMe-Xyl-(1→3)-Glc(1→4)]-[Quin-(1→2)]-Quin(1→2)-4-OSO_3Na-Xyl; R^1 = OAc; Δ^{24}
66b Frondoside A_2-7 (**2**) R = [3-OMe-Glc-(1→3)-Glc-(1→4)]-[Xyl-(1→2)]-Quin-(1→2)-4-OSO_3Na-Xyl; R^1 = OAc; Δ^{24}
66c Ds-Penaustroside A R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H
66d Ds-Penaustroside B R = [3-*O*-Me-Xyl-(1→3)-Glc-(1→4)]-[Qui-(1→2)]-Qui-(1→2)-4-OSO_3Na-Xyl; R^1 = H;

Fig. (34). Structures of non-holostane glycosides from the sea cucumbers *Cucumaria frondosa* and *Pentacta australis*

The desulfated derivative of frondoside C (**66a**) showed intense cytotoxic activity (IC_{50} = 1μg/ml) when tested in vitro against several standard mice and human tumoral cell lines (P-388, Schabel, A-549, HT-29 and Mel-28).

CONCLUSIONS

In the last five years only a few examples of asterosaponins have been published in the literature. These include new pentaglycosides from the starfishes *Labidiaster annulatus, Luidia quinaria, Psilaster cassiope* and *Culcita novaeguineae*, together with three new hexaglycosides from the Patagonian starfish *Anasterias minuta*. Cytotoxic and antifungal activities were reported for some of these saponins as well as structure-activity

correlations for the asterosaponins of *Anasterias minuta* and *Culcita novaeguineae*.

On the contrary, many new glycosides of polyhydroxylated steroids have been isolated in this period and the biological properties of most of them have been evaluated. Correlations between the number of hydroxyl groups in the aglycones and the hemolytic activity and inhibition of cell division of fertilized sea urchin eggs were established for glycosides isolated from *Asterias rathbuni, Mediaster murrayi, Ceramaster patagonicus* and *Culcita novaeguineae*. The starfish *Certonardoa semiregularis* has shown to be an extremely rich source of new glycosides of polyhydroxylated steroids: ten diglycosides and eight monoglycosides were isolated, together with twenty two new polyhydroxysteroids. Evaluation of the cytotoxicity of the individual compounds against a panel of human solid tumor cell lines revealed that the polyhydroxysteroids were more potent than the corresponding glycosides. This investigation showed that not only asterosaponins but minoritary components such as polyhydroxysterols and their glycosides may play an important role in the toxicity of starfishes.

Evaluation of the neuritogenic activity of five new monoglycosides isolated from the starfish *Linckia laevigata* demonstrated that the nature of the sugar moiety attached to the side chain seems to be an important factor for the neuritogenic activity.

During these years many new examples of sea cucumber glycosides were isolated, in particular those containing a 3β-hydroxyholost-7-ene aglycone. Some structural characteristics such as a 24-ketone conjugated with a Δ^{25} double bond as in frondoside A_2-2 and a 22-keto group in the side chains of mollisoside A and pseudostichoposide B are novel features for holothurins. Another new feature for 3β-hydroxyholost-7-ene aglycones is the presence of an acetoxyl group at C-16, present in nine glycosides isolated from *Mensamaria intercedens*. Intercedensides C, D, F, H and I also contain a 17α-hydroxy group, not characteristic for sea cucumbers of the order Dendrochirotida with exception of patagonicoside A that contains an uncommon aglycone with the simultaneous presence of two hydroxyl groups at C-12α and C-17α.

Some of the sulfated holothurins isolated in this period were evaluated for their biological activities. Holothurins isolated from the sea cucumber *Cucumaria japonica* were highly toxic and induced abnormalities in the development of sea urchin embryos. Mollisoside A, pseudostichoposide B and the intercedensides A-H showed in vitro cytotoxicity against human tumor cell lines. Liouvillosides A and B isolated from *Staurocucumis liouvillei* exerted viruscidal effect against herpes simplex virus 1. Evaluation of the antifungal activity of the sulfated hemoiedemosides A and B and patagonicoside A as well as their desulfated analogues allowed us to conclude that the aglycone structure and the presence and number of the sulfate groups at the oligosaccharide chain are important features dor their antifungal properties against the phytopathogenic fungus *Cladosporium cucumerinum*.

Much work has been done in the last thirty years on the isolation and structural characterization of saponins and polyhydroxylated steroid glycosides from starfishes and sea cucumbers. The wide spectrum of biological activities these compounds show must be related to their role in the organisms that produce them and this task must be addressed in future investigations.

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Atta-ur-Rahman (Ed.) Studies in Natural Products Chemistry, Vol. 35

3-AKYLPYRIDINIUM AND 3-ALKYLPYRIDINE COMPOUNDS FROM MARINE SPONGES, THEIR SYNTHESIS, BIOLOGICAL ACTIVITIES AND POTENTIAL USE

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ABSTRACT, During the last 30 years, a number of simple 3-alkylpyridine and 3-alkylpyridinium (3-AP) compounds have been isolated from marine sponges belonging to the order Haplosclerida, suggesting these compounds as chemical markers for systematic determination of haplosclerid sponges. They were isolated from haplosclerid sponges either as (i) monomers differing in the length, saturation, branching and termination of the alkyl chains, (ii) cyclic or linear oligomers, or (iii) a mixture of high-molecular weight polymers. What the biosynthetic pathways are in marine sponges, and how these compounds can be made by organic synthesis is also an interesting question addressed in our review. In this regard we focus particularly on organic syntheses by which selective polymerization of 3-alkylpyridinium polymers may be achieved. Structural investigation of alkylpyridinium compounds and the role played by mass spectrometry has also been reviewed. In spite of their relatively simple chemical structure, all these compounds exert a broad spectrum of biological activities. More than 40 monomeric 3-AP compounds have been isolated from marine sponges and the majority bear a nitrogenous functionality at the end of the alkyl chain. Almost all reported 3-AP monomers exert moderate cytotoxicity, in the concentration range of a few micrograms per millilitre, against certain transformed cell lines. An example of 3-alkylpyridines is niphatyne A, from the marine sponge of the genus *Niphates* which exhibits an IC_{50} of 0.5 μg/mL against several transformed cell lines. The majority of 3-AP dimers and trimers were isolated from haplosclerid sponges in cyclic forms. Haliclamines are a typical representative of this group. They inhibit the division of fertilized sea urchin eggs as well as the growth of several transformed cell lines. On the other hand cyclostellettamines are typical example of cyclic alkylpyridinium dimers that modulate muscarinic receptors. Recently an increasing number of polymeric alkylpyridinium compounds have been isolated from haplosclerid sponges. Halitoxins, amphitoxins and alkylpyridinium salts from Mediterranean sponge *Reniera sarai* are the most studied. The latter exhibit a plethora of interesting biological activities. For instance, they are able to make pores in membranes through which DNA can be

transfected into the cell, show selective cytotoxic activity against certain human cancer cells and possess non-toxic inhibitory antifouling properties against larvae of several important fouling organisms. The biological activities and potential use of alkylpyridinium polymers are discussed.

INTRODUCTION

Since the isolation of halitoxins (**74**) by Schmitz et al. in 1978 [1], interest in 3-alkylpyridine and 3-alkylpyridinium compounds (3-APs) has been maintained. These natural compounds, which occur mainly in marine sponges, are interesting from different points of view. First, they exert a large spectrum of biological activities, mainly cytotoxicity, ichthyotoxicity, inhibition of bacterial growth, and enzyme inhibition. They also have transfection and antifouling properties. 3-APs are therefore most interesting as novel potential pharmaceuticals in treating several diseases, and as gene and/or nucleotide carriers in oligonucleotide and/or gene therapy, or they can be used as active components in antifouling paints. Secondly, these compounds present a real challenge for chemists exploring new ways of chemical synthesis for synthetic 3-APs that would mimic the natural ones, in order to make them suitable for commercial production and application.

We present an overview of the structures for 3-alkylpyridine and 3-alkylpyridinium compounds that have been isolated, taking into account the structural characterization of alkylpyridinium compounds and discussing the central role played by mass spectrometry. Concerning their synthesis, both the natural pathways and organic synthesis of pyridinium alkaloids are reviewed. Synthetic aspects of 3-alkylpyridines have been taken into account only where relevant for structural modifications or for the assignment of absolute configurations. Finally, biological activities of both 3-alkylpyridine and 3-alkylpyridinium compounds and the potential use of the latter are discussed.

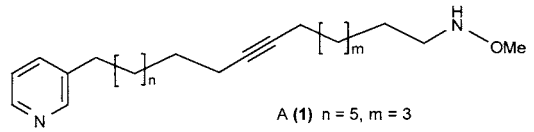
3-ALKYLPYRIDINE AND ALKYLPYRIDINIUM COMPOUNDS FROM MARINE ORGANISMS

More than 70 structurally different 3-APs have been isolated from marine sponges. It is interesting to note that they occur mainly in sponges belonging to the order Haplosclerida, and can therefore also be used as

chemical markers for the systematic determination of haplosclerid sponges [2,3]. However, not all such sponges contain 3-AP compounds. For example, only one species of *Haliclona* out of five collected in the Adriatic Sea contained 3-APs (T. Turk, unpublished data). Similar variation in the content of 3-APs has been detected even within a single sponge species collected from different geographical areas [4], suggesting that the environment can influence their secondary metabolism.

The majority of natural 3-APs isolated to date are monomeric compounds differing in the length, saturation, branching and termination of the alkyl chains. These compounds usually show cytotoxic and/or antibacterial activities at concentrations of a few micrograms per mL. A smaller number of oligomeric and polymeric 3-APs have also been isolated and partially characterized. It is clearly evident that polymeric 3-APs exert many more diverse and potent biological activities. In general, the biological activities of 3-APs (i) are higher in linear forms than in their cyclic analogues, (ii) increase with the degree of polymerization, (iii) are considerably higher in those that take the form of quaternary ammonium ions than in their neutral, tertiary amine analogues. Most of the 3-APs that have been isolated to date are listed according to their basic structural types.

The first monomeric 3-APs to be reported, bearing alkyne and *N*-methoxylamine functionalities at the alkyl chain, were isolated from a methanolic extract of the Pacific sponge *Niphates sp.*, and were called niphatynes (**1**,**2**), Fig. (**1**), [5]. From the same sponge niphatesines (**3-10**), Fig. (**2**) with an amino, *N*-methoxylamine, or oxime methyl ether functionality at the end of the alkyl chain were subsequently purified [6,7]. Assuming the gross structure of niphatesine D to have been correctly assigned, Romeril et al. [8] cast doubts on the correct assignment of the S-absolute stereochemistry of **6** based on optical rotation. The stereochemistry was first assigned by Rao and Reddy [9] by the synthesis of **6** and then confirmed by Bracher and Papke [10].

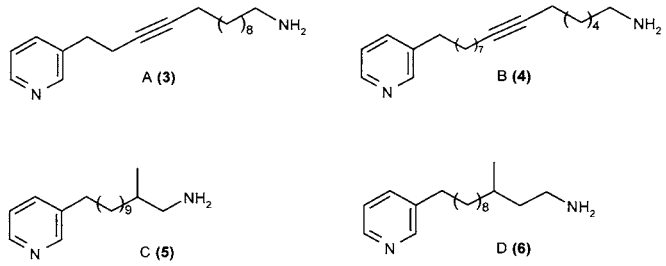


A (**1**) n = 5, m = 3

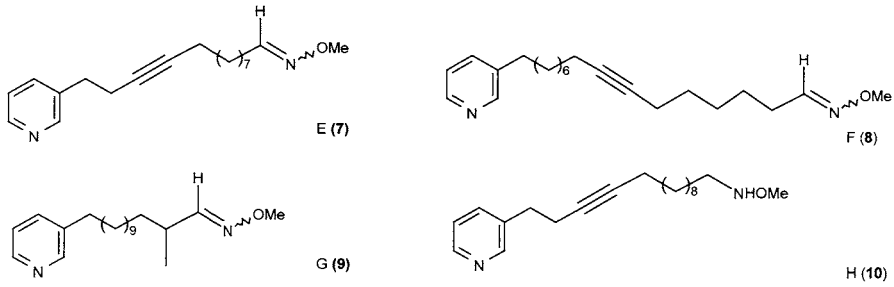
B (**2**) n = 7, m = 1

Quiñoà and Crews (1987)

Fig. (1). Niphatynes A and B (**1, 2**)



Kobayashi, Murayama, Kosuge, Kanda, Ishibashi, Kobayashi, Ohizumi, Ohta, Nozoe, and Sasaki (1990)

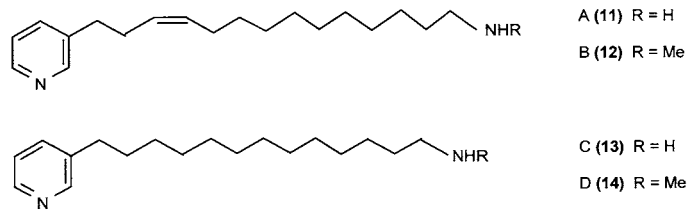


Kobayashi, Zeng, Ishibashi, Shigemori, Sasaki, and Mikami (1992)

Fig. (2). Niphatesines A-H **(3-10)**

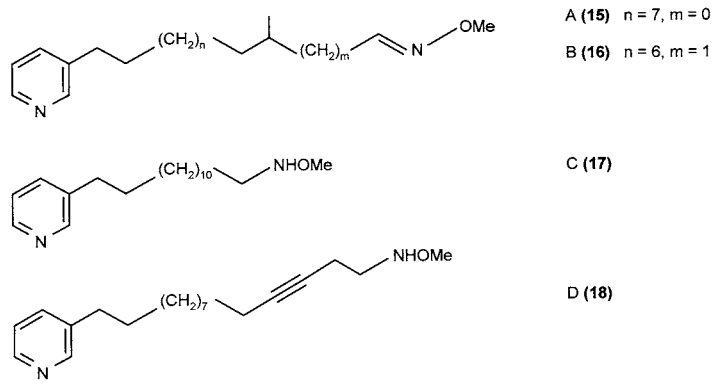
Theonelladins A-D (**11-14**), Fig. (**3**) are structurally closely related to niphatesines A and B (**3,4**), and were therefore surprisingly isolated from a non-haplosclerid sponge *Theonella swinhoei* [11]. They were followed by ikimines A-D (**15-18**), Fig. (**4**), from an unidentified Micronesian marine sponge [12]. All these structures were elucidated on the basis of spectroscopic data.

However the structure proposed for ikimine B (**16**) by Carroll and Scheuer [12] has recently been demonstrated [8] not to be consistent with the NMR data, since **16**, obtained by total synthesis, shows different spectra.



Kobayashi, Murayama, Ohizumi, Sasaki, Ohta, and Nozoe (1989)

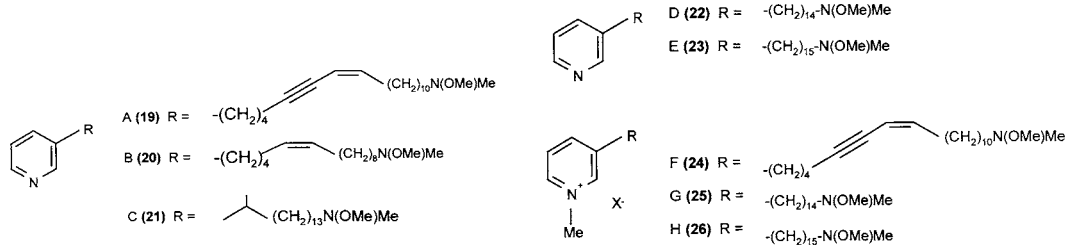
Fig. (3). Theonelladins A-D **(11-14)**



Carroll and Scheuer (1990)

Fig. (4). Ikimines A-D **(15-18)**

Xestamines **(19-26),** Fig. (**5**) are structurally related to niphatynes A and B (**1,2**), and bear an *N*-methoxyl-*N*-methylamino terminus. They were isolated from the haplosclerid sponges *Xestospongia wiedenmayeri* [13] and *Calyx podatypa* [14], and their structures elucidated by a combination of spectral and chemical methods.

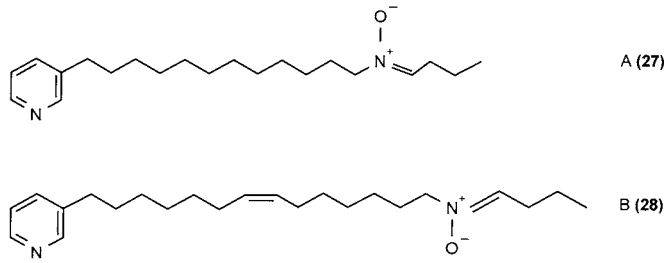


Sakemi, Totton, and Sun (1990)

Stierle and Faulkner (1991)

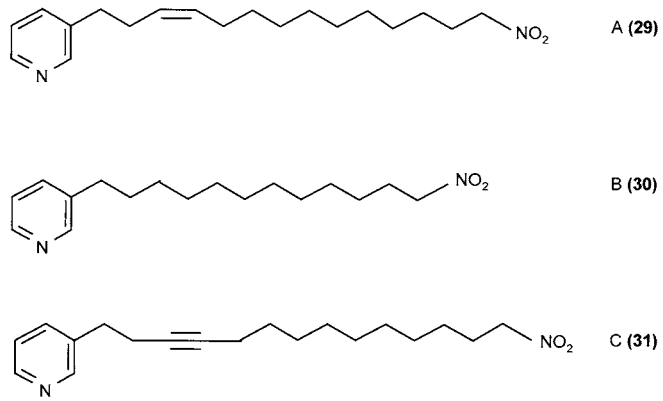
Fig. (5). Xestamines A-H **(19-26)**

Other examples of monomeric 3-APs are cribrochalinamine oxides (**27, 28**) Fig. (**6**) from the sponge *Cribrochalina* sp., two β-substituted pyridine alkaloids bearing a rare azomethine *N*-oxide function in the side chain [15]. Untenines are nitroalkyl pyridine derivatives (**29-31**), Fig. (**7**) from the sponge *Callyspongia* sp. [16].



Matsunaga, Shinoda, and Fusetani (1993)

Fig. (6). Cribrochalinamine oxides A and B (**27, 28**)

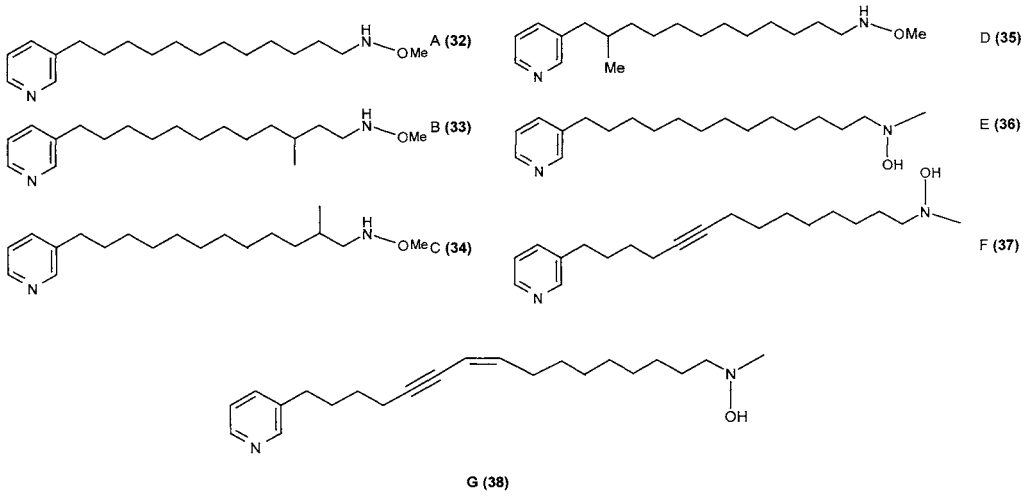


Wang, Kuramoto, Uemura, Yamada, Yamaguchi, and Yazawa (1996)

Fig. (7). Untenines A-C (**29-31**)

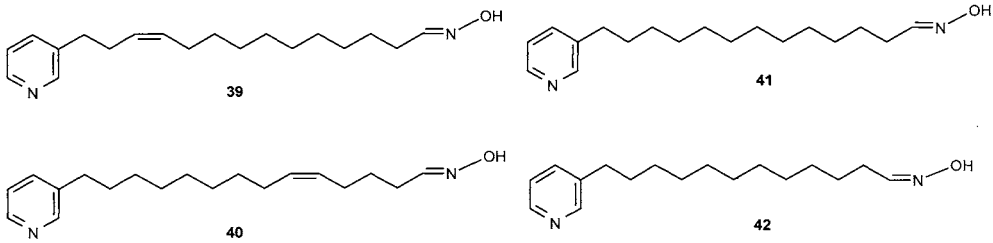
Hachijodines (**32-38**), Fig. (**8**), are also 3-AP monomers, isolated from haplosclerid sponges of the genera *Xestospongia* and *Amphimedon*, but terminate in *N*-methoxylamino or *N*-hydroxyl-*N*-methylamino group [3].

Amphimedon sp. is also a source of unnamed monomeric 3-APs (**39-42**) Fig. (**9**) terminating in the oxime group [17].



Tsukamoto, Takahashi, Matsunaga, Fusetani, and van Soest (2000)

Fig. (8). Hachijodines A-G **(32-38)**

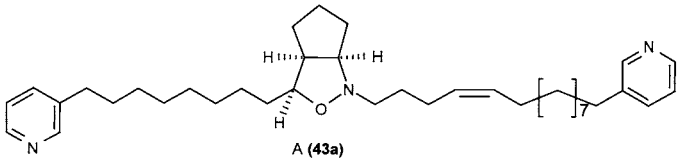


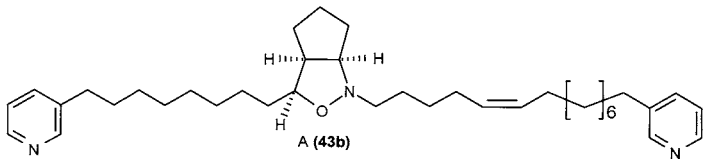
Hirano, Kubota, Tsuda, Mikami, and Kobayashi (2000)

Fig. (9). Unnamed 3-alkylpyridinium compounds from the sponge *Amphimedon* sp. (**39-42**)

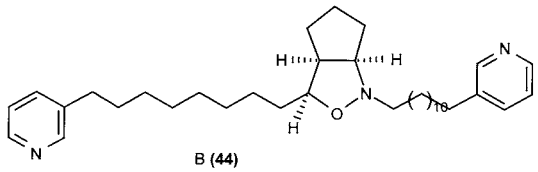
In 1999 an interesting 3-AP alkaloid possessing a unique bicyclic ring system and a *cis*-cyclopent[*c*]isoxazolidine moiety, pyrinodemin A (**43**), Fig. (**10**), was obtained from the sponge *Amphimedon* sp. [18].
More varieties of pyrinodemins (B (**44**), C (**45**), and D (**46**)), Fig. (**10**) were isolated from the same sponge a year later [17]. A revised structure

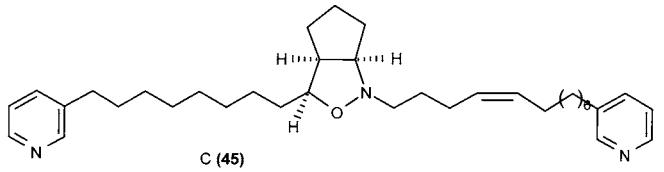
of pyrinodemin A (**43b**), shown in Fig. (**10**), has been later proposed by Baldwin et al. (2001) by total synthesis [19].

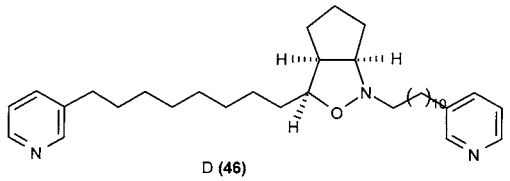




Tsuda, Hirano, Kubota, and Kobayashi (1999, for **43a**)
Baldwin, Romeril, Lee, Claridge (2001, for **43b**)



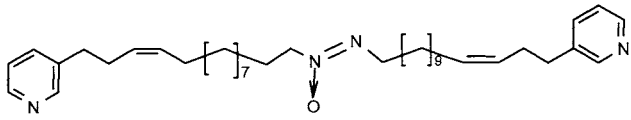




Hirano, Kubota, Tsuda, Mikami, and Kobayashi (2000)

Fig. (10). Pyrinodemins A-D **(43-46)**

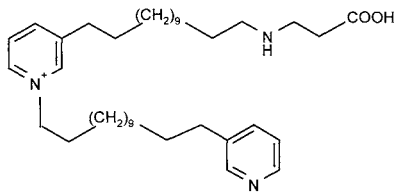
The isolation of pyrinodemins (**43-46**) was followed by the discovery of a new bicyclic 3-AP from the sponge *Cribrochalina* sp. [20]. This compound, pyrinadine A (**47**), Fig. (**11**), possesses an unprecedented azoxy moiety in the middle of the alkyl chain. The geometry of the diazo double bond was deduced to be *Z* from the UV absorbance maximum, and its position was established by ESI MS/MS analysis on **47** and on its reductive degradation product.



Kariya, Kubota, Fromont, Kobayashi (2006)

Fig. (11). Pyrinadine A **(47)**

Another interesting example of a linear 3-AP dimer is viscosaline (**48**), Fig. (**12**), a 1,3-dialkylpyridinium compound with a β-alanine moiety covalently bound to one alkyl chain. It was recently purified from the arctic sponge *Haliclona viscosa* [21], and proposed as a biosynthetic precursor of cyclostellettamines (**53-63**), Fig. (**14**) and related molecules.

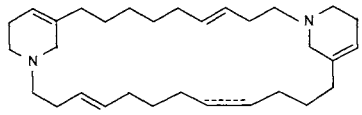


Volk and Köck (2004)

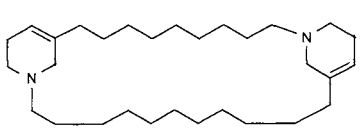
Fig. (12). Viscosaline **(48).**

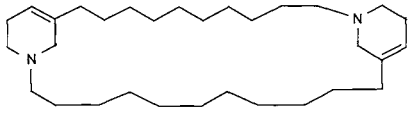
Besides linear dimeric 3-APs, different structural types of N-cyclic related compounds have been found in haplosclerid sponges so far. The first are haliclamines (**49-52**), Fig. (**13**), tetrahydropyridines linked through long alkyl chains, that were isolated from both the Okinawan

sponge of the genus *Haliclona* [22], and from the arctic sponge *Haliclona viscosa* [4].



Fusetani, Yasumuro, Matsunaga, and Hirota (1989)

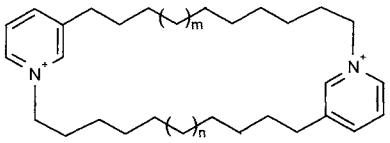




Volk, Lippert, Lichte, and Köck (2004)

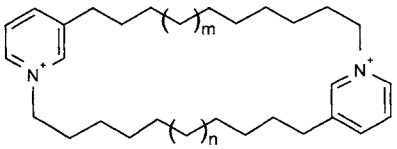
Fig. (13). Haliclamines A-D **(49-52)**

Cyclic dimeric 3-APs are exemplified by cyclostellettamines (**53-63**), Fig. (**14**), and dihydrocyclostellettamines (**64, 65**), Fig. (**15**). These compounds have been isolated from a different collection of sponges, *Stelletta maxima* (most probably from its encrusted "contaminant" – *Haliclona*) [23], *Xestospongia* sp. [24], and *Pachychalina* sp. [25].



A (**53**) n = 3, m = 3
B (**54**) n = 3, m = 4
C (**55**) n = 4, m = 4
D (**56**) n = 3, m = 5
E (**57**) n = 4, m = 5
F (**58**) n = 5, m = 5

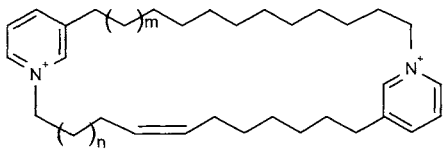
Fusetani, Asai, Matsunaga, Honda, and Yasumuro (1994)



G (**59**) n = 2, m = 3
H (**60**) n = 1, m = 3
I (**61**) n = 1, m = 4
K (**62**) n = 1, m = 5
L (**63**) n = 2, m = 5

De Oliveira, Grube, Köck, Berlinck, Macedo, Ferreira, and Hajdu (2004)

Fig. (14). Cyclostellettamines A-L **(53-63)**

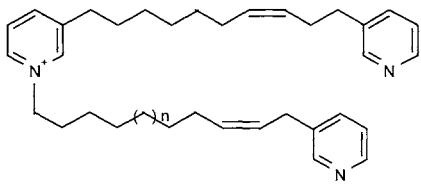


D **(64)** n = 4, Δ^{13}, m = 1
E **(65)** n = 4, Δ^{13}, m = 2

Oku, Nagai, Shindoh, Terada, van Soest, Matsunaga, and Fusetani (2004)

Fig. (15). Dehydrocyclostellettamines D and E **(64, 65)**

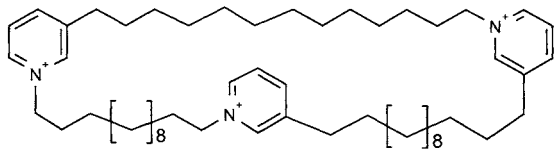
Tricyclic 3-APs can also be found in haplosclerid sponges. Examples of these compounds are linear niphatoxins **(66, 67),** Fig. **(16)** from the sponge *Niphates* sp., [26], and cyclic viscosamine **(68),** Fig. **(17)** from the Arctic species *Haliclona viscosa* [27].



A **(66)** n = 2
B **(67)** n = 3

Talpir, Rudi, Ilan, and Kashman (1992)

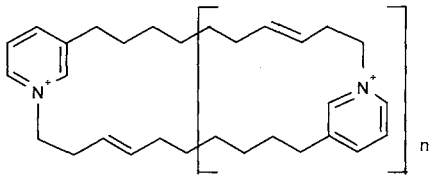
Fig. (16). Niphatoxins A and B **(66, 67)**



Volk and Köck (2003)

Fig. (17). Viscosamine **(68)**

Very recently, the first cyclic tetrameric, pentameric, and hexameric 3-APs, with C_{10} alkyl chains, have been isolated in a mixture from the sponge of the genus *Haliclona* [28]. The structure of these compounds, cyclohaliclonamines A-E **(69-73)**, is shown in Fig. **(18)**.

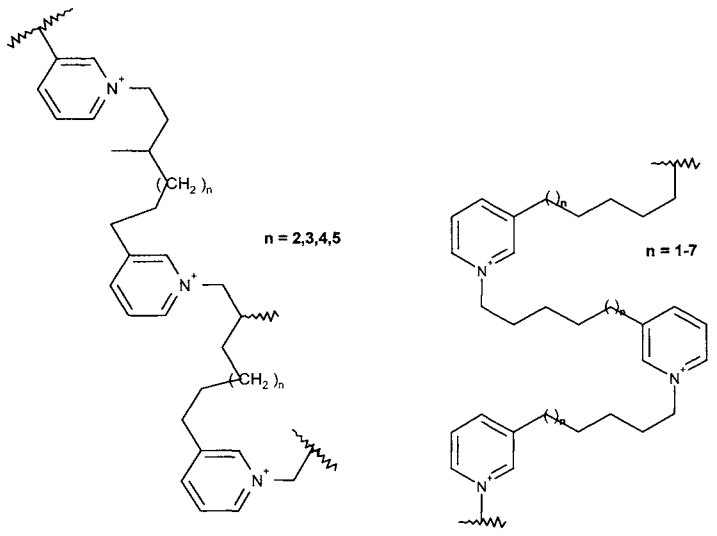


Teruya, Kobayashi, Suenaga, and Kigoshi (2006)

Fig. (18). Cyclohaliclonamines A-E **(69-73)**

With the exception of cyclohaliclonamines (**69-73**), all 3-APs with higher molecular weight isolated so far were determined structurally as linear polymers. As in the case of cyclohaliclonamines, their isolation can be rather problematic, since they often exist as a mixture of polymers with the same basic structure, but with different molecular weights. Furthermore, their polarity usually increases with the degree of oligomerization, leading to the formation of non-covalently associated supramolecular aggregates and rendering the separation of different molecular weight oligomers and polymers even more difficult. The polymeric 3-APs reported to date are composed of monomeric subunits with either different (halitoxin (**74**), amphitoxin (**75**)), or the same basic structure (EGF-active factors (**76**), poly-APS (**77**)). They all exert a broad spectrum of potent biological activities.

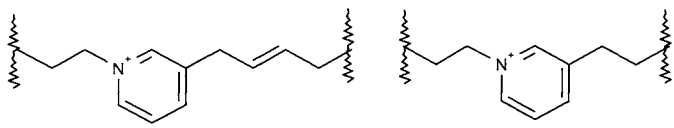
Halitoxin (**74**), Fig. (**19**), was the first 3-AP polymer to be isolated and structurally characterized [1], and exists as a mixture of high molecular weight pyridinium salts in several sponges of the genus *Haliclona*. It is composed of 3-AP units connected by saturated, methyl-branched alkyl chains composed of 8 to 11 carbon atoms, and its major fraction is in the molecular weight range of 500-1000 Da. Halitoxins with the same basic structure, but with a different distribution of molecular weights (the major peak around 2000 Da) were isolated also from the sponge *Amphimedon (Haliclona) viridis* [29], while its unbranched variant was found in *Callyspongia ridleyi* [30].



Schmitz, Hollenbeak, and Campbell (1978) Scott, Whyment, Foster, Gordon, Milne, and Jaspars (2000)

Fig. (19). Halitoxin **(74)**

Another high molecular weight pyridinium salt, amphitoxin (**75**) Fig. (**20**), isolated from the sponge *Amphimedon compressa*, is a polymer based on randomly sequenced 3-alkyl and 3-alkenyl pyridinium units in an overall ratio of 1:1 [31]. It was purified to the stage of two fractions with molecular weight ranges of 1000-3000 (38%) and 3000-10000 Da (62%). In 2001, a mixture of two closely related polymeric 3-AP homologues, identified as halitoxin (**74**) and amphitoxin (**75**), was isolated from *Amphimedon viridis* [32].

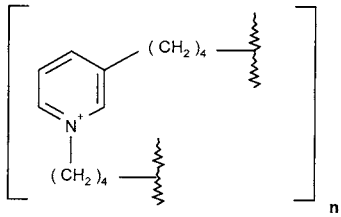


Albrizio, Ciminiello, Fattorusso, Magno, and Pawlik (1995)

Fig. (20). Amphitoxin **(75)**

The next group of 3-AP polymers comprises the EGF-active factors (**76**), Fig. (**21**) from the sponge *Callyspongia fibrosa* [33]. They are

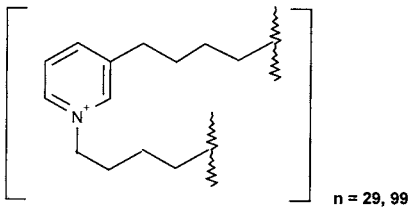
composed of pyridinium units linked head-to-tail through straight C_8 alkyl chains, and contain at least eight monomer units.



Davies-Coleman, Faulkner, Dubowchik, Roth, Polson, and Fairchild (1993)

Fig. (21). EGF-active factors **(76)**

Polymeric 3-AP salts (poly-APS (**77**), Fig. (**22**)), with the same basic structure as EGF active-factors but with a considerably higher degree of polymerization, were isolated from the Mediterranean sponge *Reniera (Haliclona) sarai* [34]. In contrast to all other known 3-APs, that are soluble exclusively in organic solvents, poly-APS are only soluble in water, which is the consequence of their high degree of polymerization. Although this feature renders their purification and structural characterization rather difficult, it also confers on them a tremendous biological potential. Poly-APS exhibit a broad spectrum of biological activities, some of which are interesting also from the pharmaceutical and industrial points of view. These biological activities and their possible applications are therefore presented in greater detail in the corresponding section of this review.



Sepčić, Guella, Mancini, Pietra, Dalla Serra, Menestrina, Tubbs, Maček, and Turk (1997)

Fig. (22). Poly-APS **(77)**

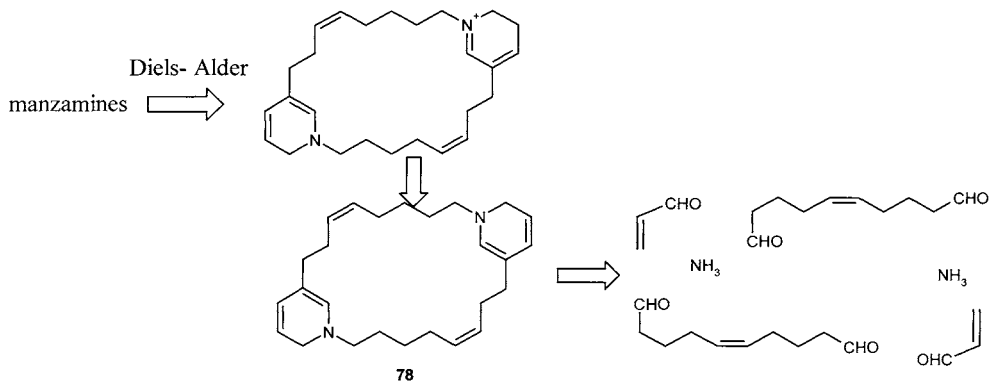
NATURAL PATHWAYS AND ORGANIC SYNTHESES LEADING TO ALKYLPYRIDINIUM COMPOUNDS

Biosynthesis

Polycyclic amine alkaloids (3-alkylpiperidine alkaloids) containing a complex skeleton with several macrocyclic rings have been isolated from marine sponges. They include manzamines, sarains, haliclamine A and ingenamines. Their structures, synthesis and biochemical aspects have been recently reviewed [35]. Our focus, therefore, is on the biogenetic origin of the bis-pyridinium macrocycle and on the role of precursors in their biosynthesis, as summarized in the retrosynthetic analysis for manzamines demonstrated schematically in Fig.(**23**).

According to the inspired proposal of the biogenetic pathway by Baldwin and Whitehead [36-38], the relatively simple bis-dihydropyridinium cycles **78,** deriving from ammonia, a C_{10} di-aldehyde, and a C_3 acrolein equivalent, are considered to be the precursors of complex molecules like manzamine. These units are first assembled into a 3-alkylpiperidine monomer that can either polymerize to give the oligomeric halitoxins or dimerize to give a bis(3-alkylpiperidine) macrocycle. Bis(3-alkylpiperidine) macrocycles can undergo various transformations, including intramolecular [4+2] cycloaddition reactions, to generate the polycyclic skeletons found in many of these complex alkaloids.

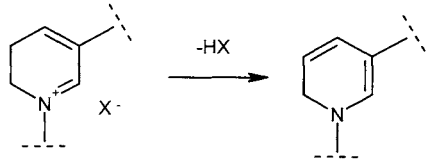
The biomimetic synthesis of this and related compounds has been completed using the bis-pyridinium cycle as a synthetic precursor of the corresponding cycle **78**. The study has confirmed the previously proposed biogenetic hypothesis [38].



Baldwin, Claridge, Culshaw, Heupel, Lee, Spring and Whitehead (1999)

Fig. (23). Proposed biosynthesis of manzamine alkaloids

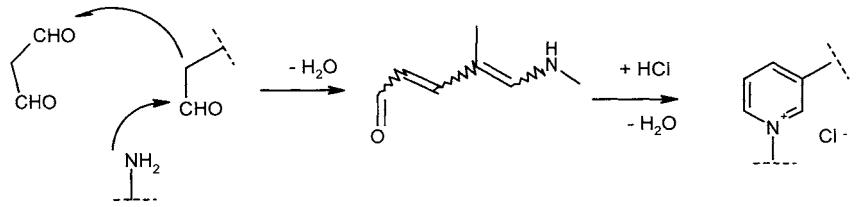
Further insights into the biosynthesis of manzamines came from the synthetic studies made on the conversion of 3-alkyl-dihydropyridinium salts to the unstable 3-alkyldihydropyridine reported in Fig.(**24**) [39]. Similar compounds were used in model studies towards a biomimetic synthesis of the alkaloids keramaphidin A and halicyclamine A [40].



Gil, Gateau-Olesker, Wong, Chernatova, Marazano and Das (1995).

Fig. (24). Conversion of 3-alkyldihydropyiridinium salts to 3-alkylduhydropyridine

New insights into the biogenesis of pyridinium compounds from sponges come from a further report by Kaiser et al. [41]. Their proposal, differing from the Baldwin and Whitehead hypothesis, involves the condensation under acid conditions of malondialdehyde and long chain aminodialdehydes (deriving from the metabolism of fatty acids), to give a pyridinium ring as illustrated in Fig. (**25**). Later such a proposed biogenesis has been experimentally proved by the synthesis of halicyclamine- and manzamine-type compounds [42].



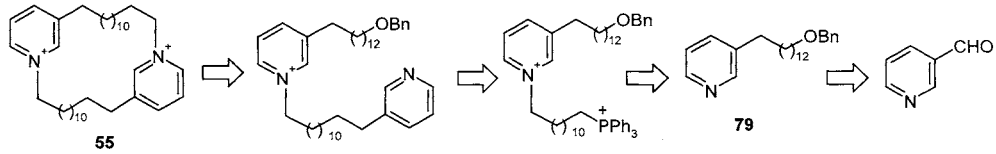
Kaiser, Billot, Gateau-Olesker, Marazano and Das (1998)

Fig. (25). Biogenetic pathway to pyridinium compounds proposed by Marazano

Chemical synthesis

Cyclostellettamines

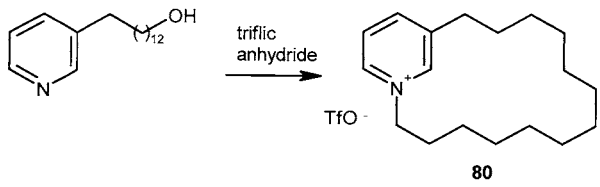
The symmetric C_{13}/C_{13} cyclostellettamine C (**55**), which constitutes the major component of cyclostellettamines A-F (**53-58**), was first prepared by the researchers who had previously isolated and structurally characterized these metabolites [43]. As shown in Fig. (**26**), a stepwise strategy in constructing the ring has been used here, involving the connection of a phosphonized alkyl group with the nitrogen of the O-protected alkylpyridine **79** and condensation with a second pyridyl group by Wittig reaction.



Anan, Seki, Noshiro, Honda, Yasumuro, Ozasa, and Fusetani (1996)

Fig. (26). Retrosynthetic strategy for cyclostellettamine C (**55**)

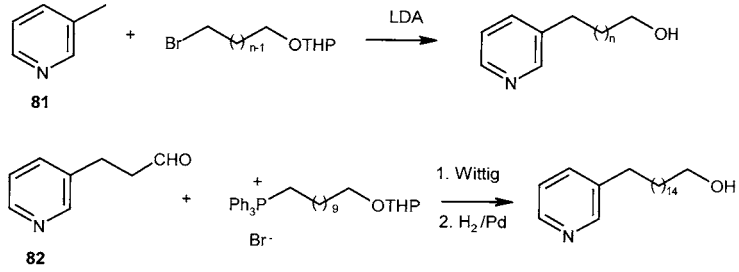
This synthetic investigation confirmed the structure of the metabolite by comparison of fast atom bombardment-mass spectrometry (FAB-MS) spectra for the natural and synthetic cyclostellettamine C and for the monomeric synthetic compound **80** obtained by cyclization as indicated in Fig. (**27**). Additional confirmation came by comparing the bioactivities shown by synthetic dimer **55**, mono-pyridinium **80** and natural cyclostellettamine C as antagonist agents for muscarinic receptors [43].



Anan, Seki, Noshiro, Honda, Yasumuro, Ozasa, and Fusetani (1996)

Fig. (27). Synthesis of mono-pyridinium cyclic compound **57**

Later, Baldwin and co-workers reported an efficient synthesis of the whole series of cyclostellettamines A-F [44]. The appropriate monomeric 3-alkylpyridine was prepared, as illustrated in Fig. (**28**), from 3-methylpyridine (**81**), or from 3-pyridin-3-ylpropanal (**82**) when tridecan-1,13-diol was unavailable as a commercial reagent.

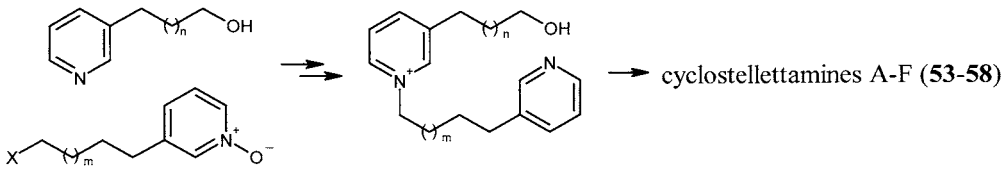


Baldwin, Spring, Atkinson and Lee (1998)

Fig. (28). Synthesis of the 3-alkylpyridines as precursors of cyclostellettamines A-F

The strategy adopted by Baldwin, first used in the synthesis of a precursor of haliclamine A (**49**) [45], consists in assembling the pyridine rings by two controlled quaternization reactions of two 3-alkylpyridine subunits. In comparison to the induction of head-to tail oligomerization by the traditional approach used in the synthesis of EGF-active pyridinium alkaloids from *Callyspongia fibrosa* [33], this is a more efficient approach for introducing a controlled number of pyridinium subunits into the structure of oligo- and polymeric pyridinium compounds. It relies on the nucleophilic substitution of one pyridine subunit with a masked leaving group at the terminal alkyl position by another *N*-protected pyridinium subunit bearing a leaving group at the terminal position of the 3-alkyl

chain. In the sequence adopted by Baldwin, the *N*-oxide function is the protective group of the pyridinium subunit and a leaving group X (i.e halogen atom, or a mesilate group for Morimoto) is present at the terminal of the alkyl chain to act as a "protected/activated" subunit as depicted in Fig. (**29**). In order to complete the reacting system, another "unprotected/ non-activated" subunit with a free pyridine moiety and a hydroxyl group at the terminal position of its 3-alkyl chain must be present.



Baldwin, Spring, Atkinson and Lee (1998)

Fig. (29). Strategy adopted for the synthesis of cyclostellettamines A-F

In the meantime, Wanner and Koomen reported their synthetic sequence to cyclostellettamines A-F and related bis(3-alkylpyridinium) macrocycles [46]. The strategy is reminiscent of the Baldwin one reported in Fig. (**28**), but involves protection of the pyridine nitrogen as a *p*-methoxybenzyl derivative, and cyclization of the iodides under conditions of high dilution, with the advantage that it requires no chromatographic separation of the pyridinium salts. Hence, such a synthetic method provides a controlled entry to a variety of symmetric and asymmetric bis pyridinium macrocycles in good yield.

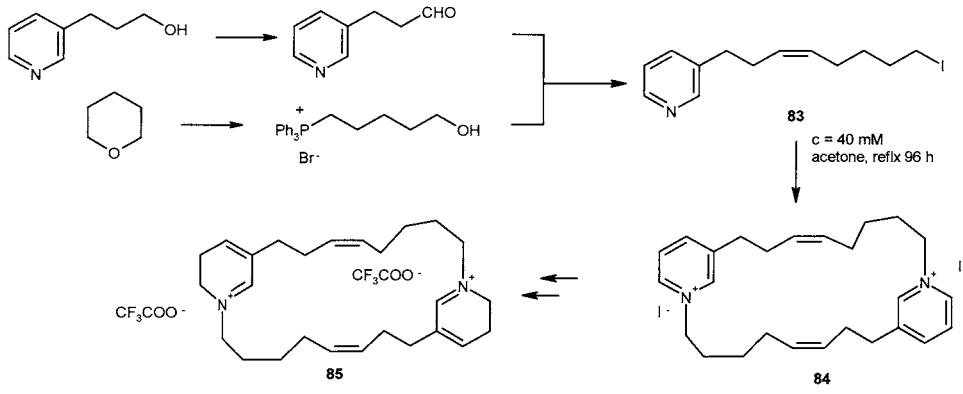
Recently, the isolation in very low amounts (only 1.5 mg as a mixture) of the new cyclostellettamines H-L (**60-63**), [25] has been reported, together with the synthesis of these alkaloids [47]. The preparative strategy is achieved following the procedure developed by Baldwin [46], with the aim of obtaining further material for biological investigations [25].

Other dimeric, oligomeric and polymeric pyridinium compounds

Direct cyclization has been performed on 3-substituted pyridines bearing a terminal leaving group on the alkyl chain. This approach gives simple access to dimeric or polymeric pyridinium by controlling the degree of polymerisation, although it provides moderate amounts of several

symmetrical dimers, together with tri-, tetra- and higher oligomers, depending on the reaction conditions used.

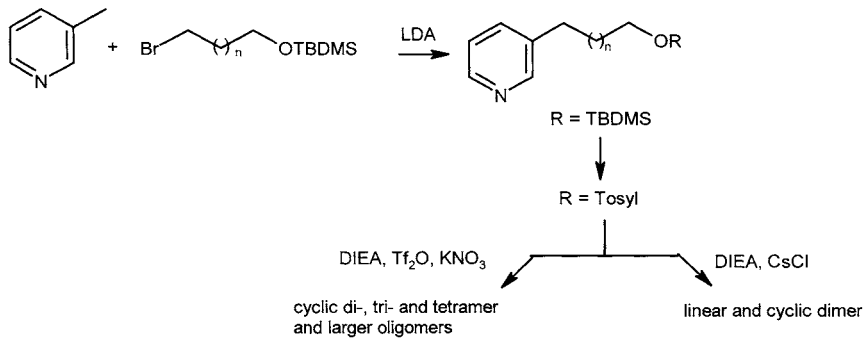
Baldwin has also reported model studies of routes towards the synthesis of manzamines, relying on a biomimetic approach. Thus, the cyclic dimer **84** has been synthesized by refluxing the iodide derivative **83**. It is worth noting that **84** is thought to be the biogenetic precursor of the bis-dihydropyridinium species **85**, which in turn is a plausible biogenetic precursor of manzamine B. By a comprehensive investigation of the effects on the reaction yield of many parameters such as leaving group, solvent, temperature and concentration, the optimized conditions listed in Fig. (**30**) have been obtained [48]. Later, in the description of the biomimetic synthesis of the manzamine-related alkaloid keramaphidin B, Baldwin reported an improved access to dimer **84** [38]. Based on the observation that iodide derivative **83** polymerized easily in the condensed phase and to the moderate yield of cyclodimerization, an alternative and better protocol was investigated. It uses the stable monomeric 3-alkyl tosylate and *in situ* generation of **83** by refluxing in butan-2-one, affording the end-product **84**. Based on two separate S_N2 reactions, the cyclodimerization is optimized by minimizing the intermolecular closure which would be favoured by high substrate concentration. From a practical point of view, the sequence requires slow addition of the tosylate solution in butan-2-one to a refluxing solution of sodium iodide in the same solvent, which leads to the best yield of cyclic dimer **84**.



Baldwin, Claridge, Culshaw, Heupel, Smrckova and Whitehead (1996)

Fig. (30). Synthesis of the cyclic dimer **84**, a plausible biogenetic precursor of manzamine B

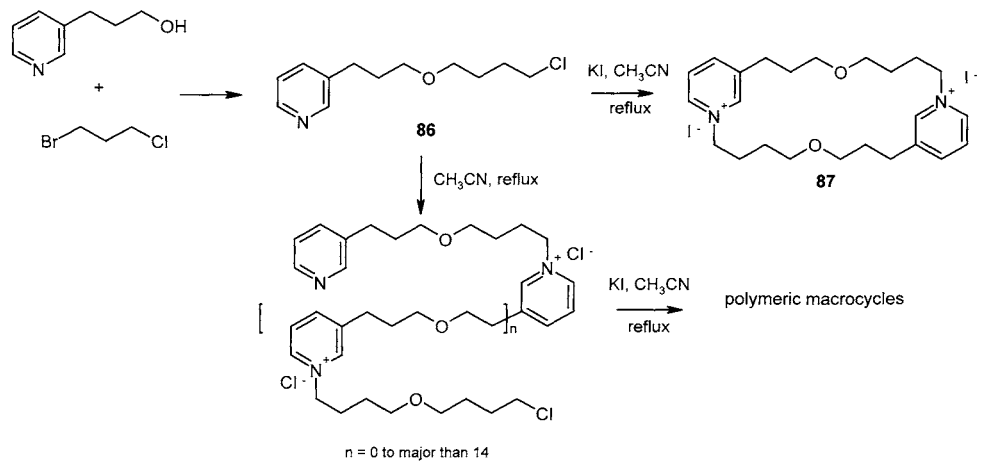
Concurrently with the report on polymeric pyridinium compounds isolated from the Micronesian sponge *Callyspongia fibrosa* [33], Faulkner described the preparation of cyclic pyridinium dimer, trimer, tetramer and oligomers, in order to confirm the structure of the natural compounds and with the aim of producing a large quantity of material for *in vivo* bioassays. The synthetic alkylpyridinium salts were obtained by inducing a head-to-tail cyclic oligomerization in refluxing dichloromethane containing diisopropylethylamine (DIEA), as shown in Fig. (**31**). For the isolation step, the best separations of such salts have been obtained on silica by elution with saturated aqueous KNO_3/ acetonitrile.



Davies-Coleman, Faulkner, Dubowchik, Roth, Polson and Fairchild (1993)

Fig. (31). Di-, tri-, tetra- and oligomeric alkylpyridinium compounds related to natural EGF-active factors from *Callyspongia fibrosa*

Model synthetic studies directed to producing natural macrocyclic compounds were realized by Gil et al. [49]. As a convenient model, the readily accessible substrate **86** was used. It was made available by phase-transfer catalyzed condensation of commercial 3-(3-pyridyl)-1-propanol and 1-bromo-4-chlorobutane and, on refluxing in acetonitrile, furnished different products depending on reaction times. In this way, cyclic dimer **87** was obtained as the iodine salt in the presence of potassium iodide, and a mixture of linear or cyclic polymers by prolonged time, as summarized in Fig. (**32**). Dimers and oligomers were purified chromatographically over alumina by gradient elution with dichloromethane/methanol, and a mixture of higher polymers was obtained in multi-gram quantities.

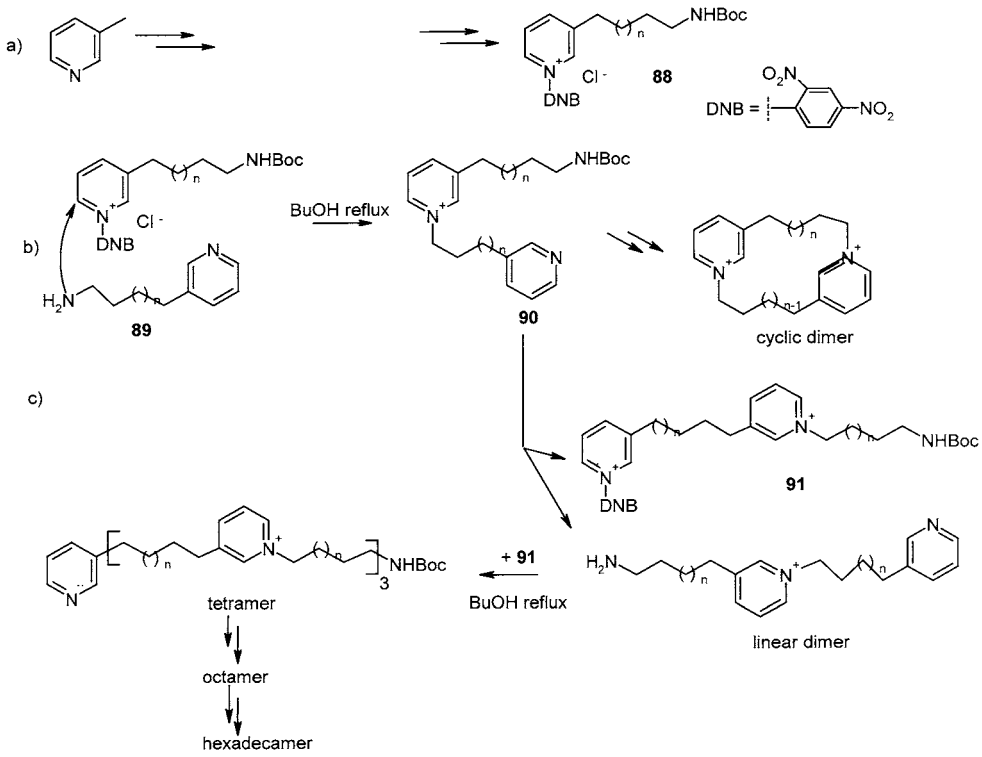


Gil, Gateau-Olesker, Wong, Chernatova, Marazano and Das (1995)

Fig. (32). Model synthetic studies toward natural macrocyclic alkylpyridinium alkaloids

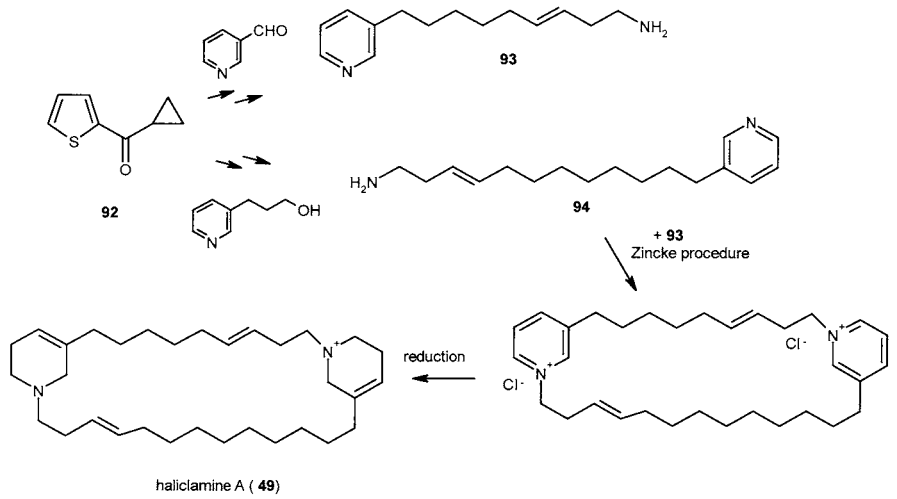
Kaiser et al. have reported a general entry for the selective synthesis of dimeric macrocycles like cyclostellettamines and for polymeric natural products [41]. It uses the Zincke reaction by which it is possible to control the number of units in a 3-alkylpyridinium polymer. As summarized in Fig. (**33**), the reaction of the free amine **89** with the Zincke salt **88** gives the dimer **90** (route b) which, after terminal amine deprotection and DNB functionalization at the *N*-pyridine centre, gives the cyclic dimer, as in the synthesis of cyclostellettamine B. Otherwise, compound **90** furnishes both the protected dimer **91** and the free linear dimer, which, refluxed together in butanol, give the linear tetramer (route c). By the same iterative sequence, the linear octamer was obtained from the tetramer, and from the latter the hexadecamer.

The procedure can also be applied in the case of substrates **87** and **88** bearing different chains. This is the case for the synthesis of the asymmetrical bis-pyridinium macrocycle, a precursor of haliclamine A [50]. As illustrated in Fig. (**34**), the 3-alkylpyridine derivatives **93** and **94**, obtained by the commercially available thiophene **92**, lead to the dimeric macrocycle *via* the Zincke procedure, which affords obtaining haliclamine A (**49**) by reduction.



Kaiser, Billot, Gateau-Olesker, Marazano and Das (1998)

Fig. (33). Iterative synthetic sequence based on the Zincke method for the selective access to dimers and higher 3-alkylpyridinium oligomers

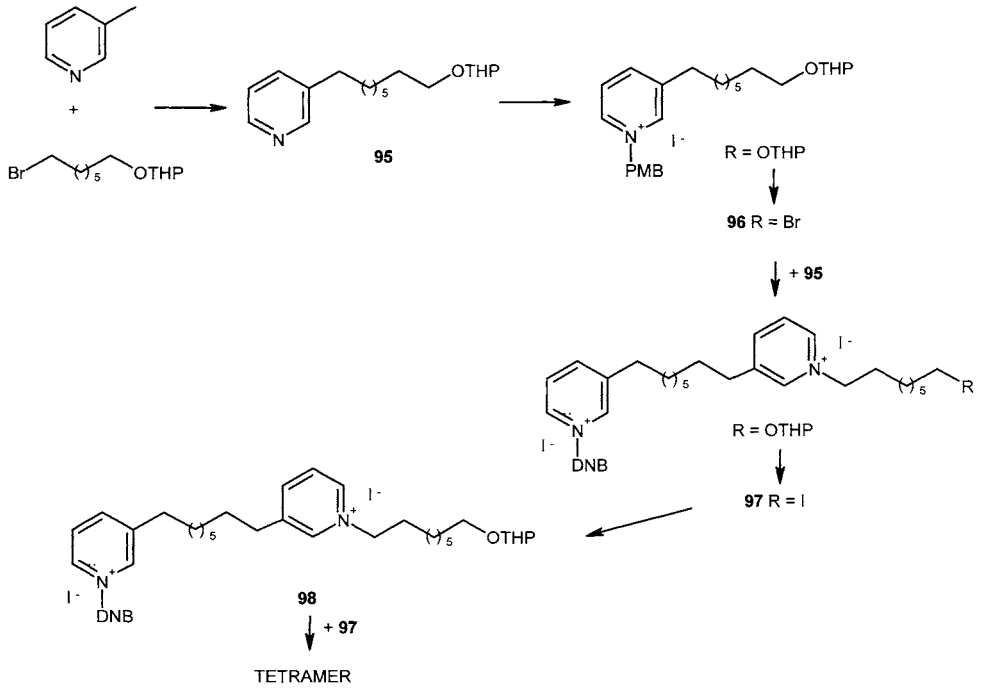


Michelliza, Al-Mourabit, Gateau-Olesker and Marazano (2002)

Fig. (34). Synthesis of haliclamine A through a dimeric macrocycle precursor obtained by Zincke procedure

The appealing strategy employing an N-protected/C-activated and an N-unprotected/C-unactivated subunit adopted by Morimoto and Baldwin for the synthesis of dimeric macrocycles, has been extended to the synthesis of linear oligomers related to poly-APS (**77**) from *Reniera sarai* [51]. In this case, as reported in Fig. (**35**), the monomeric 3-octyl pyridine **95**, obtained by standard methods starting from 3-methylpyridine, is *N*-protected by as the PMB-derivative to avoid intramolecular cyclization, and activated on the alkyl chain by conversion to bromide **96**, to give a linear dimeric compound. A portion of the latter is then converted to iodide **97**, whereas the other is *N*-deprotected to dimer **98**, able to give nucleophilic substitution on iodide **97**, thus yielding the corresponding tetramer.

This sequence applied to the synthesis of a linear tetramer provides a general access to a wide range of linear oligo- and polymeric pyridinium compounds and represents an useful alternative to the Zincke procedure proposed by Kaiser et al. [41].



Mancini, Sicurelli, Guella, Turk, Maček and Sepčić (2004)

Fig. (35). Synthesis of oligomeric analogues of poly-APS (*77*) from *Reniera sarai*

STRUCTURAL CHARACTERIZATION OF ALKYLPYRIDINIUM COMPOUNDS

Structural assignments and purity determination of the macrocyclic and/or linear oligo- and polymeric pyridinium compounds represent a peculiar aspect in the study of these compounds, due to the presence of a repetitive alkylpyridinium subunit in their polycationic structure.

NMR measurements have resulted in a diagnostic to differentiate pyridine and pyridinium moieties and to define the presence of branched or unsaturated alkyl chains, as for example in the cases of halitoxin (**74**) [1] or amphitoxin (**75**) [31], respectively. However they are of limited value for establishing the number of linked subunits, since natural 3-AP alkaloids are usually isolated as mixtures of oligomers and polymers, that

are difficult to separate. In addition, while in pure linear oligomers integration of the corresponding proton signals allowed a measure of the number of pyridinium ring *versus* neutral pyridine rings, this criterion is not applicable to cyclic compounds and to mixtures of different oligomers and polymers [41].

Mass spectrometry is a more useful technique for establishing the structures of these compounds. The FAB mass spectra were decisive in the studies of bis-pyridinium macrocyles, including cyclostellettamines. During the characterization of the synthetic cyclostelletamine C (**55**), FAB-MS analysis showed prominent peaks which were assigned to $[M/2]^+$, and to $[M+CF_3COO]^+$ due to the presence of the trifluoroacetate anion. In the negative ion-mode, the $[M-H]^-$ parent ion was detected, and such an ion was thought to be produced by a Hoffman-type elimination occurring during the FAB ionization [25].

Baldwin reported the detection of the $[M-I]^+$ ion in FAB mass spectra of synthetic cyclostellettamines obtained as iodide salts. Unequivocal proof of their structures was obtained by high resolution atomic pressure chemical ionisation (APCI)-MS experiments on the $[M+H]^+$ pseudo-molecular ion of the corresponding uncharged bis-tethrahydropyridine obtained by reduction of cyclostellettamines [44].

In contrast to dimeric macrocycles, larger oligomers do not give good FAB or electrospray mass spectral data [33]. However, in the case of the synthetic tetramer and octamer, Kaiser et al. obtained acceptable electrospray MS data after exchange of the chloride with BF_4^- anion by precipitation with $AgBF_4$ [41].

For natural poly-APS alkaloids (**77**), matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) spectrometric analysis showed that the compounds comprised a mixture of two polymers with molecular weights centred at around 5520 Da and 18900 Da and with a low degree of polydispersity [34].

Due to the recognized importance of mass spectrometry in the structure elucidation of alkylpyridinium compounds, a detailed MS analysis of synthetic cyclostellettamines H-L (**60-63**) has recently been reported [46], by using powerful analytical techniques such as high resolution electrospray ionisation (ESI)-MS and with fragmentation experiments carried out by ESI atmospheric pressure ionization-collision induced dissociation (ESI-API-CID)-MS measurements. This analysis allowed a reliable identification of the alkyl chain lengths of cyclostellettamines, and is proposed as a suitable systematic methodology

in the forthcoming structure elucidation of such complex classes of compounds.

Finally, the supra-molecular structure of poly-APS (**77**) has been also studied by dynamic light scattering (DLS) measurements. These data suggest that in aqueous solution poly-APS are mainly organized as non-covalently bound aggregates, an interesting feature which might be responsible for the wide range of biological activities reported on these compounds [34].

BIOLOGICAL ACTIVITIES OF 3-ALKYLPYRIDINIUM AND 3-ALKYLPYRIDINE COMPOUNDS

3-alkylpyridinium and 3-alkylpyridine compounds possess different biological activities which depend largely on the degree of polymerization. In general, 3-AP monomers, dimers and trimers possess greater antimicrobial activity, while higher polymers show more pronounced cytolytic, cytotoxic and antifouling activities. However, as it will be discussed further, this is not an absolute rule. As mentioned before, during over the last 30 years a number of 3-AP compounds have been isolated from marine sponges belonging mostly to order Haplosclerida. In spite of their relatively simple chemical structure, all these compounds exert a broad spectrum of biological activities, which are listed and discussed below. It must be emphasized, however, that while most of the different 3-APs have been structurally characterized in great detail, not much is known about their biological activities and mechanisms of action. Frequently, only one type of biological assay was carried out for a particular compound. The most widely employed are cytotoxicity assays on different normal or transformed cell lines, or antimicrobial assays. Notable exceptions are the reports on different biological activities of 3-alkylpyridinium polymers from the Mediterranean sponge *Reniera sarai*, and studies on the related polymeric halitoxins (**74**) and amphitoxins (**75**) from the Caribbean marine sponges *Amphimedon* (*Haliclona*) *viridis* and *Amphimedon compressa* (*Haliclona rubra*). These reports will be reviewed later in greater details.

Compounds with cytolytic or membrane permeabilizing activity

The groups of both Schmitz and Berlinck reported on the haemolytic activity of halitoxins (**74**) and their lethality to mice. These two activities increase with increasing molecular weights (degree of polymerization) [1,29]. The group of Berlinck reported a dose-dependant haemolysis obtained by polymeric halitoxins, with an ED_{50} of about 2.2 ng/mL. This value was about 500 times lower than that reported by Schmitz' group.

Electrophysiological actions of halitoxins on cell membranes were studied in detail by Scott et al. [30], using primary cultures of rat dorsal root ganglion neurons, as well as artificial lipid bilayers. In cell cultures, halitoxin caused irreversible membrane potential depolarization, decreased input resistance, and inhibited evoked action potentials. The toxin also evoked a calcium influx and its release from intracellular stores. Halitoxin also induced a channel-like activity in artificial lipid bilayers composed of phosphatidylcholine and cholesterol. Poly-APS (**77**) [34] are structurally related to halitoxins (**77**), and also show moderate haemolytic activity [52]. The cytolytic activity of 3-AP polymers was initially thought to be due to their detergent properties. These molecules possess a positively charged pyridinium ring ("polar head") and aliphatic moieties ("nonpolar tails"), and hence form supramolecular aggregates or micelle-like structures in aqueous solutions. This was established, at least for poly-APS [34]. The mechanism of haemolysis induced by poly-APS has been studied in details by Malovrh et al., [53]. They found that the haemolysis is similar in many aspects to that obtained with cationic detergents such as cetylpyridinium chloride or cetyltrimethylammonium bromide. However, poly-APS, at concentrations below their critical micellar concentration produce discrete membrane lesions with an average diameter of 5.8 nm, as determined by osmoprotectants. This indicates that the lysis is due to the formation of pores with defined diameter and not simply due to the detergent-disruptive effect of these molecules. In fact, results obtained with halitoxin isolated from *Callyspongia ridleyi* [30], as well as recent results with poly-APS [54,55], clearly show that at low concentrations, these molecules form transient but defined membrane pores which could be used for the cell DNA transfection.

At present, there are no reports indicating a cytolytic activity of natural 3-AP monomers and oligomers, and it appears that the ability to permeabilize cells is closely associated with those 3-AP compounds

which are polymeric in nature. However, according to the structures of some monomeric and oligomeric alkylpyridines and their structural similarity to certain neutral or cationic surfactants, they might be expected to be cytolytically quite active.

Cytotoxic activity

Almost all reported 3-AP monomers exert moderate cytotoxicity against certain transformed cell lines in the concentration range of a few micrograms per millilitre. Surprisingly, xestamines A-C (**19-21**) from *Xestospongia widenmayeri* [13] were inactive against P-388 cells *in vitro*, despite their structural resemblance to niphatyne A (**1**), which has an IC_{50} of 0.5 μg/mL against the same cell line [5]. The most potent cytotoxic activity belongs to pyrinodemins (**43-46**), whose IC_{50}s are within the range of a few ng/mL [17,18]. The cytotoxic activities of 3-AP monomers, oligomers and polymers are listed in the Table 1.

Haliclamines A (**49**) and B (**50**) – tetrahydropyridines linked through C_9 and C_{12} alkyl chains, – inhibit the division of fertilized sea urchin eggs, as well as the growth of transformed cell lines [22]. Another example of cyclic alkylpyridinium compounds, cyclostellettamines and dehydrocyclostellettamines (**53-65**), were reported to inhibit muscarinic acetylcholine receptors [23], as well as histone deacetylase enzymes [22], that are being considered as therapeutic targets for treating cancer, and the growth of transformed cell lines [24]. Examples of linear 3-AP dimers, pyrinodemins (**43-46**) [17,18], show 10 to 100 fold greater cytotoxicity against transformed cell lines than the 3-AP monomers.

This activity is comparable to that of the only reported linear 3-AP trimers, niphatoxins (**66, 67**) [26]. A polymeric alkylpyridinium compound, halitoxin (**74**), was also found to be cytotoxic to transformed cells [1]. Poly-APS (**77**) are also cytotoxic, although there is negligible difference between their activity towards normal (IC_{50} = 0.4 μg/mL) and transformed (IC_{50} = 0.3 μg/mL) cells [52]. These values are about 10 to 15 times lower than those of halitoxin [1].

Table 1. Cytotoxic activity of 3-alkylpyridine and 3-alkylpyridinium compounds from marine sponges on different cell lines.

Compound	L1210 cells IC_{50} (µg/mL)	KB cells IC_{50} (µg/mL)	HeLa cells IC_{50} (µg/mL)	P388 cells IC_{50} (µg/mL)
monomeric alkylpyridines				
niphatesine A **(3)** [6]	3. 0			
niphatesine B **(4)** [6]	0. 72			
niphatesine C **(5)** [6]	4. 5			
niphatesine D **(6)** [6]	0. 95			
niphatesine E **(7)** [7]	>10	>10		
niphatesine F **(8)** [7]	>10	>10		
niphatesine G **(9)** [7]	7. 9	>10		
niphatesine H **(10)** [7]	1. 9	6. 0		
niphatyne A **(1)** [5]				0. 5
theonelladine A **(11)** [11]	4. 7	10		
theonelladine B **(12)** [11]	1. 0	3. 6		
theonelladine C **(13)** [11]	3. 6	10		
theonelladine D **(14)** [11]	1. 6	5. 2		
Ikimine A **(15)** [7,12]	5. 4 [7]	5. 0[12]		
Ikimine B **(16)** [12]		7. 0		
Ikimine C **(17)** [12]		5. 0		
hachijodine A **(32)** [3]	2.2			
hachijodine B **(33)** [3]	2.2			
hachijodine C **(34)** [3]	2.2			
hachijodine D **(35)** [3]	2.2			
hachijodine E **(36)** [3]	2.3			
hachijodine F **(37)** [3]	1.0			
hachijodine G **(38)** [3]	1.0			
Oligomeric alkylpyridines				
haliclamine A **(49)**[22]	1. 5			0. 75
haliclamine B **(50)**[22]	0. 9			0. 39
niphatoxins A **(66)** and B **(67)** [26]				0. 1
pyrinadine A **(47)** [20]	2. 0	1. 0		
pyrinodemin A **(43)** [18]	0. 058	0. 5		
pyrinodemin B **(44)** [17]	0. 070			
pyrinodemin C **(45)** [17]	0. 060			
pyrinodemin D **(46)** [17]	0. 080			
cyclostellettamine A **(53)** [24]			0.9	1.06
cyclostellettamine G **(59)** [24]			1.3	1.2
dehydrocyclostellettamine D **(64)** [24]			0.31	0.67
dehydrocyclostellettamine E **(65)** [24]			0.96	0.69
polymeric alkylpyridines				
halitoxin **(74)** [1,29]		5-7 [1]		
Poly-APS **(77)** [52]			0. 3	

KB = human epidermoid carcinoma cells **HeLa** = human cervical carcinoma cells
L1210 = murine lymphoma cells **P388** = mouse leukaemia cells

Antimicrobial activity

There are few reports of antimicrobial activity for alkylpyridinium compounds. However, the numbers are difficult to compare since authors have been using different bacterial strains or fungi in their assays. Overall, it appears that monomers are superior in antimicrobial activity over to oligomers and polymers, but again, this is not a general rule.

Isolation of xestamines D-H (**22-26**) from *Calyx podatypa* yielded two fractions, xestamines and their corresponding *N*-methylpyridinium salts [14]. It is interesting that these quaternary pyridinium salts exerted a moderate antibacterial activity against G^+ bacteria, that was about 40 times greater than the activity of the corresponding tertiary amines.

Halitoxins (**74**) and amphitoxins (**75**) exhibited a broad spectrum of biological activities. Halitoxin showed antibacterial activity towards two $Gram^+$ species, *Bacillus subtilis* and *Streptococcus pyogenes* [1,29]. Kelman and co-workers [32] have assessed the antimicrobial activity of the halitoxin/amphitoxin mixture that was isolated from the Red Sea marine sponge *Amphimedon viridis*. The mixture was tested against ecologically relevant bacteria and resulted in zero activity against sponge-associated bacteria, but strong inhibition was found against most strains isolated from seawater. This selective inhibition could play an important ecological role in preventing microfouling and in defence against potentially pathogenic marine bacteria. A similar study was carried out with haliclamines **(49-52)** isolated from the Arctic sponge *Haliclona viscosa* [4]. The extracts and pure compounds from this sponge were tested against five bacterial strains from natural habitat of the sponge. Two strains were isolated from the water column, two from the nearby rocky substrate and one from the sediment. The bacterial strains were identified as *Psychrobacter* spp., *Planococcus* spp., *Colwellia* spp., *Pseudoalteromonas* spp., and *Polaribacter* spp. The sponge extracts were tested at natural concentrations and haliclamines C (**51**) and D (**52**) were tested at 5 mg/mL. Haliclamines showed a strong inhibition against *Polaribacter* and very strong activity against *Planococcus*.

Some alkylpyridinium compounds also show antifungal activity. Xestamines A-F **(19-24)** from *Calyx podatypa* were active against *Candida albicans* [14]. Haliclamines A (**49**) and B (**50**) were also reported to possess antifungal activity against *Mortierella ramannianus* [22].

Antifouling activity

An excellent review paper on compounds from marine sponges with antifouling activity was written by Fusetani [56]. A recent review on the antifouling activity of 3-AP compounds, with special emphasis on the polymeric alkylpyridinium salts (poly-APS, **77**) and their antifouling activity, was also recently published [57]. So far, poly-APS are the only 3-APs for which anti micro- and macro-fouling activity has been studied intensively.

Inhibition of the settlement of *Balanus amphitrite* cypris larvae by poly-APS was tested. In addition, stage II nauplii of *B. amphitrite*, together with some other ecologically relevant planktonic organisms (microalga *Tetraselmis suecica* and larvae of the edible mussel *Mytillus galloprovincialis*), were used in toxicity assays [58]. Poly-APS have also been tested for their potential anti-microfouling activity, for example their ability to prevent the formation of a biofilm on submerged surfaces under laboratory conditions [59].

Antifouling activity of poly-APS, tested on *Balanus amphitrite* cypris larvae, showed an EC_{50} of 0.27 μg/mL which was higher than the corresponding activity of the crude extract obtained from *R. sarai* (EC_{50} = 1.46 μg/mL). When compared to booster biocides like Zinc Omadine® and Copper Omadine®, poly-APS were about ten fold less effective in preventing fouling of barnacle larvae. However, poly-APS were non-toxic when compared to booster biocides which showed significant toxicity. The non-toxic antifouling activity of poly-APS was further substantiated by toxicity assays against *B. amphitrite* nauplii in the swimming inhibition assay, and in the naupliar toxicity assay. The lack of toxicity associated with settlement inhibition by poly-APS was attributed to the reversibility of the latter. In this test, cypris larvae were exposed for 72 hr to poly-APS at the concentration that caused 100% settlement inhibition. They were then rinsed and placed into fresh seawater, where their settlement was monitored. After 120 hr the cyprids were able to settle at a rate not significantly different from that of untreated larvae. In a bivalve acute toxicity test, poly-APS were also considerably less toxic against *Mytilus galloprovincialis* larvae (veliger and trocophora) than tested booster biocides in a bivalve acute toxicity test.

Poly-APS also inhibit microfouling by bacteria, fungi, and microalgae [59]. In aqueous solutions, poly-APS behave similarly as cationic detergents that are classified as quaternary ammonium compounds. These compounds are examples of synthetic surfactants used in microbial adhesion studies and are well-known antiseptic agents in toothpaste or/and mouth wash. Poly-APS are lytic to different cell lines, but, according to our results, they do not significantly inhibit growth of terrestrial bacteria [51,52]. On the other hand, according to laboratory assays, these compounds are quite effective against marine bacteria. Poly-APS show inhibition of marine bacterial growth in the range of 0.1 –1 μg/mL [59]. Similar selective antibacterial activity has been previously reported for the amphitoxin (**75**)/halitoxin (**74**) mixture [32].

Furthermore, poly-APS also prevent microfouling by fungi and algae, albeit at rather higher concentrations than booster biocides. They inhibit settlement of diatoms and growth of the microalga *Tetraselmis suecica*. The IC_{50} value for the growth of *T. suecica* was 10.66 μg/mL, a concentration that is about 450 times higher than that for the most effective booster biocide, copper pyrithione [58].

The only other reported 3-AP compounds with antimicrofouling activity are untenines A-C (**29-31**), monomeric nitroalkyl pyridine alkaloids from Okinawan marine sponge *Callyspongia* sp. Their IC_{100} values against the marine bacterium *Phodospirillum salexigens* were 3.0, 6.1 and 5.8 μg/cm^2 respectively [16].

3-Alkylpyridinium compounds as enzyme inhibitors and modulators of various receptors

There are only a few reports on enzyme inhibitory inhibition activity by alkylpyridinium compounds. Cyclostellettamine G (**59**) and dehydrocyclostellettamines D (**64**) and E (**65**) from the marine sponge *Xestospongia sp.* were reported to inhibit histone deacetylase (HDAC) [24], and poly-APS (**77**) from *Reniera sarai* irreversibly inhibit various types of cholinesterases [34,52,60,61] and moderately inhibit intracellular phosphatases [51]. The inhibition of HDAC by four different cyclostellettamines was in the μM range; dehydrocyclostellettamine D (**64**) possessing the highest inhibitory activity had an IC_{50} of 17 μM [24]. Poly-APS inhibit various types of cholinesterases, acetylcholinesterase (AChE) from neuromuscular junction being the most sensitive. This anti-

AChE activity of poly-APS has been studied in detail [60]. Pyridinium derivatives are well-known inhibitors of cholinesterases; they act as competitive inhibitors, binding at the catalytic anionic site at the bottom of the enzyme gorge or as non-competitive inhibitors, binding at the peripheral anionic site at the rim of the gorge, thus slowing or preventing the entrance of acetylcholine into the gorge. However, the kinetics of AChE inhibition by poly-APS is complex and comprise several successive phases ending in irreversible inhibition of the enzyme. The irreversible phase is probably accounted for by aggregation and precipitation of enzyme-inhibitor complexes [61]. It is noteworthy that inhibition of AChE has been also reported for a similar compound, – halitoxin (**74**), but it was never studied in detail [62]. Poly-APS were not able to inhibit enzymes like trypsin or acid and alkaline phosphatases [61], but there are some indications that they are able to inhibit certain types of cell phosphatases [51].

3-AP compounds can also bind and modulate the activity of certain receptors. Oligomeric 3-APs, cyclostellettamines A-F (**53-58**), are known to act as antagonists of muscarinic acetylcholine receptors. The IC_{50} binding values range from 26- to 474 μg/mL, and their efficiency decreases with the length of the alkyl chains linking the pyridinium rings [23].

EGF-active factors (**76**), that are structurally similar to poly-APS, prevent binding of epidermal growth factors to their receptors on cell membranes, resulting in decreased DNA synthesis and cell proliferation. These compounds also bind to insulin receptors, albeit with about 10 fold lesser affinity [33].

Effects of 3-alkylpyridinium compounds *in vivo*

There are only few reports on the *in vivo* effects of alkylpyridinium compounds. Most only report the LD_{50} (toxicity data) on different organisms. The notable exceptions are halitoxin (**74**) and poly-APS (**77**). The former was reported to be moderately lethal to mice with *i.v.* LD_{50} of 1.4 mg/kg. The intoxicated mice were cyanotic and died of respiratory arrest. Blood samples showed a partial haemolysis, and lethal effects were therefore attributed to the haemolytic action of halitoxin [29]. On the

other hand, the lethal action of halitoxin could be also linked to its neurotoxic activity, observed by the same authors on isolated crustacean nerve preparation. Their experiments showed a rapid blockade of action potentials conduction, with a slow and partial depolarization. The total blockade of conduction was only partially reversible and without full recovery of the control spike and resting potential. In principle, this activity could also be linked to the non-specific action of halitoxin towards the cell membranes, which prevents nerve conduction and kills the experimental animal by respiratory paralysis, as already reported by Kaul and Daftari [63]. Berlinck et al. [29] also argued that the *in vitro* and *in vivo* cytotoxic effect reported for the crude sponge extract containing halitoxin [64] is rather a consequence of the toxin antiproliferative or antimitotic activity than due to its cytolytic activity.

The *in vivo* toxicity of poly-APS was studied by Bunc et al., [65-67]. In order to evaluate the role of AChE inhibition in toxin lethality, and to assess other possible lethal effects, *in vivo* experiments were performed on male Wistar rats, and electrocardiogram (ECG), blood pressure and breathing pattern were monitored. The results showed that none of the animals died due to the AChE-inhibitory action of poly-APS. Doses lower than 1mg/kg caused only transient bradycardia and transient prolongation of expirium. At doses above 2.7 mg/kg of poly-APS, all treated animals died, but signs were not typical of AChE inhibition. Arterial blood pressure fell to mid-circulatory pressure, and breathing stopped after a few breaths, with an increase of the residual volume. Autopsy of the experimental animals that died due to the effects of the toxin revealed that mid-size and small sized blood vessels in the heart and lungs were filled with granular brownish material with inclusions of red blood cells and platelets. Data obtained on blood samples from animals treated with poly-APS also revealed numerous thrombocyte aggregates. *In vitro*, poly-APS induced thrombocyte aggregation in a dose-dependent manner. The mechanism of aggregation was not explained but attributed to the possible non-specific (cytolytic) action towards endothelial cells. Altogether, the AChE-inhibitory effects were pronounced only at lower doses of poly-APS. With higher doses those effects were masked or covered by other, more pronounced and faster developing lethal effects of the toxin, such as platelet aggregation. It is therefore reasonable to assume that the AChE-inhibitory effects so obvious in *in vitro* experiments are not responsible for the lethal activity of the toxin.

Other examples of toxicity of alkylpyridinium compounds are ichtiotoxicity, observed by monomeric niphatoxins (**66, 67**) [26] and a mixture of polymeric halitoxins [1]. A similar polymeric 3-AP, amphitoxin, (**75**) [31] showed strong antifeeding behaviour activity in Caribbean wrasse *Thalassoma bifasciatum* at one sixth of its natural concentration, so its ichtiotoxicity is also possible. Feeding deterrence was also reported for haliclamines (**49-52**), cyclic oligomeric alkylpyridinium compounds recently isolated from the Arctic marine sponge *Haliclona viscosa.* Sponge crude extract, as well as pure compounds at their natural concentrations, caused antifeeding deterrence in the amphipod *Anonyx nugas* and in starfish *Asterias rubens* [4]. A very recent report on the activity of cyclohaliclonamines A-E (**69-73**) from an unidentified sponge of the genus *Haliclona* [28] demonstrates the toxicity of these compounds in a brine shrimp assay. An LD_{50} of 65 μg/mL is reported.

Biological activities of synthetic related alkylpyridinium and alkylpyridine compounds

A series of different macrocyclic or linear pyridinium oligomers from 3-substituted pyridines was synthesized by Marazano et al. [49], as reported in Fig. (**32**). They found that cytotoxic activity of synthetic products increases with the degree of polymerization. The growth of KB cells was moderately affected by trimers (ED_{50} = 20 μg/mL), and strongly inhibited by the mixture of water soluble higher polymers with an average of 15 pyridinium units (ED_{50} = 0.5 μg/mL). This value is about ten fold smaller than the one obtained with crude natural mixture of halitoxin (ED_{50} = 5-7 μg/mL). Synthetic cyclostellettamine-like dimer was only weakly active against KB cells (ED_{50} = 25 μg/mL).

Mancini et al. [51] reported on several biological activities of different 3-AP monomers and oligomers obtained by controlled polymerization, as reported in Fig. (**35**). Antimicrobial activity against *Bacillus subtilis* was increasing with the number of pyridinium rings and positive charges. The unprotected tetramer was the most effective compound and had about 350 fold higher activity compared to natural poly-APS. Similar trend was evident also when measuring anti-AChE activity. Higher degree of polymerization and larger number of pyridinium rings and positive charges yields more effective inhibitors. Inhibition of protein phosphatase

2A by synthetic compounds was also tested. Only two monomers and a tetramer caused a weak reversible inhibition, while natural poly-APS showed moderate inhibition with an IC_{50} of 4 μg/mL. Finally, haemolytic assays showed that albeit active, synthetic compounds are far less haemolytic compared to natural poly-APS.

POTENTIAL USE OF 3-ALKYLPYRIDINIUM COMPOUNDS

In the previous chapters we showed that 3-alkylpyridinium compounds possess a plethora of biological activities that might be of certain interest in some applications e.g. as antitumour drugs, transfection agents or components in antifouling paints. There are currently no reports of such applications for 3-AP monomers and oligomers, but some progress had been recently made in studying the possible use of polymeric 3-APs. Most of these results have not been published yet, e.g. some very promising anticancer activities obtained by poly-APS (**77**). Therefore, in this contribution we will comment only on those few already available in the scientific literature.

Poly-APS and halitoxins as transfection agents

In cell membranes both poly-APS (**77**) and halitoxin (**74**) induce transient pores [30,53,55]. These pores are large enough to allow the introduction of macromolecules such as DNA into cells. It has been shown that poly-APS are particularly effective in this regard [54]. Their polycationic nature enables them to bind and condense DNA and subsequently allow passage of large pieces of supercoiled DNA or a particular cDNA plasmid into the cell.

The transfection efficiency of halitoxin and poly-APS has been studied using HEK 293 cells and compared to the efficiency of the well-known transfection agent lipofectamine [54]. In order to achieve transfection, the DNA has to enter the cell by endocytosis or through formed pores. It is believed that 3-AP polymers cause transfection by forming pores. There

are several pieces of evidences that favour this option. Both halitoxin and poly-APS cause a dramatic change in membrane potential and the input resistance [30,55]. When halitoxin is applied, the recovery of membrane potential is dose dependant. At low concentrations (0.5 μg/mL) there is a significant recovery, while concentrations above 5 μg/mL cause a substantial decrease of membrane potential, with no significant recovery. On the contrary, with poly-APS the membrane potential could be significantly recovered even at the higher doses [54]. Both halitoxin and poly-APS evoked changes in intracellular calcium levels. Again, there were differences between the two agents. While halitoxin produced some anomalous results and no clear dose-response relation was observed, poly-APS showed a typical dose-dependency and predominantly reversible responses in evoking transient changes of intracellular Ca^{2+} in HEK 293 cells. The same effect had been already shown in primary culture of dorsal root ganglion neurons. In spite of some differences, these results clearly indicate the formation of permeable pores when cell membranes are exposed to each of these toxins. Transfection of HEK 293 cells by cDNA plasmid coding for green fluorescent protein was successful when poly-APS or halitoxin were used as transfection agents. The cDNA transfection efficiency and subsequent expression of green fluorescent protein with poly-APS or halitoxin was about 25 and 20 % as effective as lipofectamine. At used concentration of 0.5 μg/mL, poly-APS and halitoxin are therefore less effective transfection agents than lipofectamine, but they are a slightly less toxic since more than 90% of the cells survived the treatment. The authors also report that the co-transfection of cDNA is stable and cell colonies could be produced that contain the delivered cDNA and the proteins they encode [54].

The results published in the very recent paper by McLaggan et al. [68] also show that poly-APS is a highly efficient DNA condensing agent being able to form pores reversibly in membranes. This is a highly desirable characteristic for any non-viral vector having the potential for gene therapy. The authors report that poly-APS are able to transfect cells with a simple dye, lucifer yellow, and with complex macromolecules such as cDNA coding for fluorescent green protein. However, compared to lipofectamine, poly-APS were not able to transfect siRNA (small interference RNA) implying that such molecules enter cells not through the pores, but rather by endocytosis, as in the case of lipofectamine. The transfection of cells using poly-APS as transfection agent was more efficient at lower temperatures. The delivery of cDNA into cells was far

better at 12^0 C than at 21^0 C. This is just one indication for optimal transfection with a particular transfection agent but other conditions should also be optimized.

Poly-APS as antifouling compounds

An extensive review dealing with the antifouling properties of 3-AP compounds, with particular focus on poly-APS (**77**), was recently published by Sepčić and Turk [57]. As already mentioned, poly-APS show promising antimicro- and antimacrofouling activities [58,59]. It is encouraging that these compounds presumably act by a non-toxic though yet unknown or better not sufficiently understood mechanism that obviously does not harm marine organisms. One of the possible explanations is that the prevention of settlement and attachment of cyprid larvae is probably achieved by inhibition of their cholinergic system [69]. Poly-APS are strong inhibitors of AChE [60,61], therefore such a mechanism is quite plausible.

Antimicrofouling properties and the ability to form pores in biological membranes raise the possibility that poly-APS and similar molecules found in some sponges as a natural defence compounds could be also important factors of natural genetic material transfer between marine microorganisms [68].

ABBREVIATIONS

3-AP	=	3-alkylpyridine and/or 3-alkylpyridinium
AChE	=	acetylcholinesterase
API-CID-MS	=	atmospheric pressure ionization-collision induced dissociation-mass spectrometry
Bn	=	benzyl
Boc	=	*tert*-butoxycarbonyl
cDNA	=	complementary deoxyribonucleic acid
Da	=	Dalton
DIEA	=	diisopropylethylamine

DLS = dynamic light scattering
DNB = dinitrobenzyl
EC_{50} = the concentration of the active compound causing with biological effect on 50% experimental organisms
EGF = epidermal growth factor
ESI-MS = electrospray-mass spectrometry
FAB-MS = Fast Atom Bombardment-Mass Spectrometry
G^+ = Gram positive
HDAC = hystone deacetylase
HEK = human embryonic kidney cell line
IC_{50} = the concentration of the active compound causing inhibitory effects on 50% experimental organisms
LC_{50} = the concentration of the active compound causing death of 50% experimental organisms
LDA = lithium diisopropylamide
MALDI-TOF = matrix assisted laser desorption ionization-time-of-flight
MS = mass spectrometry
NMR = nuclear magnetic resonance
PMB = *para*methoxybenzyl
Poly-APS = polymeric alkylpyridinium salts
TBDMS = *tert*-butyldimethylsililbutyldimethylsilyl
THP = tetrahydropyranyl

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NOVEL DOMINO REACTIONS FOR SYNTHESIS OF BIOACTIVE DITERPENOIDS AND ALKALOIDS

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ABSTRACT: In the synthesis of relevant organic compounds such as natural products and analogues, the proportion of the number of steps coupled with the increase of complexity is now a universal paradigm to ascertain the quality and efficiency of a process. Alongwith providing accessibility to a multitude of diversified classes of natural products such as alkaloids, terpenoids, steroids and others, these criteria have been addressed by us via the application of domino processes. The acid-catalyzed intermolecular cyclization has been used as a viable synthetic tool for the stereospecific formation of different classes of polycyclic natural products.

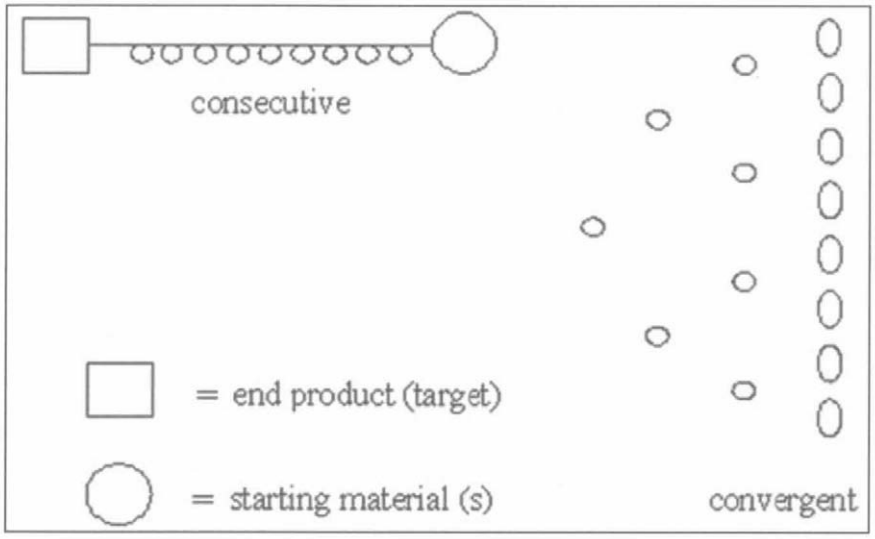
INTRODUCTION

The synthesis of bioactive natural products, agrochemicals, pharmaceuticals have evolved to allow the formation of complex molecules in a few steps, starting from simple substrates. Any synthetic procedure involves either linear retrosynthesis or disconnection approach. The linear pathway leads to a consecutive synthesis and the branching pathway leads to a convergent synthesis. In a convergent synthesis, the several pieces of a molecule are synthesized individually and the final target is sequentially assembled from the fragments. In contrast, the consecutive synthesis involves the stepwise formation of the final complex molecule. It is apparent that the convergent synthesis will have fewer consecutive steps in the overall synthesis (**Chart-1**) .

In a synthesis involving fifteen steps, if each step has a mean yield of 90%, the overall yield of final products is $(0.9)^{15} \approx 0.21$ (21%). If the largest sequence is split and divided into convergent solution, there would be only four consecutive steps ($A_1 \rightarrow B_1 \rightarrow C_1 \rightarrow D_1 \rightarrow Z$) in a perfectly convergent pathway. The yield of the final product would be $(0.9)^4 \approx 0.66$ (66%). It is difficult, however to achieve such a perfect convergence, since functional group manipulation, steric considerations, and asymmetry lead to "imperfections" [1] .

An example of a convergent synthesis used by Fuchs[2] for the synthesis of prostaglandin E_2 (PGE_2) is illustrated in **Chart-2**, while an example of consecutive synthesis used by Corey[3] for the synthesis of prostaglandin E_2 (PGE_2) is illustrated in **Chart-3**.

Chart 1 [1]



The overall yield of compound obtained by **Chart-2** was 13%[1] while the overall yield of compound obtained by **Chart-3** was 23%[3].

In this instance the consecutive synthesis gives a better yield of prostaglandin E_2 (PGE_2) than the convergent synthesis. Both consecutive and convergent strategies are attractive, and only careful consideration of each will yield the best approach for an individual target. Obviously, once it is completed a given synthesis can be continually reexamined and, in principle, improved upon.

In general, a convergent strategy is expected to produce larger amounts of the target in the fewest steps if a scheme can be devised that converges at an useful point, such as Fuch's[4] work. A symmetrical convergent strategy is often the most attractive. **However, it would be much more efficient if one could form several bonds in one sequence without isolating the intermediates, changing the reaction conditions or adding reagents**. It is obvious that this type of reaction would allow the minimization of waste thus making waste management unnecessary since compared to the stepwise reactions, the amount of solvents, reagents, adsorbents and energy would be dramatically decreased. This has led to the need for development of **domino reactions**.

Chart-2 [2]

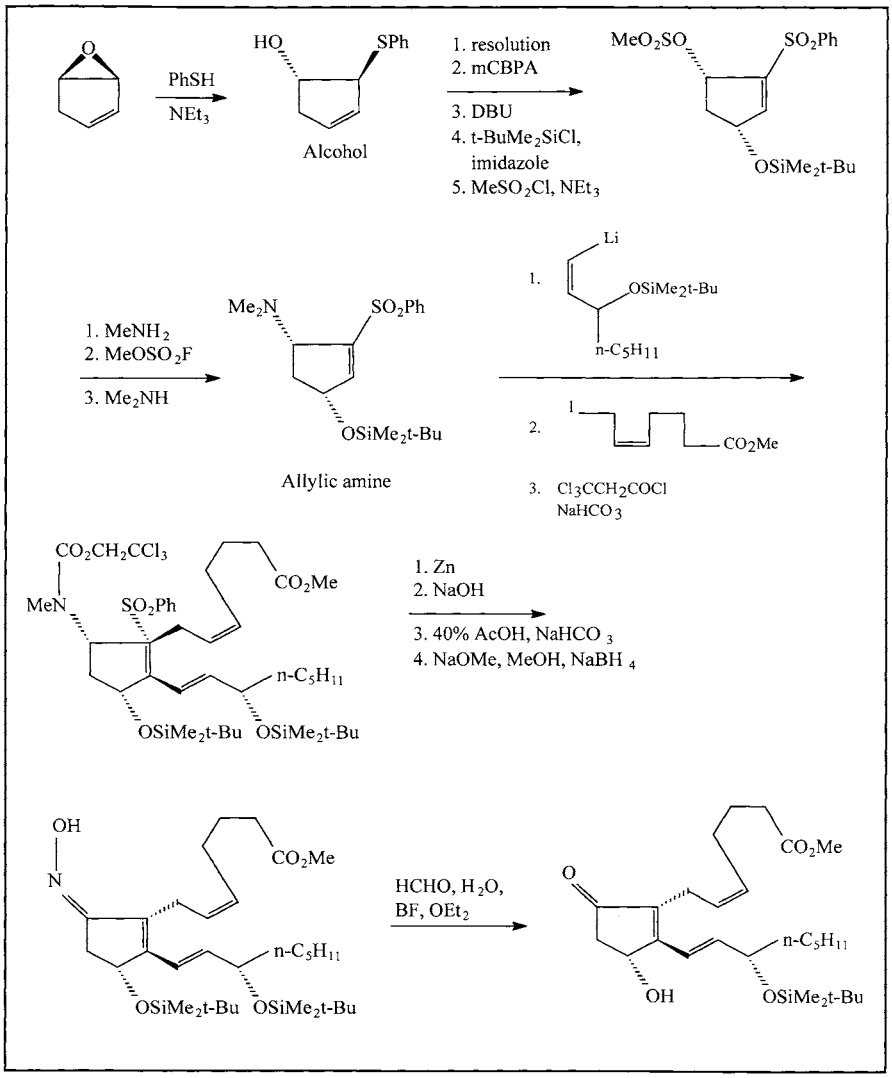
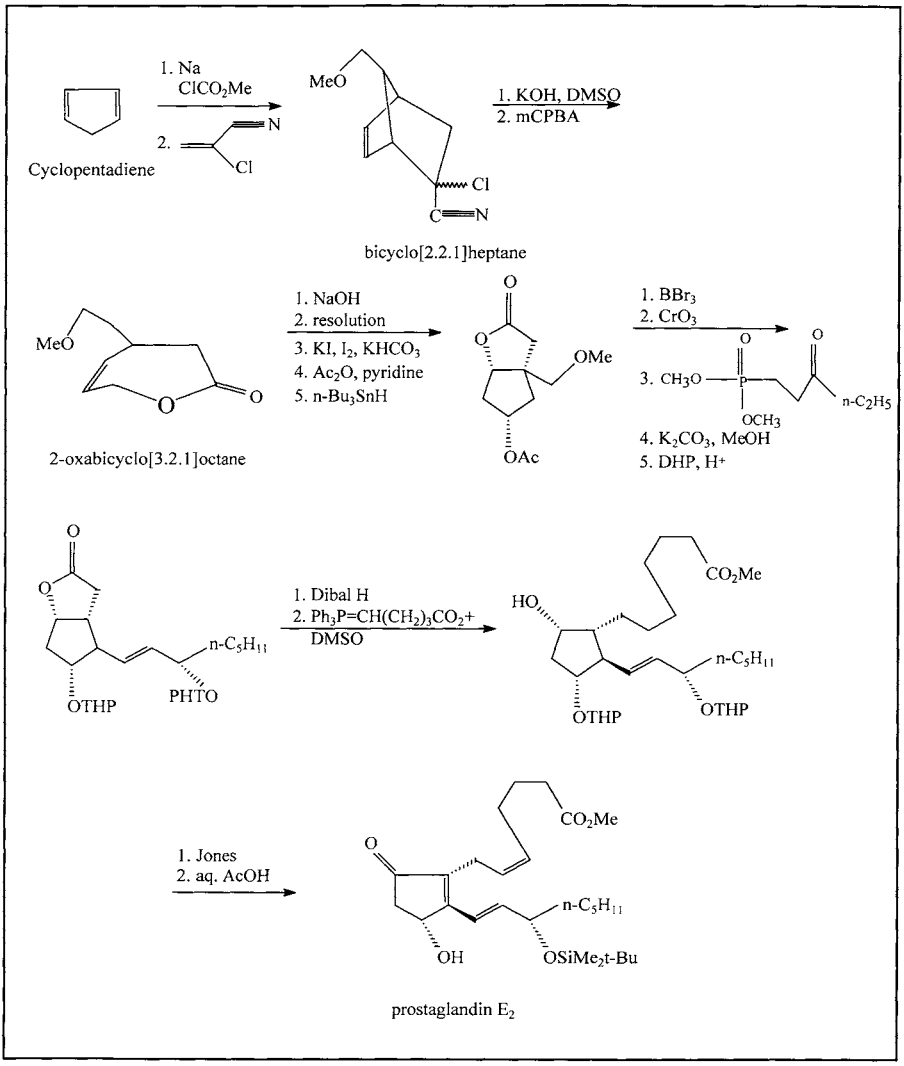

PhSH
NEt3
HO
SPh
Alcohol
1. resolution
2. mCBPA
3. DBU
4. t-BuMe2SiCl, imidazole
5. MeSO2Cl, NEt3
MeO2SO
SO2Ph
OSiMe2t-Bu
1. MeNH2
2. MeOSO2F
3. Me2NH
Me2N
SO2Ph
OSiMe2t-Bu
Allylic amine
1.
Li
OSiMe2t-Bu
n-C5H11
2.
CO2Me
3. Cl3CCH2COCl
NaHCO3
CO2CH2CCl3
MeN
SO2Ph
CO2Me
n-C5H11
OSiMe2t-Bu
OSiMe2t-Bu
1. Zn
2. NaOH
3. 40% AcOH, NaHCO3
4. NaOMe, MeOH, NaBH4
OH
N
CO2Me
n-C5H11
OSiMe2t-Bu
OSiMe2t-Bu
HCHO, H2O,
BF, OEt2
O
OH
CO2Me
n-C5H11
OSiMe2t-Bu

Chart-3 [3]


1. Na
ClCO2Me
2.
Cyclopentadiene
MeO
Cl
N
1. KOH, DMSO
2. mCPBA
bicyclo[2.2.1]heptane
MeO
O
1. NaOH
2. resolution
3. KI, I2, KHCO3
4. Ac2O, pyridine
5. n-Bu3SnH
OMe
OAc
2-oxabicyclo[3.2.1]octane
1. BBr3
2. CrO3
3. CH3O
OCH3
n-C2H5
4. K2CO3, MeOH
5. DHP, H+
n-C5H11
OTHP
PHTO
1. Dibal H
2. Ph3P=CH(CH2)3CO2+
DMSO
HO
CO2Me
1. Jones
2. aq. AcOH
OH
OSiMe2t-Bu
prostaglandin E2

The term domino reaction in organic chemistry was coined by the German researcher Dr. Lutz. F. Tietze [5] in the year 1993.

The oldest known reference of domino type of reaction was performed by Robinson [6] for the synthesis of a natural product, a bicyclic tropinone, which is a structural component of several alkaloids such as cocaine and atropine (**Chart- 4**).

The biosynthesis of fatty acids [7] and progestrone [8] are some characteristic examples of the domino type of reactions (**Charts-5 to 6**).

Chart-4 [6]

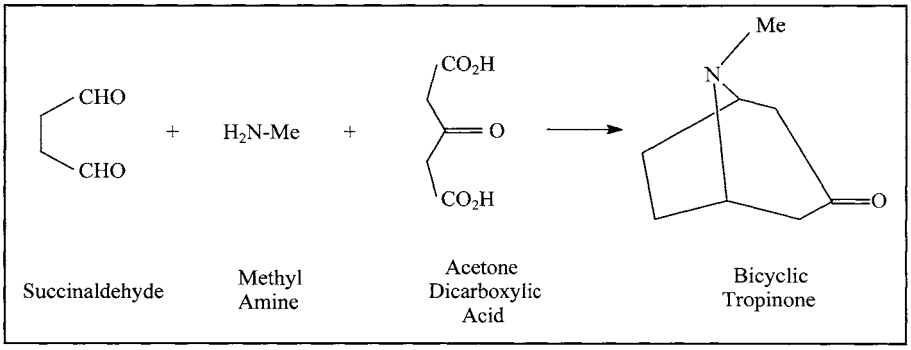


Chart-5 [7]

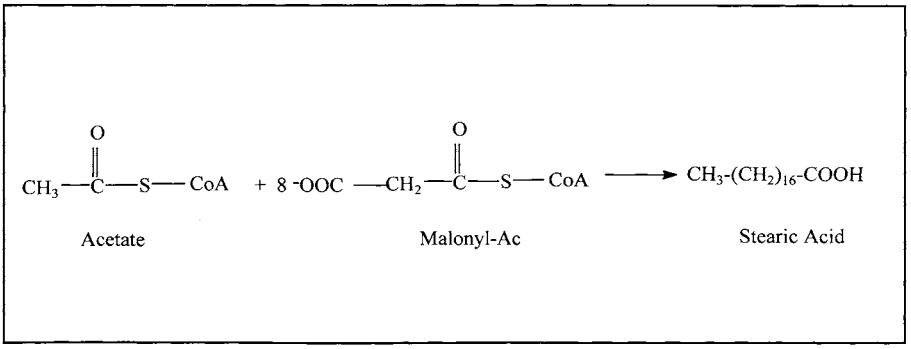
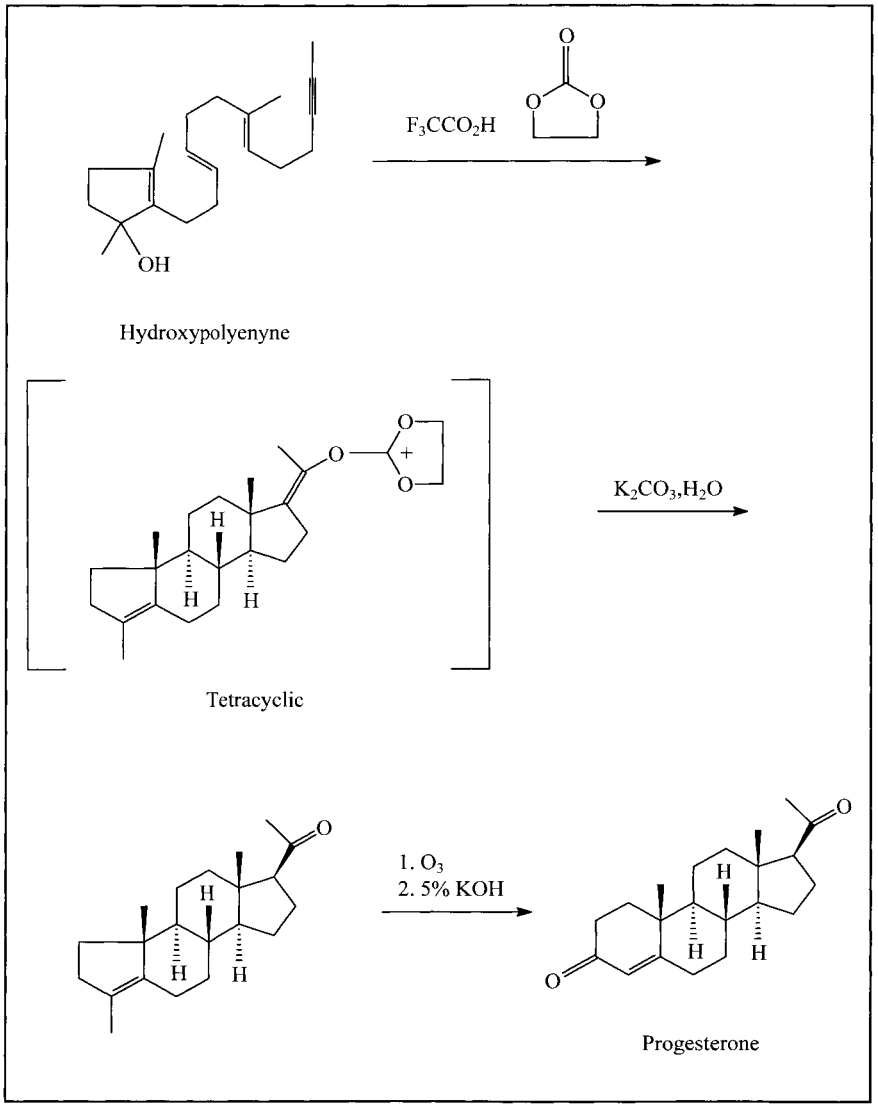


Chart-6 [8]


F3CCO2H
OH
Hydroxypolyenyne
K2CO3,H2O
Tetracyclic
1. O3
2. 5% KOH
Progesterone

CLASSIFICATION OF DOMINO REACTIONS

The criteria [5] for a reaction to be termed as a domino reaction are as follows:

A domino reaction is a process involving two or more bond transformations (usually involving the C-C bonds) which take place under the same reaction conditions without adding additional reagents and catalysts, and in which the subsequent reactions result as a consequence of the functionality formed in the previous step.

A substrate with several functionalities which undergo transformations individually in the same pot is not a domino reaction. Clearly, the preliminary formation of an intermediate such as a carbocation or a carbanion is not counted as a reaction step. On the other hand the formation of a diene by a retro-Diels-Alder reaction with a subsequent cycloaddition would be considered as a domino reaction.

The classification of domino reactions is based on the intermediate generated in the first step. Thus a clear classification, which not only allows a better understanding of the existing domino reactions, but also facilitates the invention of newer domino reactions is presented below.

There are altogether four types of domino reactions based on the intermediate generated in the first step.

i. **Cationic domino reactions**

ii. **Anionic domino reactions**

iii. **Radical domino reactions**

iv. **Pericyclic domino reactions**

CATIONIC DOMINO REACTIONS

In this process, a carbocation is formed, either formally or in reality. This cation can be formed, for example the elimination of water from alcohols, of alcohols from acetals or by oxidation of a positive particle such as a proton to an alkene or an epoxide. The carbocation then reacts with a nucleophile to form a new carbocation, that undergoes one or more comparable further transformations in a cationic-cationic process, finally being trapped by a nucleophile or stabilized by elimination of a proton.

Rearrangement reactions, in which numerous cation species appear, similarly belong to this type of reactions.

Some interesting examples of this type of reactions [9-11] are illustrated in **Charts-7 to 9**.

Chart-7 [9]

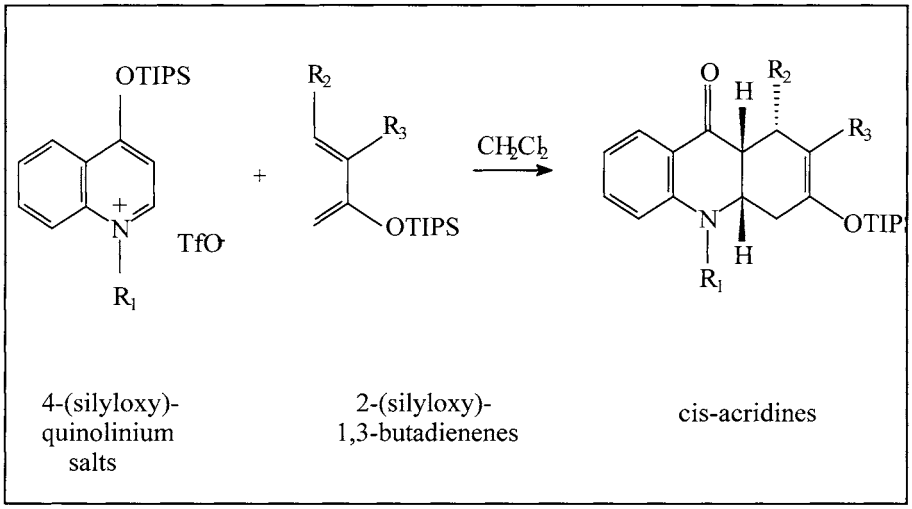


Chart-8 [10]

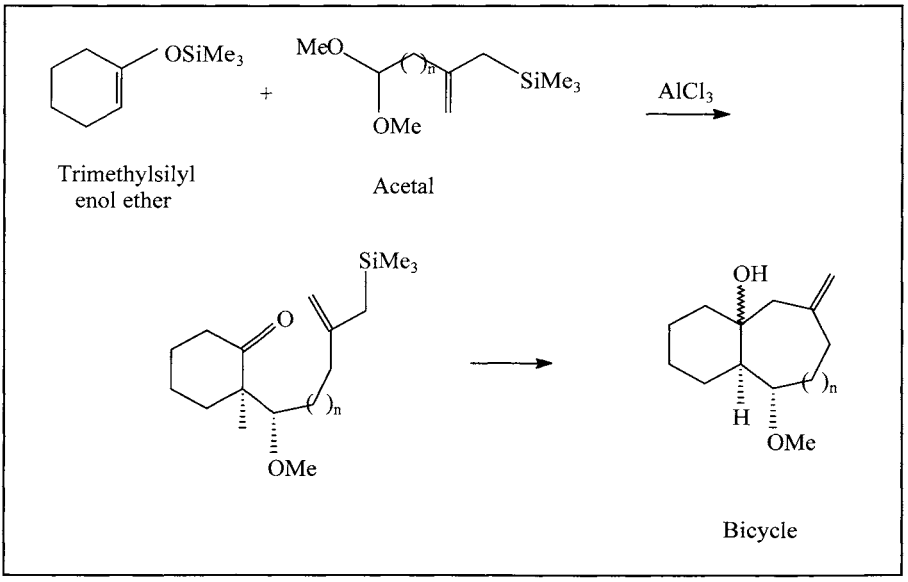
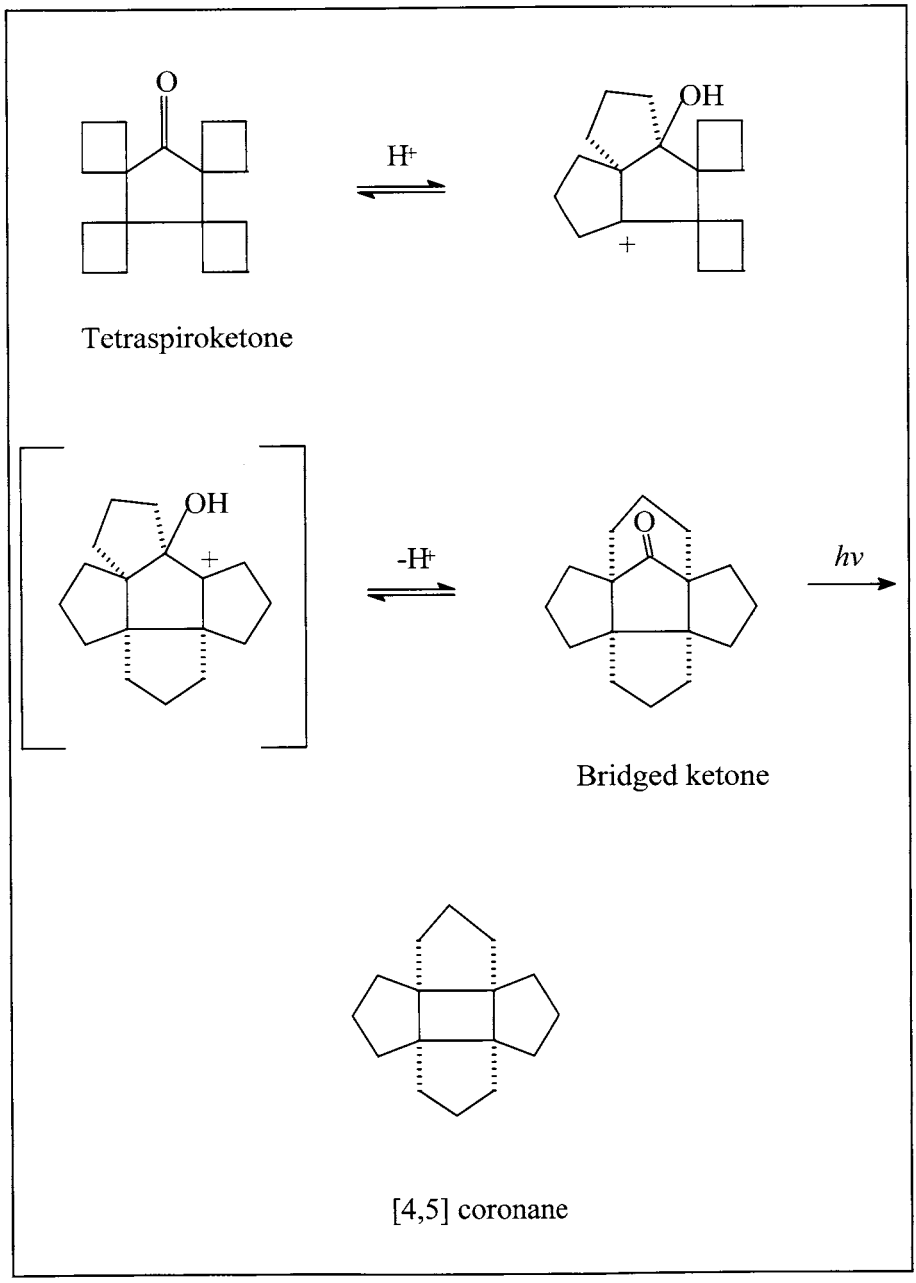


Chart-9 [11]



ANIONIC DOMINO REACTIONS

The anionic domino reactions are the most often encountered domino reactions in literature. Especially reactions combining two Michael additions.

In this type of reactions, the primary step is the formation of an anion or a nucleophile. The majority of cases involve the deprotonation of a C—H group with the formation of a carbanion, which then reacts with an electrophile to form a new anionic functionality. The sequence is complicated by reaction with an electrophile for example a proton or by elimination of an X^- group.

Some of the examples of anionic domino type of reactions [12-14] are illustrated in **Charts-10 to 12.**

Radical Domino Reactions

For those reaction sequences that start with a radical primary step, there have been found, exclusively examples of homosequence transformations. Thus an abundance of possibilities for further investigations exists in this area.

In the last decade radical domino reactions have gained importance for the synthesis of natural products. The formation of radicals can result by the reaction of halogens, phenyl-thio and phenyl selenium compounds.

Chart-10 [12]

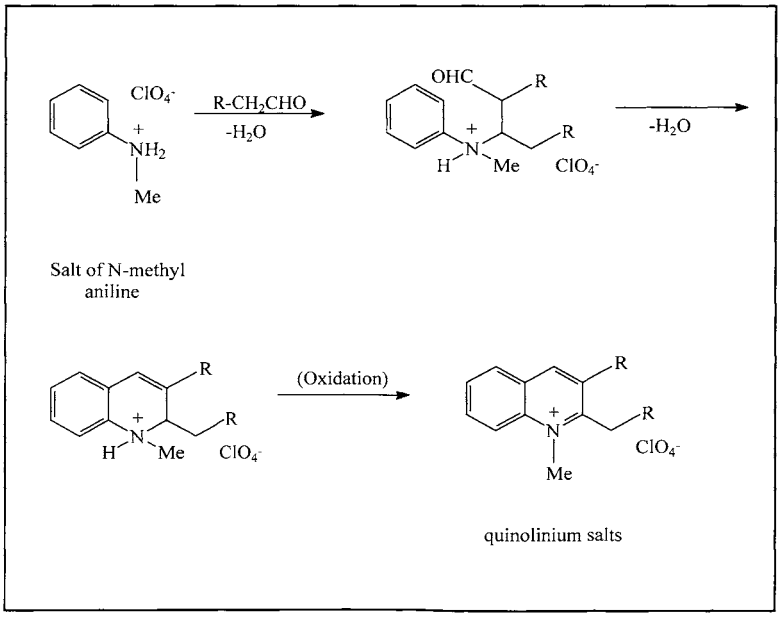


Chart-11 [13]

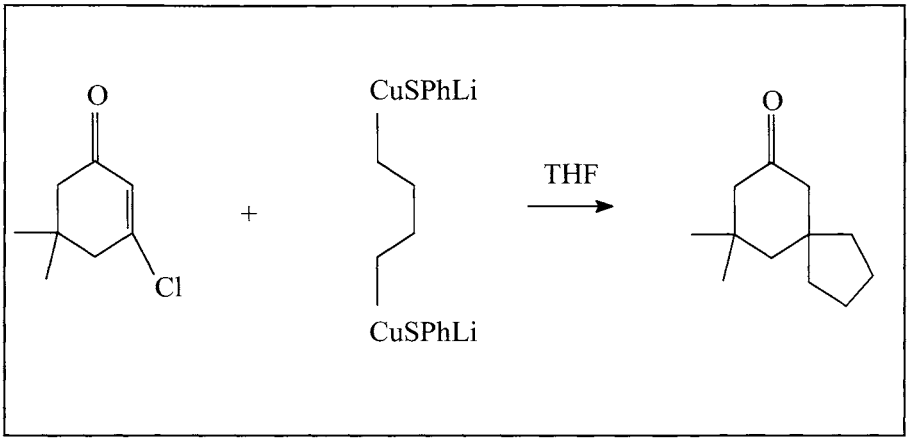
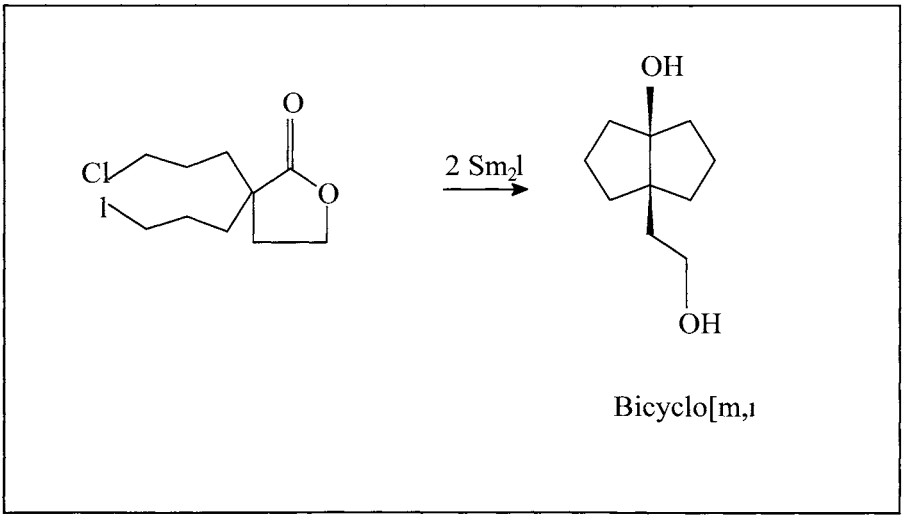


Chart-12 [14]



Some of the examples of radical domino reactions [15-17] are illustrated in **Charts- 13 to 15.**

PERICYCLIC DOMINO REACTIONS

Pericyclic reactions can be combined easily with other pericyclic reactions to give sequences. Whether inter- or intra-molecular, the all-carbon atom or the hetero-atom, normal, neutral or with inverse electron demand this reaction is one of the most efficient methods in the repertoire of the organic chemist.

Interesting examples of this type of reactions [18-20] are illustrated in **Charts-16 to 18.**

Chart-13 [15]

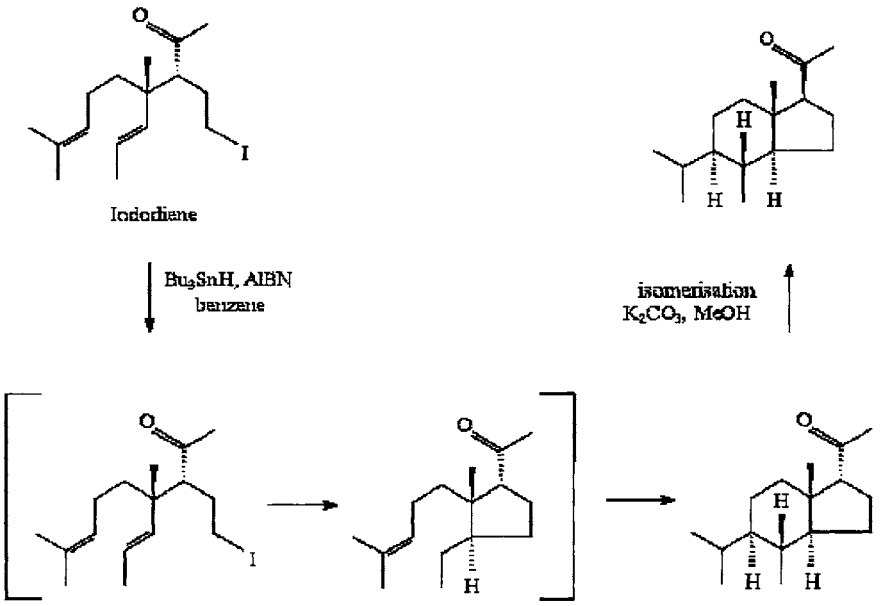


Chart-14 [16]

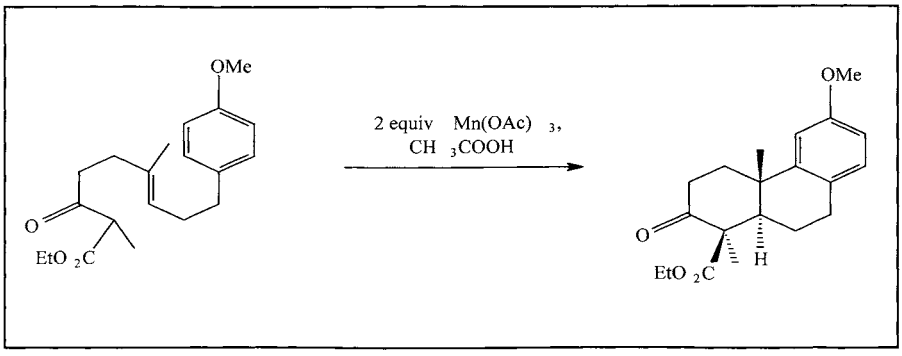


Chart-15 [17]

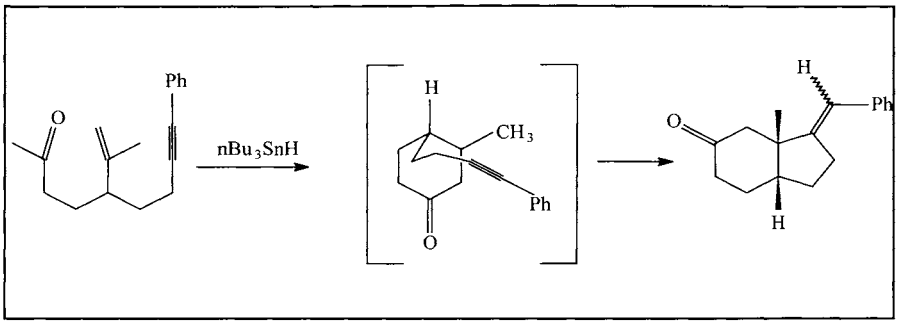


Chart-16 [18]

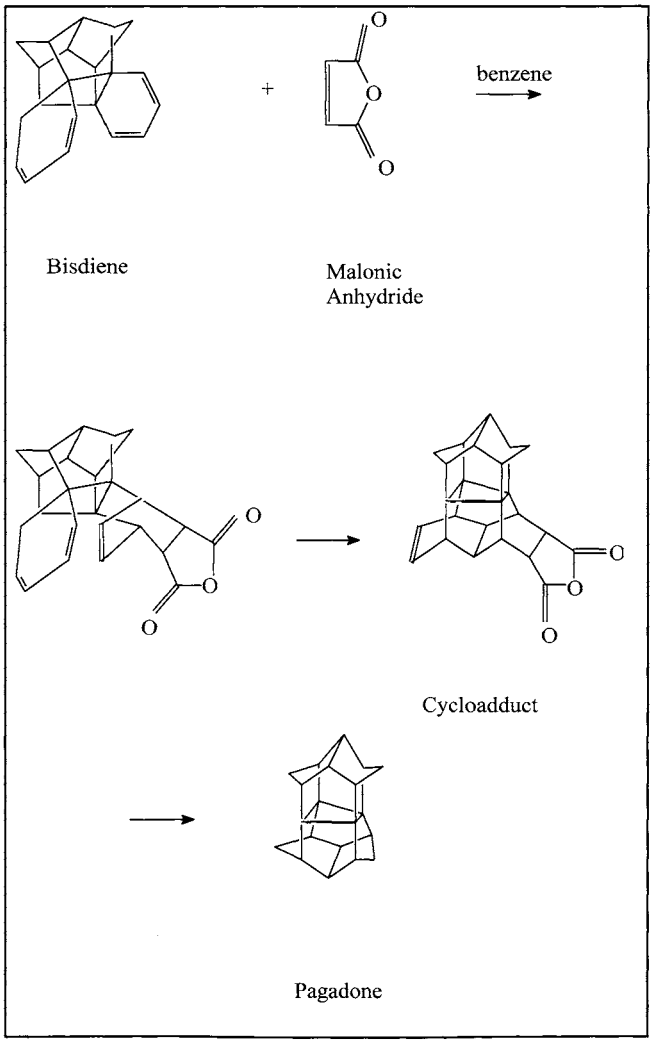


Chart-17 [19]

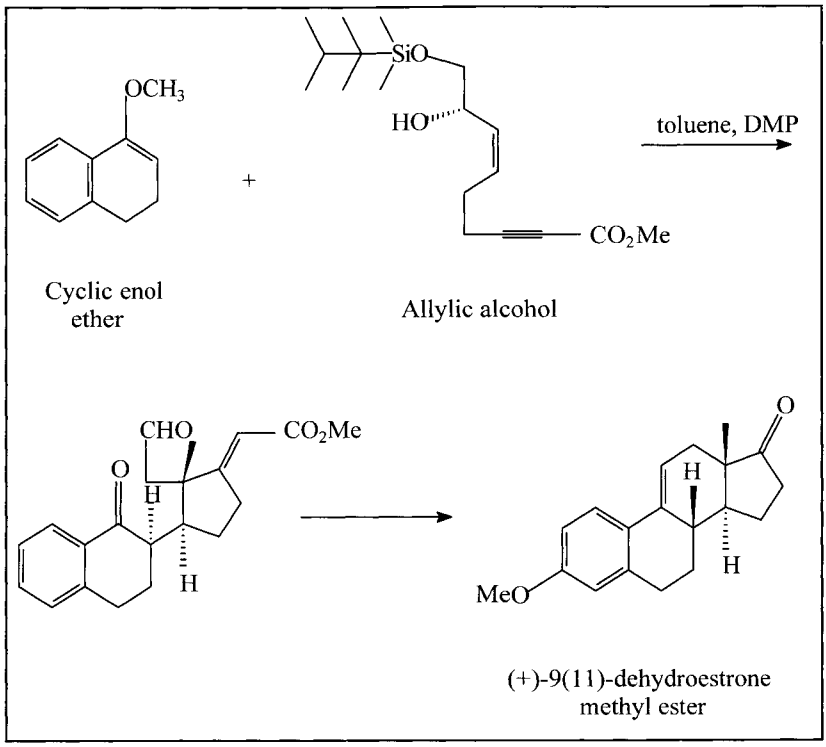
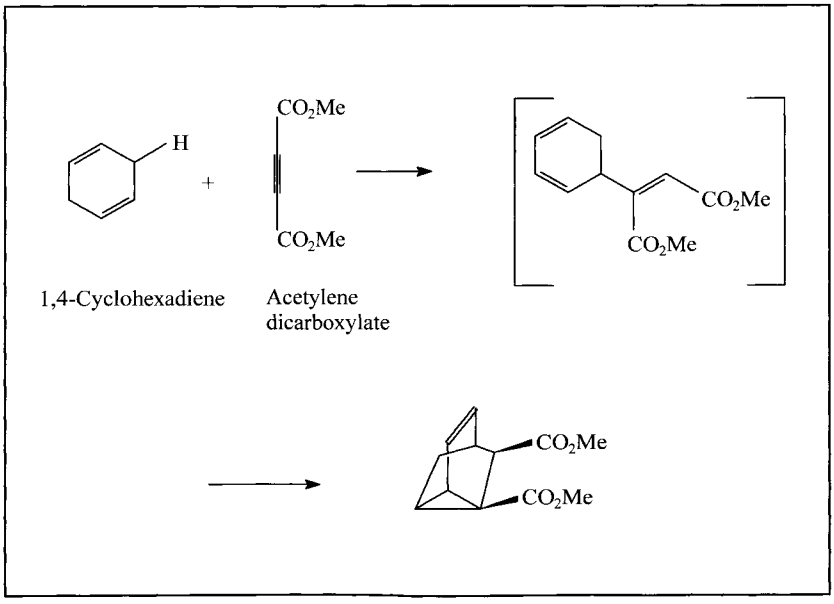


Chart-18 [20]

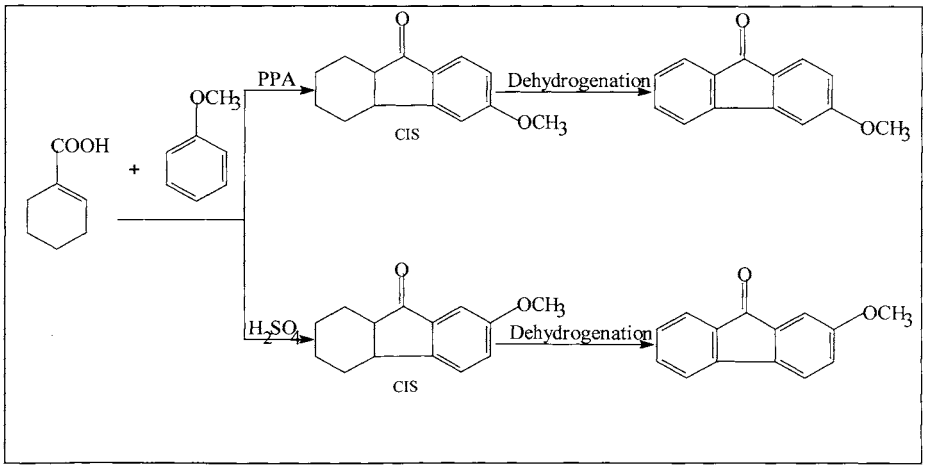


The application of domino processes has been used by us to provide accessibility to a multitude of other diverse classes of natural products such as fluorenones, alkaloids and diterpenes.

Our interest in the synthesis of fluoren-9-ones stemmed from their extensive biological activities as antiviral [21], antitumour [22] local anaesthetic [23] and trypanocidal [24] agents. Besides, some of their derivatives are now known to be natural products. It may be added that substituted hexafluorenones have attracted attention as intermediates for the synthesis of β-norditerpenoids, β-norsteroids [25], C-nor-D homosteroids [26] and the gibberellins [27]. These compounds after expansion of the B-ring, have the potential of being used for the synthesis of compounds with cis A/B ring junction such as the opium alkaloids and some bile acids. However, lack of methods of preparing these compounds have restricted their use in organic synthesis.

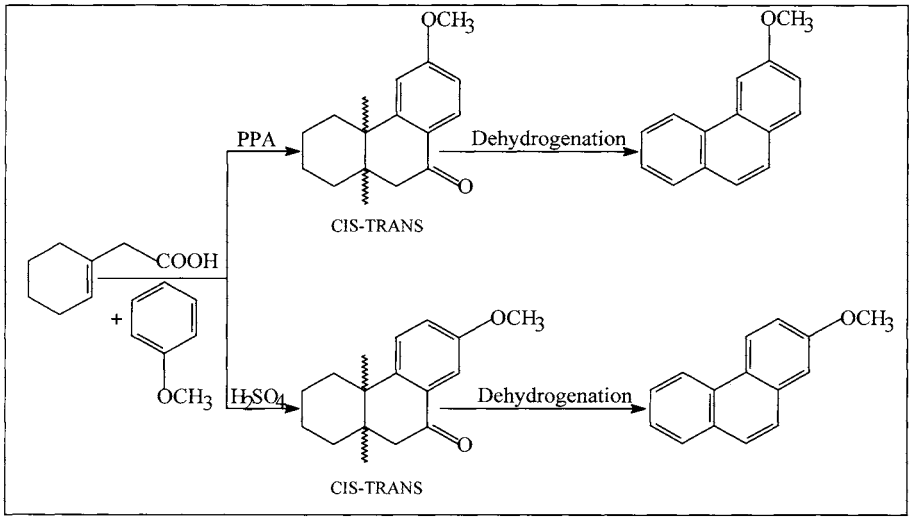
Prior to our work, fluorenones had been synthesized by methods characterized by the limited accessibility of the starting substrate [28,30,32,33] (substrate being fluorene, biphenyl-1-carboxylic acid, benzophenone, fluoranthene, cyclohexene, phenanthrene or phenylpropionic acid derivatives), a lack of stereoselectivity [28,29,31,33] and poor yields. We therefore developed a simple methodology for synthesizing fluoren-9-ones via domino acylation-cycloalkylation/alkylation-cycloacylation (**Chart-19**).

Chart-19: Formation of Fluorenones [31,34]

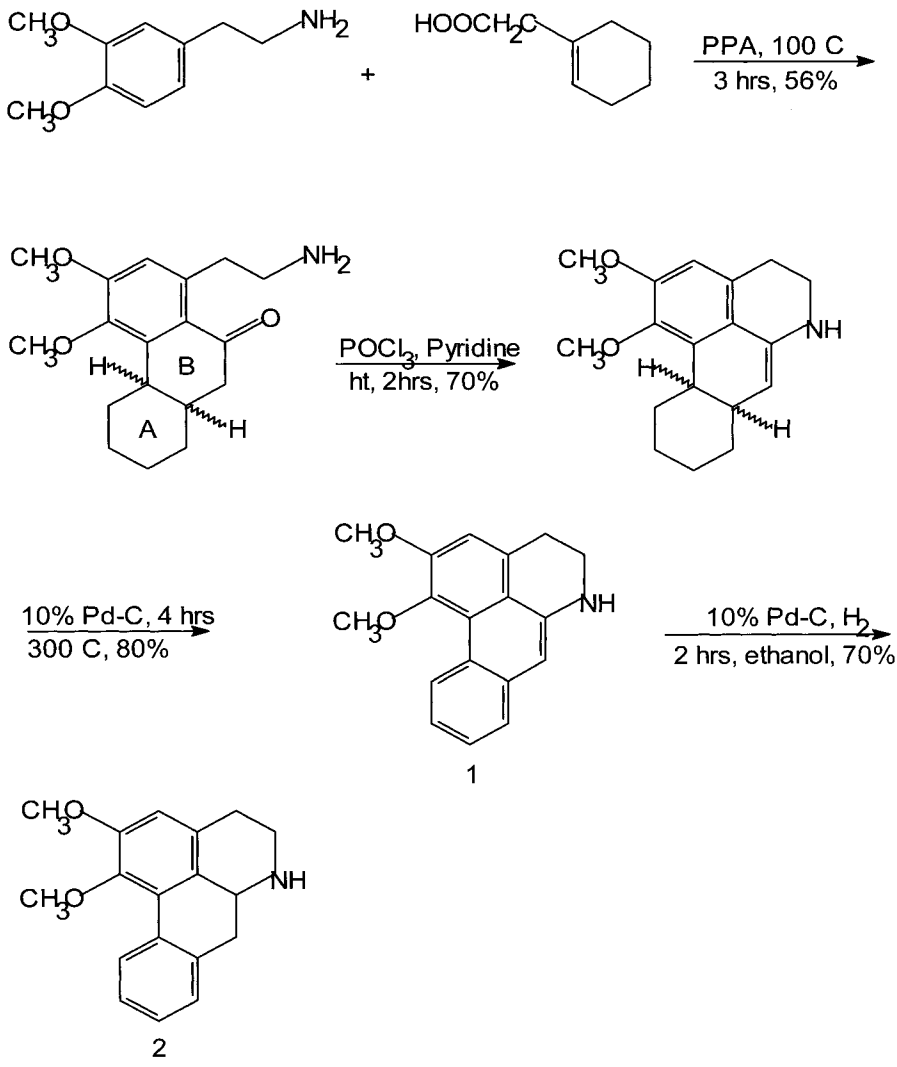


Encouraged by these results, we devised an analogous strategy for the synthesis of phenanthrenes (**Chart-20**) due to their significant existence as natural products [35], as also the presence in nature of phenanthrene skeleton in aporphinoid alkaloids [36], phenanthrene alkaloids [37], cassaine analogues [38] and diterpenes [39]. It may be noted that hexahydrophenanthren-9-ones have served as key intermediates in the synthesis of perhydrophenanthrenes [40], morphines [41], diterpenes [39] and D-homosteroids [42]. Phenanthrene derivatives are known to possess wide range of biological activity as antimalarial [43], anticancer [44] and emetic activity [45].

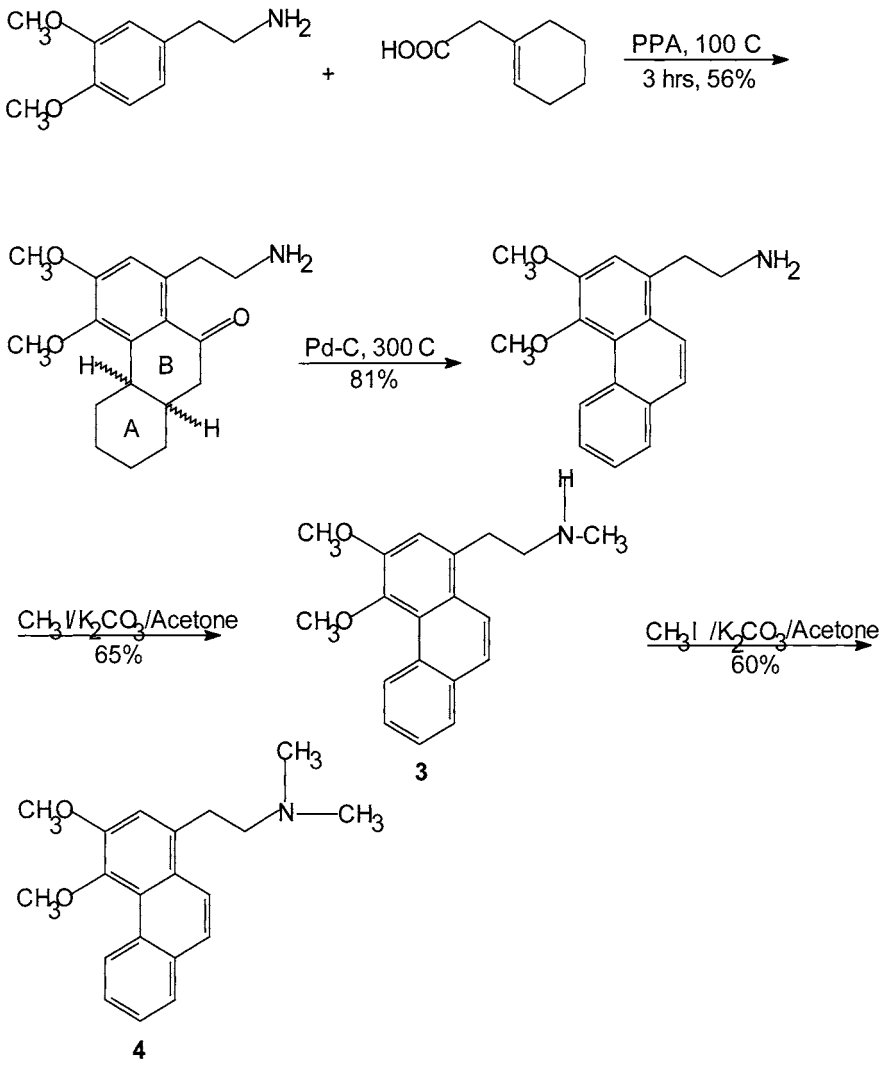
Chart-20: Formation of Phenanthrenones [46-49]



These domino reactions have also been employed by us for the total synthesis of pharmacologically important [50,51] aporphine alkaloids namely dehydronornuciferine (**1**) and (±) N-nornuciferine (**2**)(**Scheme 1**), and biologically active [52] phenanthrene alkaloids namely N-noratherosperminine (**3**) and atherosperminine (**4**)(**Scheme 2**). Incidentally, phenanthrene alkaloids are related to the aporphines, from which they can be obtained by degradative procedures [37].

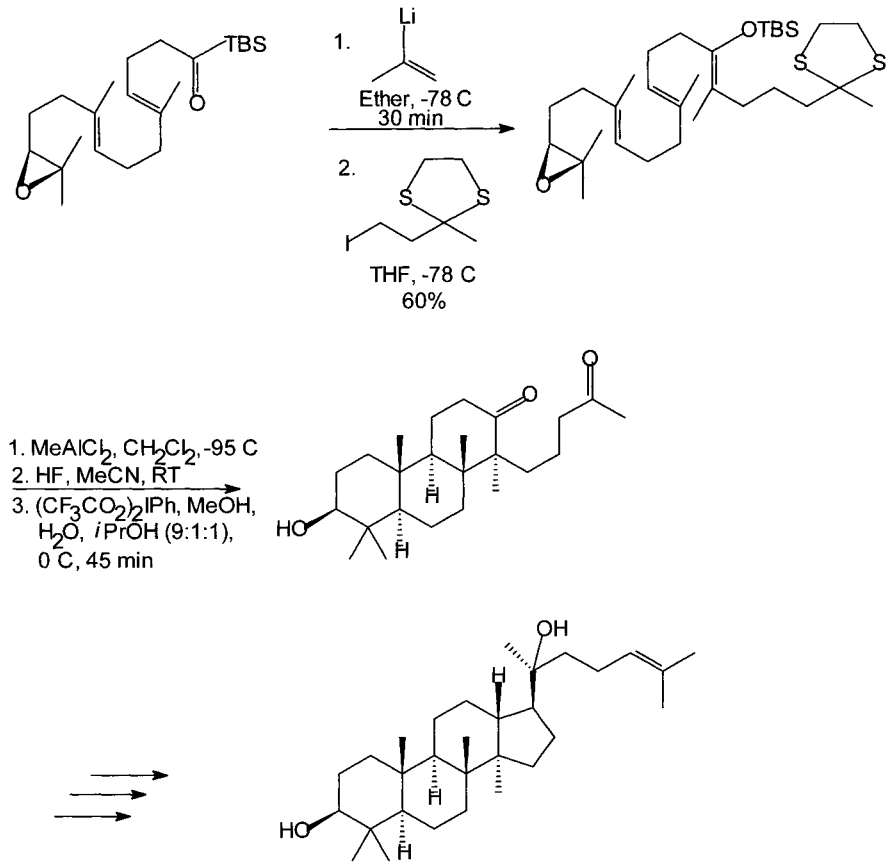


Scheme 1. Total synthesis of dehydronornuciferine (**1**) and (±) N-nornuciferine (**2**) via domino acylation-cycloalkylation.



Scheme 2. Total synthesis of N-noratherosperminine (**3**) and atherosperminine (**4**) via domino acylation-cycloalkylation.

The biomimetic cationic domino cyclization of an acyclic unsaturated substrate to give the tetracyclic scaffold of triterpenes and steroids is intensively described in the literature [53]. The concept has recently been used by Corey and co-workers to prepare enantiopure (+)-dammarenediol II in an exceptionally short way [54] (**Scheme 3**).



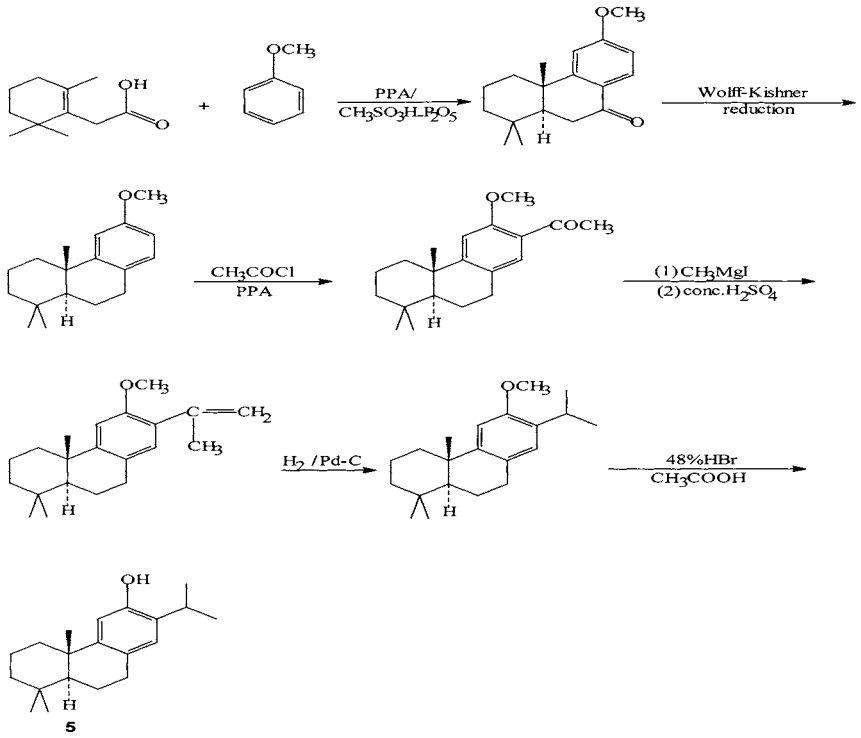
Scheme 3. Cationic domino polycyclization to the triterpene dammarenediol II

However, domino cyclization reactions had not been reported for diterpene synthesis.

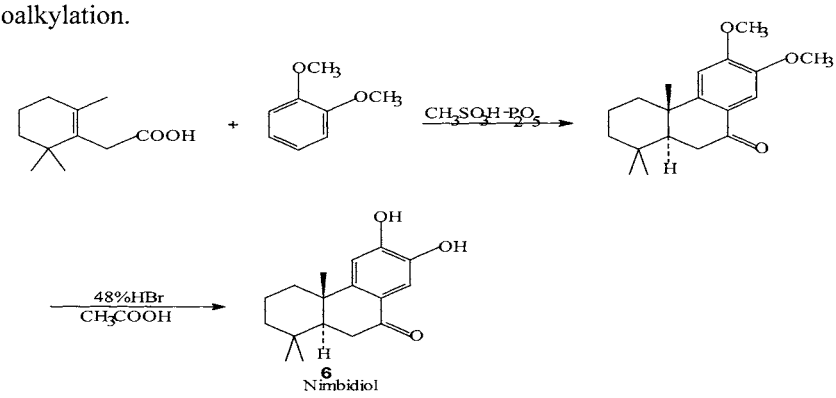
The diterpenes are C_{20} compounds biogenetically derived from geranylgeranyl pyrophosphate. The notable features of diterpene structures is the fascinating variation encountered in their skeletons and the occurrence in nature of both normal and antipodal stereochemical series.

Our work on fluorenones and alkaloids was extended to design convenient, expeditious and stereocontrolled total synthesis of several naturally occuring diterpenoids [55] namely (±)-ferruginol (**5**), (±)-nimbidiol (**6**), (±)-nimbiol (**7**), (±)-totarol (**8**) and abietatriene (**9**) (**Schemes 4 - 8**) via a concerted mechanism of domino acylation-cycloalkylation/ alkylation-cycloacylation as the principal step to construct the basic carbocyclic framework required for the trans-fused

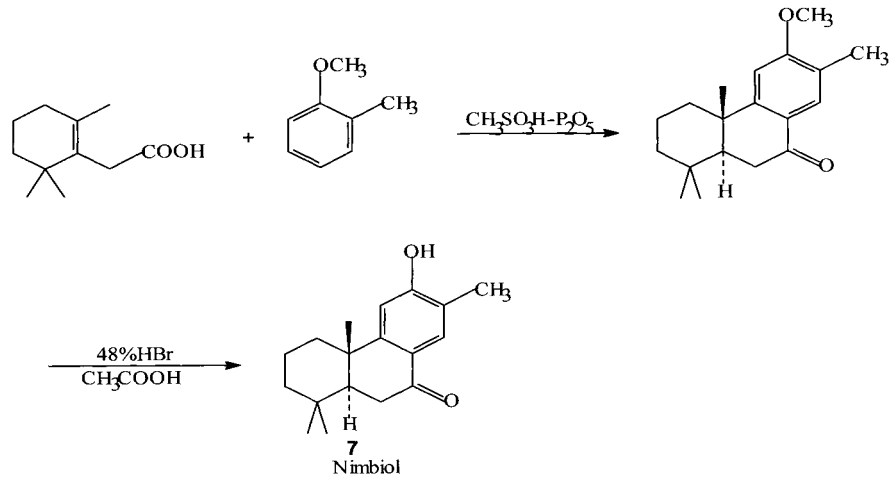
trimethyloctahydrophenanthrene nucleus starting from the readily available cyclic monoterpene citral, thus increasing efficiency over previous reported methods for total syntheses of diterpenes [56-62].



Scheme 4. Total synthesis of (±)-ferruginol (**5**) via domino acylation-cycloalkylation.

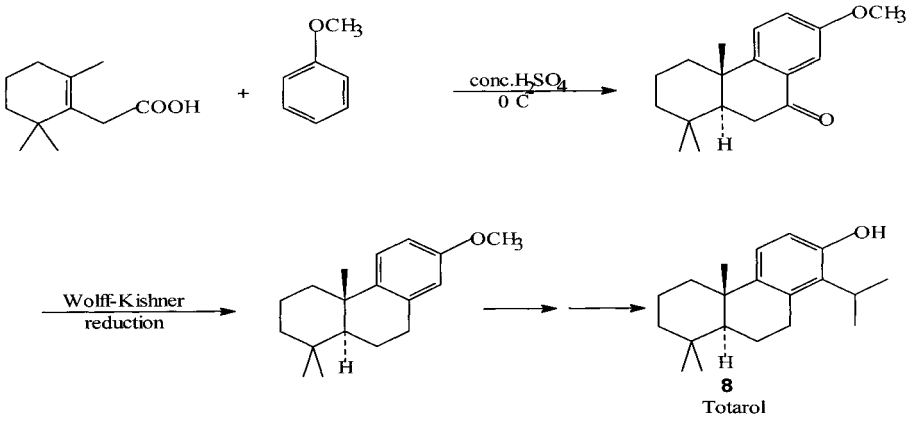


Scheme 5. Total synthesis of (±)-nimbidiol (**6**) via domino acylation-cycloalkylation.



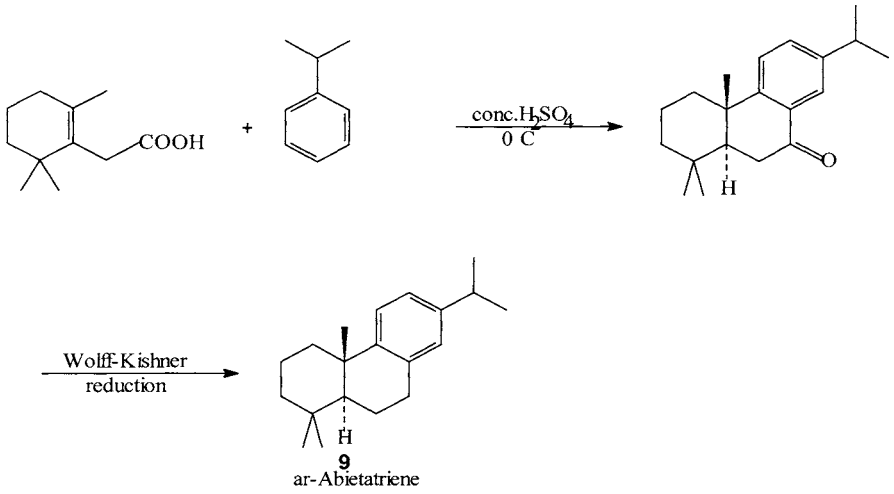
7
Nimbiol

Scheme 6. Total synthesis of (±)-nimbiol (**7**) via domino acylation-cycloalkylation.



8
Totarol

Scheme 7. Total synthesis of (±)-totarol (**8**) via domino alkylation-cycloacylation.



9
ar-Abietatriene

Scheme 8. Total synthesis of ar-abietatriene (**9**) via domino alkylation-cycloacylation.

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NATURAL PRODUCTS AS ANTIBACTERIAL AGENTS

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ABSTRACT: For thousands of years medicinal plants have played a significant role in the treatment of a wide range of medical conditions, including infectious diseases. Some naturally occurring chemical compounds serve as models for a large percentage clinically proven drugs, and many are now being re-assessed as antimicrobial agents. The primary reason for this renaissance is the fact that infectious disease remains a significant cause of morbidity and mortality worldwide, accounting for approximately 50% of all deaths in tropical countries and as much as 20% of deaths in the Americas. Despite the significant progress made in microbiology and the control of microorganisms, sporadic incidents of epidemics due to drug resistant microorganisms and previously unknown disease-causing microbes pose an enormous threat to global public health. These negative health trends call for a global initiative for the development of new strategies for the prevention and treatment of infectious disease, including natural products. Literally thousands of plant species have been tested against hundreds of bacterial strains in vitro, and many medicinal plants are active against a wide range of gram-positive and gram-negative bacteria. However, very few of these medicinal plant extracts have been tested in animal or human studies to determine safety and efficacy. This review focuses on the medicinal plants and phytochemical for which there is significant published in vitro, in vivo and clinical data available. The examples provided in this review such as St. John's wort, tree tea oil, and green tea demonstrate that even commonly used plant extracts may offer prospective new treatment of bacterial infections, including multi-drug resistant bacteria. One interesting example is St. John's wort (*Hypericum perforatum*) and its active constituent hyperforin, both of which have significant activity against MRSA in microgram concentrations. This antibacterial discovery was based on the ethnomedical use of St. John's wort to treat skin infections and wounds. Review of the published data indicates that medicinal plants offer significant potential for the development of novel antibacterial therapies and adjunct treatments (i.e. MDR pump inhibitors). However, new investigations should employ modern methodology, including using nationally recognized protocols and standards for microbial testing, the generation of minimum inhibitory concentrations, as well as standardization of the quality of plant materials used for testing.

INTRODUCTION

Reports from the World Health Organization, show that infectious diseases are a significant cause of morbidity and mortality worldwide, accounting for approximately 50% of all deaths in tropical countries [1].

Infectious and parasitic diseases remain the primary cause of pediatric mortality in most developing countries, partly as a result of the HIV/AIDS epidemic [1]. Approximately 98% of the 10.5 million pediatric deaths in 2002 were children under the age of five years, who lived in developing countries [1]. Lower respiratory tract infections, such as pneumonia, as well as diarrheal diseases and malaria account for more than 45% of all deaths in these pediatric populations [1-4]. In the adult population of developing countries, approximately 25% of the disease burden is due to infectious diseases associated with HIV/AIDS. While it may not be surprising to see such statistics in developing countries, the infectious disease mortality rates are also increasing in industrial nations, such as the United States. Death from infectious disease in the U.S.A. ranked 5th in 1981, but by 1992 had moved up to third place, an increase of 58% [2]. If one includes the statistics for HIV/AIDS, it is estimated that infectious disease is the underlying cause of death in >20% of the deaths occurring in the Americas [1].

Factors that have contributed to the global burden of infectious disease include the HIV/AIDS pandemic and resultant secondary infections, as well as an increase in antibiotic resistance in both nosocomial and community acquired infections [2-4]. Furthermore, despite the progress made in the understanding of bacteria and the control of microorganisms, sporadic incidents of epidemics have emerged due to drug resistant microorganisms and hitherto unknown disease-causing microbes pose an enormous threat to public health.

ANITIBIOTIC RESISTANCE AND NATURAL ALTERNATIVES

Shortly after the introduction of penicillin, there was the emergence of penicillin-resistant strains of *Staphylococcus aureus*, a common bacterium that makes up part of normal human bacterial flora [1, 5]. Since then, resistant strains of gonorrhea, and dysentery causing *Shigella* and *Salmonella* have also become resistant [1]. From the initial case of resistant *Staphylococcus*, the problem of antimicrobial resistance has grown into a serious threat to public health with economic, social and medical implications that are global in scope and cross all environmental and ethnic boundaries [5]. Multi drug-resistant tuberculosis (MDR-TB) is no longer confined to any one country or to those co-infected with HIV, but has appeared in locations as diverse as Eastern Europe, Africa and Asia [1]. Penicillin-resistant pneumococci and resistant malaria are on the rise, disabling and killing millions of children and adults each year [6]. As much as 60% of hospital-acquired infections are caused by multidrug-resistant microbes and these infections, many of which are caused by vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA), are now no longer confined to hospital wards but are seen at the community level as well [7]. Although most antibiotics are still active, the rapid progression of resistance

suggests that many of these drugs may not be effective for much longer. Thus, research and development of new strategies and therapies for treating bacterial infections are urgently needed.

Recently, mainstream medicine has become more receptive to the research and development of antimicrobial agents from plants. This may be due to the fact that while pharmaceutical companies usually produce two or three new antibiotics on average per year, over the past twenty years the number of new antibiotics in the research and development pipeline has begun to decline.Thus, pharmaceutical companies have become interested in the potential use of plant-based medicines as antimicrobial agents [5]. In addition, the general public has become more aware of the overuse and misuse of antibiotics. Consumers are very interested in natural medicines, including medicinal plants (herbal medicines) because they are perceived as being both safe and effective [8, 9]. Furthermore, it has been estimated that between 20-80% of the populations many developing countries use natural products almost exclusively, and consider them to be a normal part of primary healthcare [10].

NATURAL PRODUCTS WITH ANTIBACTERIAL ACTIVITIES

Plants have always played a central role in traditional systems of medicine for the prevention and treatment of disease worldwide [10, 11]. Although an in-depth history of plant medicines, for both human and veterinary purposes is beyond the scope of this review, it is important to understand that for thousands of years the traditional medicine in all countries exclusively employed naturally occurring plant medicines. For example, Hippocrates (5th century B.C.) mentions approximately 300 to 400 medicinal plants in his medical writings; Dioscorides (1st century A.D.) wrote *De Materia Medica*, a medicinal plant treatise that outlined the medical use of numerous plant species; and many hundreds of plant remedies are described in the Papyrus of Ebers, discovered in Egypt, which was written about 1550 BC [5, 11]. Furthermore, traditional Chinese medicine uses multiple plant prescriptions and has served the health needs of the Chinese population for over 5000 years [12, 13]. The Bible also described over 30 plant species, including frankincense and myrrh, which are reported to have antiseptic and healing properties [5].

There are literally thousands of published scientific papers from around the globe describing the antimicrobial activities of natural products [14-17]. A search of the PubMed database (data from 1975 to 2005, available on the Internet) produced approximately 1360 reports in the scientific and medical literature that described the antimicrobial activities of various plant species and their chemical constituents. Furthermore, a search of the Napralert database, the world's largest natural products database housed within the University of Illinois at

Chicago, shows that of the 58,850 plant species listed in the database, 6,550 species had published experimental antimicrobial activity, of which almost 4000 species had ethnomedical data supporting the use of these plants to treat infectious disease [14, 18]. The majority of the plants had activity against a range of bacteria, fungi or *Mycobacterium*.

While many natural products have been tested against hundreds of different strains of bacteria, the most common bacteria used in susceptibility tests include: *Bacillus cereus, Bacillus subtillis, Chlamydia pneumonia, Enterococcus faecalis, Escherichia coli, Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae, Klebsiella pneumoniae,* vancomycin-resistant *Enterococcus* (VRE), *Pseudomonas aeruginosa* and *Helicobacter pylori* [18, 19]. As the amount of published data describing the in vitro, in vivo and clinical antibacterial activities of natural products is so vast it could easily fill a book (or two), this review focuses only on natural products for which there is in vitro, in vivo and some clinical antibacterial data, as well as a plausible mechanism of action.

ANTIBACTERIAL ACTIVITIES OF NATURAL PRODUCTS

Staphylococcus aureus and *Staphylococcus epidermidis* are Gram-positive cocci that normally colonize the epithelial surfaces of humans [20, 21]. *S. epidermidis* is considered part of the normal human microbial flora, while *S. aureus* is usually regarded as a transient member. Colonization by either species usually does not lead to adverse events, unless the bacterium or their extracellular products penetrate the epithelial layer, which may cause serious infections such as necrotizing pneumonia, and toxic shock syndrome [22].

Literature searches of the scientific data reveals over 2500 plant species that are reported to have activity against *S. aureus,* and 13 plant species, reported be active against *S. epidermidis in vitro* [14]. In addition, there is growing evidence that specific plant species and their extracts may be of benefit for the treatment of methicillin-resistant *S. aureus* (MRSA) [23-37]. For example, in 2005 there were a number of reports of naturally occurring compounds from various *Garcinia* species that had very strong activity against MRSA [35-38]. For example, the crude methanol extract of the twigs of *Garcinia nigrolineata,* along with xanthones isolated from the twigs and the stem bark had antibacterial activity against MRSA [35]. Nigrolineaxanthone F, latisxanthone D, and brasilixanthone, Fig. **1**, showed significant activity, with a minimum inhibitory concentration of 2 μg/ml [35-36]. Ethanol extracts of bitter kola (*Garcinia kola*) were tested against *Staphylococcus aureus* [35]. The minimum inhibitory concentration (MIC) of extracts ranged from 0.08 μg/ml to 1.8 mg/ml for bitter kola, while the minimum bactericidal concentration (MBC) ranged from 0.135 mg/ml to 4.2 mg/ml [45]. Thus, the extracts were strongly active against *S. aureus*. The active

antibacterial constituents of *Garcinia kola* are reported to be four 3-8 linked biflavanoids, kolaflavanone, GB-1, GB-1a and GB-2, Fig. **2** [38].

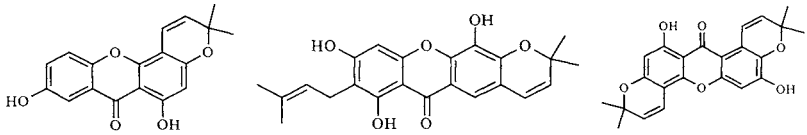


Figure 1. Structures of xanthones from *Garcinia nigrolineata* with activity against MRSA

A 50% ethanol extract of the dried fruits of *Terminalia chebula* Retz. inhibited the growth of MRSA, with a minimum inhibitory concentration (MIC) of 31.3 mg/ml [32]. This plant is native to many countries of Southeast Asia and has been used in traditional medicine to treat upper respiratory tract infections. The primary constituents of the fruit are hydrolysable tannins and components thereof, including chebulagic acid, chebulinic acid, corilagin, gallic acid, punicalagin, terchebulin, and terminalic acid [33]. Another plant, *Melaleuca alternifolia*, commonly known as tea tree is also known for its traditional use as an antimicrobial agent. In one study, 66 isolates of *Staphylococcus aureus* were

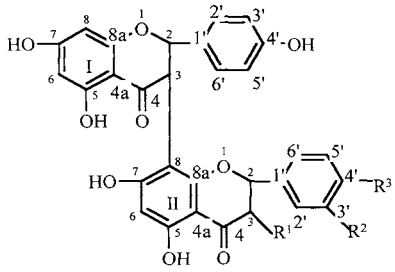


Figure 2. The structures of kolaflavanone, GB-1, GB-1a and GB-2 biflavanoids from *Garcinia kola*.

	R^1	R^2	R^3
GB-1	OH	H	OH
GB-2	OH	OH	OH
GB-1a	H	H	OH
Kolaflavanone	OH	OH	OMe

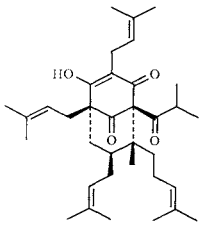
susceptible to tea tree oil in both the disc diffusion and modified broth microdilution assays [23, 24]. Of the isolates tested, 64 were MRSA and 33 were mupirocin-resistant. The MIC and the minimum bactericidal concentrations (MBC) were 0.25% and 0.50%, respectively, and suggest that tea tree oil may be useful in the treatment of MRSA infections [23, 24]. Some of the naturally occurring compounds in the oil, including 1, 8-cineol (4.5-16.5%), terpinen-4-ol (29-45%), γ-terpinene (10-28% and α-terpineol (2.7-13.0%), were also shown to reduce the growth of *S. aureus* in vitro. The bacteria treated with the tea tree oil or its active constituents appeared to have an altered morphology, as seen by electron microscopy, and suggested that the oil and its components compromise the cytoplasmic membrane [23, 24]. Interestingly, there was no development of bacterial resistance to the oil when used at a concentration of 2.5% (v/v) [31]. Thus, tea tree oil appears to be a viable

alternative as a topical agent to eradicate MRSA colonization, but resistance may eventually occur [31]. Only one published clinical trial compared the use of a combination of 4% tea tree oil (TTO) nasal ointment and 5% TTO body wash (intervention) with a standard 2% mupirocin nasal ointment and triclosan body wash (routine) for eradication of MRSA [19]. A total of 30 in-patients infected, or colonized with MRSA, were recruited and randomly assigned for treatment with TTO or standard routine care for a minimum of 3 days. Infected patients also received intravenous vancomycin and all participants were screened for MRSA carriage at 48 and 96 h after the cessation of topical treatment. Only 18 patients completed the trial. More patients in the intervention than in the control group cleared infection (5/8 versus 2/10). Two patients in the intervention group received 34 days treatment and one cleared the infection while the other remained chronically colonized. The inter-group differences were not statistically significant. This trial was too small to generate conclusive results [19].

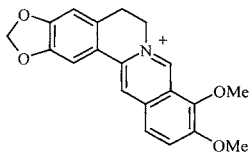
Another common plant that appears to have some activity against MRSA is *Hypericum perforatum* L., commonly known as St. John's wort. In traditional folk medicine, oily extracts of St. John's wort are used for topical treatment of wounds, burns and myalgia [39]. The lipophilic phloroglucin-derivative hyperforin, Fig. **3**, has antibacterial effects and has been shown to inhibit the growth of MRSA at a concentration of 1 μg/ml [39]. Thus, investigating known plants based on their historical medical (ethnomedical) use is a potentially useful means of discovering new alternative antimicrobial agents.

The antibacterial activity of berberine, Fig. **4**, a naturally occurring isoquinoline alkaloid isolated from a number of plants including *Coptis chinensis,*

Figures 3 and 4. Structures of hyperforin from *Hypericum perforatum* L. and berberine from *Berberis vulgaris*



Hyperforin



Berberine

Berberis vulgaris, and *Hydrastis canadensis*, is also active against *Staphylococcus aureus. in vitro.* Berberine inhibited the growth of *S. aureus*, with an MIC of 25.0 μg/ml [40]. Sub-inhibitory concentrations of berberine were potentiated by the flavones chrysosplenol-D and chrysoplenetin, Fig. **5**, from *Artemisia annua*. This potentiation appears to be due to the inhibition of an *S. aureus* multidrug resistance (MDR) pump [41].

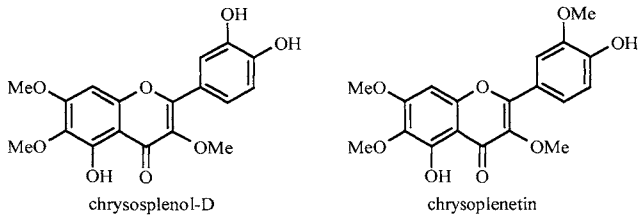


Figure 5. The structures of the flavones chrysosplenol-D and chrysoplenetin, isolated from *Artemisia annua* that enhance the antibacterial activity of berberine by inhibiting the MDR pump.

The 8-alkyl-, 8-phenyl-, 12- bromo-, 8-alkyl-12-bromo-, and 12-bromo-8-phenyl-berberine derivatives were also tested for their antimicrobial activity in vitro to evaluate structure-activity relationships. Introduction of the alkyl or phenyl group and the bromine atom into the C-8 and C-12 positions of berberine, respectively, led to significant increases of the antimicrobial activity. In both the 8-alkyl- and 8-alkyl-12-bromo-berberines, the antibacterial activity increased as the length of the aliphatic chain increased. Among the compounds tested, 12- bromo-8-*n*-hexyl-berberine (12-BHB) was 64 times more active against *S. aureus,* respectively, in comparison with berberine. 12-BHB was also found to be more active against *S. aureus* than kanamycin sulfate [42]. Interestingly, in a recent publication, the inhibitory activity of a berberine containing extract from *Coptis chinensis* rhizome was evaluated against sortase, a bacterial surface protein-anchoring transpeptidase, from *Staphylococcus aureus* ATCC 6538p and compared to that of four commercially available isoquinoline alkaloids. The biologically active constituent of *C. chinensis* extract was determined to be the isoquinoline alkaloid, berberine. Berberine was a potent inhibitor of sortase, with a median inhibitory concentration of 8.7 mg/ml [43]. These results suggest that berberine may be a possible candidate the development of a bacterial sortase inhibitor.

Of the Gram-negative bacilli, *Escherichia coli* (*E. coli*) is by far the most intensively investigated and perhaps the best understood of all bacteria [44]. The bacterium was first described in 1885 by Theodor Escherich, a German pediatrician, who discovered *E. coli* in the intestinal microflora of healthy individuals, as well as it's potential to cause disease when directly inoculated into extra-intestinal sites [44, 45]. The most common symptom of gastrointestinal diseases caused by *E. coli* is diarrhea [46]. Diarrheal diseases are a significant cause of morbidity and mortality in most developing countries, particularly in children under the age of five years [1, 14]. Thus, it is not unexpected that natural products have been used for thousands of years in traditional systems of medicine to treat a wide range of gastrointestinal ailments,

including nausea, vomiting and diarrhea caused by *E. coli*. According to searches of the Napralert database, over 1800 species of plants have been tested in vitro for their antibacterial activity against various strains of *E. coli* [14]. Examples of medicinal plants with activity against *E. coli* include common food and spice plants. The essential oils extracted from anise (*Pimpinella anisum*), angelica (*Angelica sinensis*), basil (*Ocimum basilicum*), carrot (*Daucus carota*), celery (*Apium graveolens*), cardamom (*Elettaria cardamomum*), coriander (*Coriandrum sativum*), dill weed (*Anethum graveolens*), fennel (*Foeniculum vulgare*), oregano (*Origanum vulgare*), parsley (*Petroselinum crispum*), and rosemary (*Rosmarinus officinalis*) all had activity against *E. coli in vitro* [47]. Inhibition of the growth of E. coli O:157:H7 ranged from complete with oregano oil to no inhibition with carrot oil. Oregano essential oil showed the greatest inhibition (zone, > or = 70 to 80 mm) (MIC, approximately 8 ppm). Coriander and basil were also highly inhibitory (MIC, approximately 25 to 50 ppm) [47]. These data suggest that even common food and spice plants may be of benefit to control *E coli*-induced diarrhea.

Probably one of the most well known natural products used to treat diarrheal diseases is berberine, Fig. **4** [48-54]. Intra-luminal perfusion with *E. coli* heat-stable enterotoxin (ET) reversed water and electrolyte movements from net absorption to net secretion in porcine jejunal segments [48]. The addition of berberine hydrochloride (3.2 X 10-5 M) to the perfusate reduced the jejunal secretory response of water, sodium, potassium, and chloride to ST and enhanced water and electrolyte absorption in control segments [49]. At lower concentrations (0.1 μM), berberine reduced the secretory response in ET-exposed segments, but only the decrease of sodium flux was significant [48]. In the presence of berberine, the mucosal enzyme activities of adenosine triphosphatase and disaccharidases were not significantly different between control and ET-exposed segments. Doses of 1, 2, 3, 4, 5, and 10 mg of berberine were injected into ligated loops of proximal part of the jejunum with 1 ml of ST filtrate. At doses of 2.0 or more mg/loop, berberine was effective in reducing water and electrolyte secretions induced by ET; the effect was dose-dependent [49]. Berberine has also demonstrated clinical efficacy for the treatment of secretory diarrheal diseases [50-55]. However, the quality of the trials has suffered from a lack of positive controls. Few studies have compared the efficacy of berberine with tetracycline, for the treatment of fluid loss caused by diarrhea in patients with cholera or in non-cholera diarrhea [50-52, 56]. A randomized comparison-controlled trial involving 165 patients assessed the antisecretory activity of berberine sulfate for enterotoxigenic Escherichia coli-induced (ETEC) diarrhea [52]. Patients were treated with either 400.0 mg of berberine as a single oral dose or 1200.0 mg of berberine sulfate (400.0 mg every 8 hours) for the treatment of diarrhea. In patients with ETEC diarrhea who received a

single oral dose of berberine, the mean stool volumes were significantly ($P < 0.05$) reduced less than those of controls during three consecutive 8-hour periods after treatment. At 24 hours after treatment, patients with ETEC, who were treated with berberine, had a reduced stool frequency than controls (42% versus 20%, $P < 0.05$). No adverse effects were observed in the patients receiving berberine. The results of this study suggest that berberine was an effective and safe antisecretory drug for ETEC diarrhea [52]. These data indicate that berberine is an effective antidiarrheal agent in *E coli* heat-stable enterotoxin mediated secretory diarrhea and provide a plausible mechanism for the use of berberine and berberine-containing plant-based medicines in the treatment of gastroenteritis and infectious diarrhea in developing countries [49].

Along with the bactericidal and bacteriostatic activities of medicinal plants, inhibition of the production of enterotoxins, such as verotoxin, may also be a means to control *E.coli*-induced diarrheal diseases. Verotoxin-producing *E. coli* (VTEC) are pathogenic bacteria associated with diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [57, 58]. Verotoxins (VTs) produced by these organisms produce cytopathic effects on a restricted number of cell types, including endothelial cells lining the microvasculature of the bowel and the kidney. First associated with hemorrhagic colitis and hemolytic uremic syndromes, VTEC have been shown to cause also uncomplicated watery or bloody diarrhea. Verotoxins or Shiga-like toxins are holotoxins composed of a single enzymatic A subunit of approximately 32 kDa in association with a pentamer of receptor-binding B subunit of 7.7 kDa [57, 59]. The term verotoxin originated from the cytotoxic effect on Vero cells (African green monkey kidney cells). The expression of the A- and B- subunit genes is individually regulated in that the production of verotoxin is negatively regulated at the transcriptional level by an iron-Fur protein co-repressor complex. Many factors may impact the production or release of the verotoxins, such as growth conditions, antibiotics and aeration [57]. Sakagami and co-workers [60] investigated the inhibitory effect of extracts from 21 plants collected in North and Central America on the formation of verotoxin by enterohemorrhagic *E. coli* O157:H7 (EHEC). Of the 21 methanol extracts tested, four plant species, *Limonium californicum* (Boiss.) A. Heller, *Cupressus lusitanica* Miller, *Salvia urica* Epling, and *Jusiaea peruviana* L., inhibited verotoxin formation in vitro at concentrations of 31.3-125 μg/ml. The inhibition of verotoxin formation was observed at concentrations well below the MIC (1 mg/ml). These data suggest that specific plant extracts may prevent the formation of verotoxin rather than have bactericidal effect on EHEC. Such findings suggest that the administration of active plant extracts may prevent VTEC-induced diarrhea by inhibiting or reducing the formation of verotoxin in the human intestines [60].

Another Gram-negative bacterium with gastrointestinal implications is *Helicobacter pylori* (HP). HP is a spiral or helical-shaped

aerobic bacillus that colonizes the gastric epithelial surface, and can withstand the stomach's environment by microaerophilic growth capability and high urease activity [61, 62]. The discovery of HP was one the most significant discoveries in the field of gastroenterology of the twentieth century [63]. HP-induced gastritis is now associated with duodenal ulcer disease, peptic ulcer disease, gastric carcinoma, primary gastric B cell lymphoma, ischemic heart disease and hyperemesis gravidarum [63, 64-68].

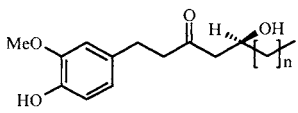
Current statistics indicate that as much as one-half of the world's population is infected with HP [1]. The infection occurs early in childhood via the feco-oral route, and transmission appears to be from person to person. HP infections are more prevalent in developing countries due to poor sanitation, over-crowded living conditions and a lack of clean water supplies [63]. Gastritis, the most common symptom of HP infections, has one salient feature, an acute or chronic inflammation of the gastric mucosa [69]. Persistent superficial gastritis predisposes to duodenal ulcer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Atrophic gastritis predisposes patients to gastric ulcer and adenocarcinoma [69]. Infection with HP is now accepted as the primary cause of peptic ulcer disease (PUD) [63]. In the United States, four to five million people have peptic ulcer disease, and the economic consequences of the disease are responsible for approximately $3 to $4 billion in annual health care costs [70]. The situation in many developing countries is even more dramatic, as HP infections, peptic ulcer disease and gastric cancer are major causes of morbidity, mortality, and healthcare expenditures [71]. The prevalence of HP infection amoung adolescents in parts of the developing world exceeds 90% by 5 years of age. For example, in Ethiopia, HP infection is acquired early in life, with 60% of 4 year-olds being infected, and almost 100% of 12-year-olds [72]. In addition to HP-induced gastritis, peptic ulcer disease and gastric carcinoma, the infection in the developing world appears to also be linked with chronic diarrhea, malnutrition and growth faltering in children, as well as predisposition to other enteric infections, including typhoid fever and cholera [71]. Furthermore, once identified, the treatment of HP within the developing world presents increased difficulties due to the frequency of antibiotic resistance as well as the frequency of recurrence after successful treatment. A good example of this is seen in Peru, where the recurrence rate of the infection is as high as 73% even after successful eradication of the infection [73]. Thus, antibiotic resistance and high re-infection rates strongly argue for the development of new therapeutic modalities to prevent and treat HP infections in developing countries.

In most developing countries, natural products are commonly used to treat gastrointestinal ailments, including gastritis, peptic ulcer disease and diarrhea [74]. Thus, considering the strong association between these conditions and HP infections, it should not be surprising

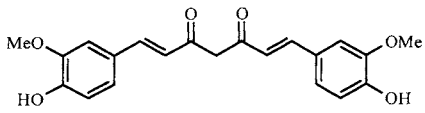
that some plant-based medicines would have activity against HP in vitro. The first investigation of the in vitro efficacy of plant extracts against HP was published in 1991 [75]. This group reported that extracts of 13 Malagasy medicinal plants were effective against a number of clinical strains of HP in vitro. In 1996, Fabry and co-workers [76] reported that a number of East African medicinal plant extracts had inhibitory effects on the growth of HP in vitro [76]. One plant, *Terminalia spinosa* was reported to be the most active, with an MIC range 62.5-500 mg/ml. Extracts of *Thymus vulgaris* (aqueous extract) and *Cinnamonum zeylanicum* (alcohol extract) were also reported to inhibit the growth of the bacterium at concentrations of 3.5 mg/ml [77].

In 1997, investigators discovered that common food plants such as garlic, soybean and fresh vegetables may be protective against HP infections [78-79]. An aqueous extract of garlic (*Allium sativum*) inhibited the growth of HP strains at concentrations of 40 μg/ml [78]. *Rabdosia trichocarpa*, a traditional remedy for gastric and stomachic complaints in Japan, was also inhibits the growth of HP in vitro [80]. A diterpene named trichorabdal A, isolated from the plant showed very strong in vitro antibacterial activity against HP, as well [80]. In 1999, the anti-HP effects of seven Turkish plant extracts, used in folk medicine for the treatment of gastric ailments including peptic ulcers, was reported [81]. Extracts of the flowers of *Cistus laurifolius*, cones of *Cedrus libani*, aerial parts of *Centaurea solstitialis* ssp. *solstitialis*, fruits of *Momordica charantia*, aerial parts of *Sambucus ebulus*, and flowering herbs of *Hypericum perforatum* were active with an MIC range of between 1.95 and 250 mg/ml [81].

In 2003, a screening of 20 plant extracts from Thailand used to treat gastrointestinal ailments reported that over 50% of the plant species tested had anti-HP activity [74]. Methanol extracts of *Myristica fragrans* (aril) inhibited the growth of all HP strains with an MIC of 12.5 μg/ml; extracts from *Barringtonia acutangula* (leaf) and *Kaempferia galanga* (rhizome) had an MIC of 25.0 μg/ml; *Cassia grandis* (leaf), *Cleome viscosa* (leaf), *Myristica fragrans* (leaf) and *Syzygium aromaticum* (leaf) had MICs of 50.0 μg/ml. Extracts with an MIC of 100.0 μg/ml included *Pouzolzia pentandra* (leaf), *Cycas siamensis* (leaf), *Litsea elliptica* (leaf) and *Melaleuca quinquenervia* (leaf) [74]. Ginger root (*Zingiber officinale*), a plant well known worldwide to treat gastrointestinal ailments also has activity against HP [82]. Methanol extracts of ginger rhizome inhibited the growth of 19 HP strains in vitro with a minimum inhibitory concentration range of 6.25-50 μg/ml. The 6-, 8-, 10-gingerols, Fig. **6**, all had varying degrees of activity, with an MIC range of 0.78 to 12.5 μg/ml and interestingly had significant activity against the *cagA*+ strains (cancer causing strains) [82].



6-gingerol (n= 4)
8-gingerol (n = 6)
10-gingerol (n = 8)

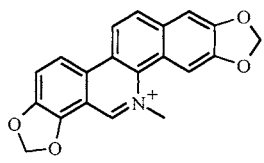


Curcumin

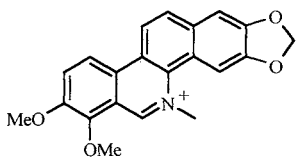
Figure 6. The structures of the gingerols from *Zingiber officinale* and curcumin from *Curcuma longa* L. with activity against *Helicobacter pylori*.

Curcumin, Fig. **6**, a polyphenolic constituent isolated from turmeric (*Curcuma longa*), and a methanol extract of the dried powdered turmeric rhizome were both active against 19 strains of HP, including five *CagA*+ strains. The MIC range was 6.25-50 μg/ml [83]. In addition, a red wine (*Vitis vinifera*) and resveratrol inhibited the growth of HP in vitro [84-85]. Resveratrol, a stilbene present in red wine had an MIC of 25 mμg/ml, while the red wine extract had an MIC range of 25-50 mg/ml [84]. Interestingly, resveratrol was more active against *Cag A*+ strains of HP than *Cag A*- strains [85].

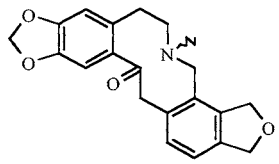
Two indigenous American plants, *Sanguinaria canadensis* and *Hydrastis canadensis*, used traditionally by the Native American Indians for the treatment of gastrointestinal ailments, are also active against HP [86]. Methanol extracts of the rhizome or suspension cell cultures of *S. canadensis* had an MIC range of 12.5-50.0 μg/ml. Three isoquinoline alkaloids were identified in the active fraction. Sanguinarine and chelerythrine, Fig. **7**, two benzophenanthridine alkaloids,



sanguinarine



chelerythrine



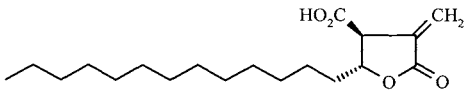
protopine

Figure 7. Isoquinoline alkaloids, sanguinarine, chelerythrine and protopine isolated from *Sangunaria canadensis* with activity against *H. pylori*.

inhibited the growth of HP, with an MIC of 50.0 and 100.0 μg/ml, respectively. Protopine, Fig. **7**, a protopine alkaloid, also inhibited the growth of the bacterium, with a MIC of 100 μg/ml. A crude methanol extract of *H. canadensis* rhizomes was very active, with an MIC of 12.5 μg/ml (range 0.78 to 25.0 μg/ml). Two isoquinoline alkaloids, berberine and β-hydrastine, were identified as the active constituents, and having an MIC of 12.5 and 100.0 μg/ml, respectively [86]. The *in vitro* susceptibility of 15 HP strains to natural products that had a history of traditional use in the treatment of GI disorders was assessed [87]. Methanol extracts of *Myristica fragrans* (seed) had a MIC of 12.5 μg/ml; *Zingiber officinale* (ginger rhizome/root) and *Rosmarinus officinalis* (rosemary leaf) had an MIC of 25 μg/ml. Methanol extracts of botanicals

with a MIC of 50 μg/ml included *Achillea millefolium* (aerial parts), *Foeniculum vulgare* (seed), *Passiflora incarnata* (aerial parts), *Origanum majorana* (herb) and a (1:1) combination of *Curcuma longa* (root) and ginger rhizome. Botanical extracts with a MIC of 100 μg/ml included *Carum carvi* (seed), *Elettaria cardamomum* (seed), *Gentiana lutea* (roots), *Juniper communis* (berry), *Lavandula angustifolia* (flowers), *Melissa officinalis* (leaves), *Mentha piperita* (leaves) and *Pimpinella anisum* (seed). Methanol extracts of *Matricaria recutita* (flowers) and *Ginkgo biloba* (leaves) had a MIC > 100 μg/ml [87]

Another traditional medicine from Iceland, a lichen (*Cetaria islandica*), is used to treat gastrointestinal ailments and has also been shown to have anti-HP activity [88]. Protolichesterinic acid, Fig. **8**, an aliphatic α-methylene-γ-lactone, was



protolichesterinic acid

Figure 8. Structure of protolichesterinic acid.

identified as one of the active constituents. The MIC range of protolichesterinic acid, in free as well as salt form, was 16 to 64 μg/ml. The antibacterial activity of Gosyuyu, a crude extract from the fruit of *Evodia rutaecarpa*, a Chinese herbal medicine, has also been tested for activity against HP *in vitro* [89]. Two compounds were identified as the active constituents and were the quinolone alkaloids, 1-methyl-2- [(Z)-8-tridecenyl]-4-(1H)-quinolone and 1-methyl-2-[(Z)-7- tridecenyl]-4-(1H)-quinolone. The minimum inhibitory concentration (MIC) of these compounds against reference strains and clinically isolated HP strains were <0.05 μg/ml, that was similar to the MIC of amoxicillin and clarithromycin [89]. Furthermore, tea (*Camellia sinensis*) and rosemary (*Rosmarinus officinalis* L.) extracts inhibit HP urease in vitro. Green tea extract (GTE) showed the strongest inhibition of HP urease, with an MIC of 13 μg/ml. Active principles were identified as catechins, with the hydroxyl group of 5(')-position appearing important for urease inhibition [90]. Finally, in a recent report, screening of 70 medicinal plants from Greece, led to the discovery of a number of plants with anti-HP activity. Extracts of *Anthemis melanolepis*, *Cerastium candidissimum, Chamomilla recutita, Conyza albida, Dittrichia viscosa, Origanum vulgare* and *Stachys alopecuros* were active against one standard strain and 15 clinical isolates of HP [91].

The in vivo model of choice for HP infections is the Mongolian gerbil model that was developed by a group of Japanese researchers. This model was a major advancement and has accelerated investigations of the anti-HP activities of natural products. A number of botanical extracts have been tested in this animal model and have shown significant activity. For example, treatment of HP-inoculated Mongolian

gerbils with green tea extract in drinking water at the concentrations of 500, 1000 and 2000 ppm for 6 weeks, suppressed gastritis and HP load in a dose-dependent manner [90]. The effect of a garlic extract on HP-induced gastritis in Mongolian gerbils was also reported [92]. Garlic extract was fed to animals in rations at doses of 1, 2 and 4% in the diet from four hours after HP inoculation until the end of the experiment, at week six. Administration of the garlic extract to the gerbils reduced HP-induced gastritis in a dose-dependent manner, and was significant at a dose of 4% of the diet. The numbers of hemorrhagic spots in the glandular stomach and the microscopic score for gastritis were significantly reduced from 19.2+/-15.6 and 5.9+/-0.8 in control gerbils to 8.1+/- 11.2 and 4.2+/-1.5, respectively, by 4% garlic extract treatment. However, HP bacterial load was not altered by the garlic extract treatment [92].

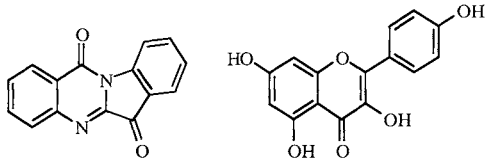


Figure 9. Tryptanthrin and kaempferol, isolated from *Polygonum tinctorium.*

The effect of tryptanthrin and kaempferol, Fig. **9**, two compounds isolated from *Polygonum tinctorium* were assessed in HP-infected Mongolian gerbils. Mongolian gerbils were inoculated with HP strain ATCC 43504, and after 4 weeks, the infected gerbils were treated with tryptanthrin and/or kaempferol, by gastric lavage, twice a day for 10 days. The results showed that administration of tryptanthrin and/or kaempferol significantly reduced bacterial load in the gerbils' stomachs [93].

In terms of human studies, three clinical trials were found that tested the efficacy of natural products for the eradication of HP. Two of the studies tested different preparations of garlic bulbs (*Allium sativum*), and one tested an extract of cinnamon [19].

In the first study, a group of 20 HP-infected patients suffering from dyspeptic complaints for more than two months were treated for two weeks with capsules containing an oil macerate of garlic (275 mg, three times a day). This treatment was compared with the combination of garlic in combination with omeprazole (20 mg, twice a day) for the eradication of HP [94]. All subjects underwent endoscopy before and one month after the end of treatment, and the presence of HP in biopsy specimens was confirmed by the urease test and by microscopy. Symptom scores and degree of gastritis, as judged by histological examination, were recorded pre- and post-treatment. Neither intervention resulted in the elimination of the organism, change in the severity of gastritis or a significant change in symptom scores (9.2 ± 1.55 versus 8.7

± 1.70 in the garlic oil group, 9.0 ± 1.49 versus 8.5 ± 1.51 in the garlic oil plus omeprazole group). The study was not randomized, probably not patient-blinded and the sample size was small [19].

In the second clinical trial, preparations of fresh garlic or capsaicin-containing peppers were tested for their ability to reduce the bacterial load and symptoms in a crossover trial involving 12 individuals infected with HP [95]. Test substances were included in morning, noon and evening, Mexican-style meals. Subjects participated in a minimum of 3 trial days (negative and positive controls and one experimental ingredient). During each test meal participants received one intervention: garlic (10 freshly sliced cloves), capsaicin (six freshly sliced jalapeno peppers), two tablets of bismuth subsalicylate (Pepto-Bismol, positive control) or no additions (negative control). The urea breath test was performed before the first meal of the day, before the evening meal and the morning after each test day. The results were used to evaluate the effectiveness of the therapies. Ten subjects received garlic, six received jalapenos and 11 subjects received bismuth. Neither garlic nor jalapenos had any effect on urease levels (median urease activity pre- and post-garlic 28.5 versus 39.8 and jalapenos 43.7 versus 46.6; $P > 0.8$) but there was a marked reduction after ingestion of bismuth (55.8 versus 14.3; $P < 0.001$). Two patients experienced nausea and diarrhea graded as severe after eating the jalapenos and 70% of those eating garlic complained of taste disturbance and body odor. This study had a number of flaws including a lack of randomization and small sample size [19, 96].

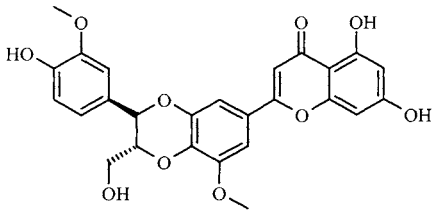
Finally, the effects of treatment with an extract of cinnamon (*Cinnamonum cassia*) were assessed in 23 patients who were undergoing gastroscopy and had a positive urea breath test for HP [19, 97]. Thirty-two patients were randomly allocated in a 2:1 ratio to the study and control groups but only 23 were included in the final analysis. Fifteen patients received 80 mg/day of the cinnamon preparation and eight patients received placebo for four weeks. Breath tests were repeated at the end of the trial period. There were some increased and some decreased urea breath test values in both groups, but overall mean values (pre- and post-cinnamon treatment 22.1 versus 24.4, placebo 23.9 versus 25.9) showed no significant changes [19, 97].

NATURAL PRODUCTS AS MULTIDRUG RESISTANCE PUMP (MDR) INHIBITORS

Bacterial resistance to antibiotics continues to be a significant problem, as microorganisms appear able to develop resistance to new drugs as rapidly as they are introduced. One of the primary means by which microbes become resistant is through the development or enhancement of methods for the extrusion of antibiotics out of the cell via multi-drug resistance (MDR) pumps [98]. The function of microbial MDRs remains a hotly debated subject given the very broad substrate specificities of

some MDRs. For example, resistance-nodulation-cell division-type (RND) pumps can extrude all classes of amphipathic compounds (cationic, neutral, and anionic), thus making it difficult to develop a strategy to develop possible inhibitor substrates for MDRs. However, it is well known that amphipathic cations appear to be the preferred substrates of MDRs. These substances are extruded by MDRs of all five known families [99]. Unlike other substances, amphipathic cations accumulate in the cell driven by the membrane potential, however these compounds are also highly hydrated and do not permeate the membrane as readily as neutral compounds, making it feasible to design an MDR inhibitor. Interestingly, Gram-negative bacteria have an outer membrane barrier that slows the penetration of virtually all amphipathic molecules, and transenvelope MDRs of the RND type pump and EmrAB-type extrude their substrates across this outer membrane barrier [99]. The presence of MDR sensors that regulate the expression of specific MDR pumps suggests that defense against external toxins is the function of MDRs pumps [99].

Recently, it has been demonstrated that medicinal plants contain MDR pump inhibitors that actually enhance the activity of their own natural antimicrobial compounds. Plant amphipathic cations, such as berberine, have been shown to be good MDR substrates. Recent research has shown that in addition to berberine, *Berberis* species produce 5'-methoxyhydnocarpin-D, Fig. **10**, an MDR inhibitor that enhances the action of this compound. In addition, isoflavones isolated from *Lupinus argenteus* were found to enhance the antibacterial activity of a-linolenic acid, also found in the same plant [99].



5'-methoxyhydnocarpin-D

Figure 10. The structure of 5'-methoxyhydnocarpin-D, an MDR inhibitor isolated from Berberis species that enhances the antibacterial activity of berberine.

A new method of bioactivity-directed fractionation, based on multidrug resistant pump (MDR) inhibition in *Staphylococcus aureus*, was reported for medicinal plants. This work resulted in the isolation, from berberine-containing *Berberis* species, two compounds that are themselves devoid of antibacterial activity, but that form potent synergistic couples with a sub-inhibitory concentration of berberine. The bacterial MDR pump inhibitors were identified as the flavonolignan 2 and the porphyrin 3 [98]. The isoflavones not only enhanced the antibacterial activity of the natural product, berberine, Fig. **4**, but also the activity of synthetic

fluoroquinoline antibiotic norfloxacin [98]. In another study by Stermitz and co-workers [100], bioassay-guided fractionation of an extract of *Artemisia annua* L. (Asteraceae) was conducted in order to assess the possible presence in the plant material of inhibitors of bacterial MDR pumps. Fractions were tested for *Staphylococcus aureus* growth inhibition in the presence of a sub-inhibitory concentration of berberine. The active fractions yielded the flavones chrysosplenol- D and chrysoplenetin Fig. **5**, which themselves had very weak growth inhibitory action, but when combined with berberine were very active. In comparison with work on other flavonols, it is likely that potentiation is due to the inhibition of an *S. aureus* MDR pump. These same two flavonols were earlier reported to potentiate the activity of artemisinin against *Plasmodium falciparum* [100].

ABBREVIATIONS

12-BHB	=	12-bromo-8-*n*-hexyl berberine
AIDS	=	Acquired immune deficiency virus
EC	=	*Escherichia coli*
ET	=	enterotoxin
ETEC	=	enterotoxigenic *Escherichia coli*
GTE	=	green tea extract
HP	=	*Helicobacter pylori*
HIV	=	human immunodeficiency virus
MDR	=	multidrug resistant
MDR-TB	=	multidrug resistant tuberculosis
MIC	=	minimum inhibitory concentration
MRSA	=	multidrug resistant *Staphylococcus aureus*
RND	=	resistance-nodulation-cell-division-type
TTO	=	tea tree oil
VRE	=	Vancomycin resistant *Enterococcus*
VT	=	verotoxin
VTEC	=	verotoxin-producing *E. coli*

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ANTI-INFECTIOUS ACTIVITY IN THE ANTHEMIDEAE TRIBE

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ABSTRACT: Infectious diseases account for one third of all deaths worldwide. The spread of multidrug-resistant strains of microbes makes it necessary to discover new classes of antimicrobials and compounds that inhibit these resistance mechanisms. In the past few decades, the search for new anti-infective agents has occupied many research groups in the field of ethnopharmacology. Many focus on determining the antimicrobial activity of plant extracts found in folk medicine, essential oils or isolated compounds. The Anthemideae tribe of the Asteraceae comprises a large number of species that have been and are still used as medicinal plants, particularly in folk medicine. In the present review, we analyze the past, present and future of medicinal plants of the Anthemideae tribe, both as potential antimicrobial crude drugs as well as a source for natural compounds that act as new anti-infectious agents.

INTRODUCTION

In spite of the substantial advances that have been made in synthetic organic chemistry, plant products still remain an integral part of modern therapeutics, with substances derived from higher plants comprising approximately 25% of prescribed medicines. Indeed, in many areas of the world, natural drugs are of primary importance. Infectious diseases account for one third of all deaths worldwide. Although antimicrobial agents have played important roles in modern medicine and are well developed, antibiotic resistance of microorganisms has increased rapidly shortening the useful lifetimes of currently available agents. The spread of multidrug-resistant strains of microbes makes it necessary to discover new classes of antimicrobials and compounds that inhibit these resistance mechanisms. At present, no single chemical entity plant-derived antibacterials are used clinically, and this chemically diverse group deserves consideration. In the past few decades, the search for new anti-infectious agents has occupied many research groups in the field of

ethnopharmacology [1,2]. Many focus on determining the antimicrobial activity of plant extracts found in folk medicine. The use of higher plants and their preparations to treat infections is an age-old practice and in times past possibly the only method available. Today, interest in plants with antimicrobial properties has revived. First, plants have exceptional ability to produce cytotoxic agents, and second, there is an ecological rationale that natural antimicrobial products should be presented or synthesised *de novo* in plants following microbial attack to protect the producer from pathogenic microbes in its environment.

The Anthemideae tribe of the Asteraceae comprises a large number of species that have been and are still used as medicinal plants, particularly in folk medicine. Most of them are used empirically by rural and indigenous populations, commonly as infusions or decoctions, for medicinal purposes. Due to the increasing interest in natural products, the urban use of some folk medicinal plants as herbal remedies is also increasing. In the present chapter, we analyze the past, present (1985-2005) and future of medicinal plants of the Anthemideae tribe, both as potential antimicrobial crude drugs, as well as a source for natural compounds. Some of these compounds were isolated after previously detecting antimicrobial activity on the part of the plant.

CRUDE EXTRACTS

A review of the literature concerning the evaluation of plant extracts of the Anthemideae tribe reveals that many studies into their bacterial, antifungal and antiviral activities have been carried out in recent years. These reports concern mainly the genus *Baccharis* and *Helichrysum*.

Nostro *et al.* [3] studied the antibacterial activity of *Helichrysum italicum* (Roth) Don (ethanolic extract) against oral streptococci (*Streptococcus mutans*, *Streptococcus salivarius* and *Streptococcus sanguis*) and its influence on cell-surface hydrophobicity, *in vitro* sucrose-dependent adherence to glass surface and cellular aggregation of *Streptococcus mutans*. The results indicate that all streptococci were susceptible to ethanolic extract. More recently, the biovariability of *Helichrysum italicum* grown wild in Calabria and Sardinia (Italy) was reported [4]. The antibacterial activity of extracts from this plant showed performed best on the Gram (+) bacteria, particularly *Micrococcus luteus*. Moreover, the antifungal activity of extracts was also tested, producing important results particularly on the phytopathogic fungus *Pythrium*

ultimum. The anti-herpes simplex virus type I (HSV-1) activity of diethyl ether extract from the flowering tops of this plant were also investigated [5]. The extract showed significant antiviral activity at concentrations ranging from 400 to 100 μg/ml. Guarino and Sciarrillo [6] evaluated the antiviral activity against HSV-1 *in vitro* of another *Helichrysum* species, *Helichrysum litoreum* Guss., a Campania medicinal plant reported to have antibacterial properties. The crude aqueous extract from the leaves of *Helichrysum litoreum* at a concentration of 1.35 mg/ml showed significant antiviral activity on HSV-1 in human lung fibroblasts, as demonstrated by the absence of cytopathic effect (CPE).

Baccharis dracunculifolia DC from the genus *Baccharis*, is a native plant from Brazil, and the most important botanical origin for the production of green propolis (Brazilian propolis) by honeybees. Propolis and plant secretions are derived from three species most frequently mentioned as botanical sources of the bee glue in Brazil, including *Baccharis dracunculifolia* [7]. Recently, Leitao *et al.* [8] demonstrated that extracts of this plant produced a basteriostatic effect on *Streptococcus mutans* cultures. Propolis samples collected in the dry and rainy season from an experimental apiary located in cerrado vegetation in Brazil were investigated by Santos *et al.* [9]. Antibacterial assays were performed by the method of dilution of an ethanol extract of propolis in agar, and showed that all 16 actinomycete strains tested were inhibited by propolis concentrations of 0.1 to 0.25%. The growth inhibition of 6 *Fusobacterium* species and 16-black pigmented anaerobes were observed at concentrations of 0.05 to 0.1%

In an antimicrobial screening of plants used in traditional medicine in San Juan province, Argentina, the hexane and dichloromethane extracts of another *Baccharis* species, *Baccharis grisebachii* Hieron., showed the broadest spectrum of action against fungi, inhibiting all of the tested dermatophytes [10]. Regarding antibacterial activity, both extracts of *Baccharis grisebachii* were active on methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, with minimum inhibitory concentrations (MIC) from 125 to 500 μg/ml. Fungicidal activity of wild plants growing in Mexico was evaluated against the mould species *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium expansum*, *Fusarium moniliforme* and *Fusarium poae* [11,12]. Two mould species were controlled by *Baccharis glutinosa* Pers.

Other *Baccharis* species with antiviral properties also included *Baccharis teindalensis* L. and *Baccharis genistelloides* (Lam.) Pers.

which were active against HSV-1 and the vesicular stomatitis virus (VSV) [13,14], and *Baccharis trinervis* Pers., a medicinal plant from Ecuador, which exhibit potent anti-human immunodeficiency virus (HIV) activity *in vitro* [15].

Other anti-infectious genera from the Anthemideae tribe are the genera *Eupatorium* and *Acanthospermum*. *Acanthospermum hispidum* DC. appears to be promising for the isolation of active constituents to develop phytomedicines with antimicrobial properties. The ethanolic and ethyl acetate extracts of this plant showed varying degrees of activity against a wide range of pathogenic bacteria [16,17]. The activity resided mostly in the polar fractions of the alcoholic extract, with only slight activity in the non-polar fractions.

Gupta *et al.* [18] examined petroleum ether and methanolic extracts of the leaves of *Eupatorium ayapana* L. for their antimicrobial activity. The petroleum ether extract showed higher antibacterial and antifungal activity than the methanolic extract. The petroleum ether extract showed a broad spectrum of activity against all the bacterial strains and fungi at the tested concentrations (250-1000 μg/ml), except for *Shigella dysenteriae*. Antibacterial screening of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of *Eupatorium glandulosum* Michx. leaves also showed a broad spectrum of inhibitory activity against Gram (+) and (-) pathogenic bacteria [19].

Chomnawang *et al.* [20] investigated the antimicrobial activity of Thai medicinal plants against *Propionilbacterium acnes* and *Staphylococcus epidermis*, the ethiologic agents of acne vulgaris. Among these, *Eupatorium odoratum* L. had strong inhibitory effects. Thirty six medicinal plants from Sri Lanka have been also screened for antifungal activity against *Cladosporium cladosporioides* [21]. Extracts of *Eupatorium riparium* Regel. have shown significant antifungal activity.

Other genera from the Anthemideae tribe also showed a broad spectrum of antibacterial and antifungal activity. Ramezani *et al.* [22] reported the antimicrobial activities of methanol extracts from four species of *Artemisia* from Iran, *Artemisia diffusa* Krasch. ex Poljak, *Artemisia oliveriana* J. May ex DC., *Artemisia scoparia* Waldst. et Kit. and *Artemisia turanica* Krasch. All extracts were effective against 2 Gram (+) bacteria *Bacillus subtilis* and *Staphylococcus aureus*. None of the extracts showed antibacterial activity against *Escherichia coli*, while *Pseudomonas aeruginosa* was inhibited by *Artemisia oliveriana* and *Artemisia turanica* extracts. Antifungal activity was observed only for

Artemisia scoparia extract. One hundred medicinal plant extracts from British Columbia were screened for antifungal activity against 9 fungal species [23]. The extracts with the greatest fungal inhibition were prepared from the aerial parts of *Artemisia ludoviciana* Nutt. and *Artemisia tridendata* L. Examples of other antifungal *Artemisia* species also included methanol and aqueous extracts of *Artemisia verlotorum* Lamotte. which were active against *Saprolegnia ferax* [24].

The extracts of aerial parts of four *Achillea* species, *Achillea clavennae* L., *Achillea holoserica* (Burm. F.) Greuter, *Achillea lingulata* Waldst. et Kit. and *Achillea millefolium* L. have been tested for antimicrobial activity against 5 bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella enteritidis*) and 2 fungi (*Aspergillus niger* and *Candida albicans*) [25]. Extracts of all four species possessed a broad spectrum of antimicrobial activity against all tested strains.

The water-soluble and water-insoluble parts of the methanol extract of *Achillea sintenisii* Hub. Mor. were assayed for their antimicrobial activities against 12 bacteria and 2 yeasts [26]. No activity was exhibited by the water-soluble subfraction, whereas the water-insoluble subfraction of the methanol extract was found to be active against some of the test microorganisms studied.

Inflorescence extract in 45% ethanol of *Centaurea rupestris* L. showed significant antifungal activity against the dermatophytes *Epidermophyton floccosum*, *Microsporum gypseum* and *Trichophyton mentagrophytes* [27]. The same extract showed weak bactericidal activity against *Bacillus anthracis*, while leaf extract in 90% ethanol possessed significant antibacterial activity against *Streptococcus faecalis*. Examples of another antifungal *Centaurea* species also included *Centaurea pallescens* Del. which showed 95% inhibition of spore germination in phytopathogenic fungi [28].

Rangel *et al.* [29] evaluated the antimicrobial activity of the aerial parts of *Pseudognaphalium moritzianum* (Klatt) Badillo. Ethanol and acetone extracts showed activity against *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*, while the aqueous extract was only active towards *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Another genus related to *Pseudognaphalium* from the Anthemideae tribe also showed a broad spectrum of antimicrobial activity. These reports concerned the genus *Gnaphalium* from different

geographical locations. Villagomez-Iborra *et al.* [30] investigated the antibacterial activity of the hexane, dichloromethane, ethyl acetate and methanol extracts of the flowers, leaves and stems of three Mexican *Gnaphalium* species: *Gnaphalium oxyphyllum* DC. var. *oxyphyllum*, *Gnaphalium liebmanii* var. *monticola* (Mc Vaugh) Nash. and *Gnaphalium viscosum* H.B.K. The hexane extracts showed the higher inhibitions in all cases. *Gnaphalium oxyphyllum* var. *oxyphyllum* flower extract exhibited the widest spectrum of activity.

Eighteen crude extracts from six different plant species used in Mexican traditional medicine for the treatment of respiratory infections were evaluated for potential antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli* and *Candida albicans* [31]. The extracts of *Gnaphalium oxyphyllum* DC. and *Gnaphalium americanum* Mill. possessed strong antimicrobial activity against the pathogens tested.

Decoctions of four plants used for the treatment of different infections by indigenous groups of the Peruvian Amazon were also evaluated for antimicrobial activity by the "stroke" method in agar plates [32]. The microorganisms tested included *Staphylococcus aureus*, *Escherichia coli*, *Salmonella gallinarum*, *Klebsiella pneumoniae*, *Candida albicans*, *Pseudomonas aeruginosa* and *Mycobacterium gordonae*. The decoction of *Gnaphalium spicatum* Lam. showed antimicrobial activity against *Staphylococcus aureus*.

Ethanol and aqueous extracts of twenty Palestinian plant species used in folk medicine were investigated for their antimicrobial activities against 5 bacterial species (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) and one yeast (*Candida albicans*) [33]. The most antimicrobially active plant was *Gnaphalium rupreste* Rafin. Examples of another antibacterial *Gnaphalium* species also included *Gnaphalium gracile* H.B.K. which inhibited the growth of *Staphylococcus aureus* [34].

The aqueous/methanol fraction of the dichloromethane extract of *Carthamus lanatus* L. exhibited a high rate of antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* [35], while crude dichloromethane extracts of the aerial parts and roots of *Leontopodium alpinum* Cass. exhibited significant growth inhibition of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes* [36].

Petrovic *et al.* [37] investigated the antibacterial activity of the water, ethanol and ethyl acetate extracts of *Cichorium intybus* L. Water extract inhibited *Agrobacterium radiobacter* spp. *tumefaciens*, *Erwinia carotovora*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. The plant also showed antifungal activity against phytopathogenic fungi [28].

Holetz *et al.* [38] reported the antimicrobial screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Two members of the Anthemideae tribe, *Tanacetum vulgare* L. and *Arctium lappa* L. presented some degree of antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

Examples of other antibacterial crude extracts from the Anthemideae tribe also included methanol extracts of the leaves of *Tagetes terniflora* Kunth [39], the benzene fraction of *Vernonia cinerea* L. which exhibited a broad spectrum of antibacterial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa* [40], *Aster ageratoides* Turcz. [41] and *Xanthium strumarium* L. extracts which were active against several strains of bacteria, yeasts and fungi [42].

Other genera from the Anthemideae tribe also showed a broad spectrum of antifungal activity. Plants from the genus *Pterocaulon*, known as quitoco, are used to treat problems popularly diagnosed as mycoses which may have a fungic ethiology. In order to validate this traditional practice, the crude methanol extracts and fractions from the aerial parts of three species of *Pterocaulon* growing in southern Brazil, *Pterocaulon alopecuroides* (Lam.) DC., *Pterocaulon balansae* Chodat. and *Pterocaulon polystachyum* DC., were analyzed for *in vitro* antifungal activity against a panel of standardized and clinical opportunistic pathogenic yeasts and filamentous fungi, including dermatophytes [43]. The crude methanol extract of *Pterocaulon alopecuroides* was the most active.

Batawila *et al.* [44] investigated the fungicide activity of a threatened species of Togo flora, *Conyza aegyptiaca* (L.) Ait. var. *lineuriloba* (DC.) O. Hoffm. Extracts of this plant presented fungicide and fungistatic activity on *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Candida zeylanoides*.

Aqueous extracts of ten medicinal plants were tested for their ability to inhibit *Microsporum canis* and *Trichophyton rubrum*, the ethiological agents of dermal fungal infections in humans [45]. Aqueous extract from the leaves of *Inula viscosa* Ait. (=*Dittrichia viscosa* (L.) Greuter) produced detectable antifungal activity against these

dermatophytes. This extract was also active against *Candida* species [46] and HIV, HSV-1 and VSV [47,48].

Trovato *et al.* [49] reported the antimycotic activity of aqueous, ethanol and petroleum ether extracts of *Matricaria recutita* L. These extracts inhibited *in vitro* strains of *Candida albicans* isolated from clinical samples obtained in the course of acute vaginitis.

Examples of other antifungal crude extracts from the Anthemideae tribe also included the ethanol extract of *Erigeron breviscapus* (Vant) Hand-Mazz whole plant [50], chloroform, ethanol and ethyl acetate extracts of *Cynara scolymus* L. leaves [51], the hexane extract of *Senecio vulgaris* L. which showed significant activity against *Trichophyton tonsurans* [52], and the petroleum ether and dichloromethane extracts of the leaves and flowers of *Senecio pampae* L. which showed antifungal activity against *Fusarium oxysporum* and *Trichoderma viride* [53].

Other genera from the Anthemideae tribe also showed a broad spectrum of antiviral activity. Extracts of eight taxa from the genus *Echinacea* were found to have antiviral activity against HSV-1 *in vitro* [54]. The most potent inhibitors of HSV-1 were *Echinacea pallida* Bins, Baum & Arnason var. *sanguinea* (Nutt.) Gandhi & Thomas 70% ethanol inflorescence extract and *Echinacea purpurea* (L.) Moench hexane root extract.

Ooi *et al.* [55] investigated the *in vitro* antiviral activity of aqueous and ethanol extracts of *Youngia japonica* (L.) DC. against the respiratory syncytial virus, the influenza A virus and HSV-1 by CPE reduction assay. The ethanol extract exhibited antiviral activity against the respiratory syncytial virus in Hep-2 cells, but did not show any activity against the other viruses tested.

Examples of other antiviral crude extracts from the Anthemideae tribe included the ethanol extract of *Santolina oblongifolia* Boiss. [56], and the hot water extract from *Stevia rebaudiana* Bertoni which showed anti-human rotavirus activity [57]. Another *Santolina* species, *Santolina etrusca* (Lacaita) Marchi et Dam., was also active against *Saprolegnia ferax* [24].

Another antiviral species from the Anthemideae tribe is *Bidens pilosa* L. var. *minor* (Blume) Sherff which significantly inhibited the replication of HSV-1 at a concentration of 100 μg/ml [58]. The ethanol extract of *Bidens pilosa* L. also showed a broad spectrum of antibacterial activity [59]. From another *Bidens* species, *Bidens cernua* L., a new complex antibiotic preparation known as cerbiden was obtained [60].

High cerbiden activity against *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida parapsitosis* and *Candida guilliermondii* sensitive and resistant to nystatin, amphotericin C and clotrimazole was detected.

ESSENTIAL OILS

Volatile or essential oils, as their name implies, are volatile in steam and are generally complex mixtures of hydrocarbons and oxygenated compounds derived from these hydrocarbons. The odour and taste of volatile oils is mainly determined by these oxygenated constituents. In chemical structure, most essential oils are terpenoids in origin. Testing and evaluation of the antimicrobial activity of essential oils is difficult because of their volatility, their water insolubility and their complexity. However, in recent years a large number of studies have been conducted on the antimicrobial activity of essential oils from the Anthemideae tribe. These reports concerned mainly the genera *Achillea* and *Artemisia*.

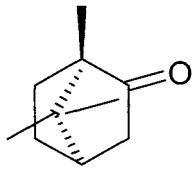

Fig. (1). Structure of camphor

Bezic *et al.* [61] investigated the antibacterial and antifungal activities of the essential oil of *Achillea clavennae*, a rare European plant. The activity was more pronounced against Gram (-) and fungal organisms that against Gram (+) bacteria. *Achillea clavennae* essential oil was found to possess antimicrobial activity against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and all fungal organisms tested (*Aspergillus niger*, *Aspergillus fumigatus* and *Candida albicans*). The major constituents of the essential oil were found to be camphor, Fig. **(1)**, myrcene, 1,8-cineole, Fig. **(2)**, β-caryophyllene, Fig. **(3)** and linalol. More recently, the essential oil of *Achillea clavennae* was also investigated for

its antibacterial activity against some respiratory tract pathogens [62]. Maximum activity was observed against *Klebsiella pneumoniae* and penicillin-susceptible and penicillin-resistant *Streptococcus pneumoniae*. The oil also exhibited strong activity against Gram (-) *Haemophilus influenzae* and *Pseudomonas aeruginosa*. Gram (+) *Streptococcus pyogenes* was the most resistant to the oil.

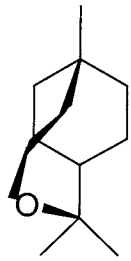

Fig. (2). Structure of 1,8-cineole

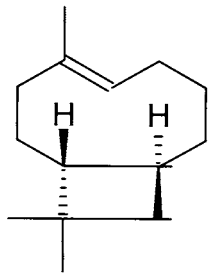

Fig. (3). Structure of β-caryophyllene

The essential oil from another *Achillea* species, *Achillea sintenisii*, was assayed for its antimicrobial activities against 12 bacteria and 2 yeasts [26]. The oil was found to be active against some of the test microorganisms studied. The analysis of the oil revealed that the main components, e.g., camphor, Fig. **(1)** and eucalyptol, possessed appreciable activity against *Candida albicans* and *Clostridium perfringens*. The antimicrobial activities of the essential oils of *Achillea setacea* W. & K. and *Achillea teretifolia* Willd. were individually evaluated against 14

microorganisms [63]. Both oils exhibited inhibitory effects on *Clostridium perfringens*, *Acinetobacter lwoffii* and *Candida albicans*, with a range of MIC values ranging from 0.28 to 2.25 mg/ml. Camphor, Fig. **(1)** and its derivatives borneol, Fig **(4)**, terpinen-4-ol and 1,8-cineole, Fig. **(2)** can be considered as the main antimicrobial constituents of the oils studied.

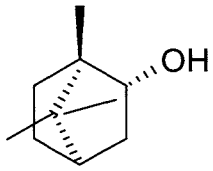

Fig. (4). Structure of borneol

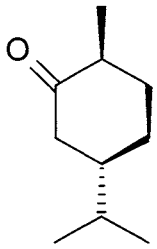

Fig. (5). Structure of β-thujone

Baser *et al.* [64] examined the antimicrobial activity of the essential oil of *Achillea multifida* (DC.) Boiss. using a micro-dilution assay. The results showed the inhibition (MIC = 62.5-250 μg/ml) of human pathogenic bacteria and yeasts. Sabinene, β-thujone, Fig. **(5)** and camphor, Fig. **(1)** were characterized as the main constituents. The antimicrobial activity of the essential oil of another *Achillea* species, *Achillea millefolium* L. subsp. *millefolium* L. Afan was also investigated [65]. The oil showed activity against *Streptococcus pneumoniae*, *Clostridium perfringens*, *Candida albicans*, *Mycobacterium smegmatis*, *Acinetobacter lwoffii* and *Candida kruser*. Eucalyptol, camphor, Fig. **(1)**,

α-terpineol, β-pinene, Fig. **(6)** and borneol, Fig. **(4)** were the principal components. The chemical composition of the essential oils of three Greek *Achillea* species, *Achillea holoserica*, *Achillea taygetea* Boiss. & Heldr. and *Achillea fraasii* Schultz Bip was determined [66]. Camphor, Fig. **(1)**, borneol, Fig. **(4)** and 1,8-cineole, Fig. **(2)** were found to be the major components of the oils. The *in vitro* antimicrobial activity of these essential oils was evaluated against 6 bacteria, indicating that the first is totally inactive while the other two possess moderate to strong activities, mainly against the Gram (-) strains. The essential oil of *Achillea fraasii* was also active against the tested pathogenic fungi.

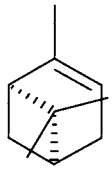

Fig. (6). Structure of β-pinene

Karamenderes *et al.* [67] reported the composition and antimicrobial activity of the essential oils obtained from *Achillea millefolium* L. subsp. *pannonica* (Scheele) Hayek, *Achillea millefolium* subsp. *millefolium*, *Achillea arithmifolia* Waldst & Kitt and *Achillea kotschyi* Boiss. subsp. *kotschyi*, four *Achillea* species from Turkey. 1,8-cineole, Fig. **(2)**, artemisia alcohol and ascaridole were identified as major components. The essential oils showed antibacterial and antifungal effects even with low concentrations. The essential oil of the flowering tops of another *Achillea* species, *Achillea fragantissimum* (Forsk.) Sch. Bip growing in Sinai, was analyzed [68]. Santolina alcohol, α- and β-thujone, Fig. **(5)** and artemisia ketone account for approximately 80% of the oil. The oil showed marked antimicrobial activities against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*.

Other species from the *Achillea* genus also showed a broad spectrum of antibacterial activity. Senatore *et al.* [69] investigated the composition of the essential oil from the aerial parts of *Achillea falcata* L.

growing wild in Lebanon, which has been shown to possess inhibitory activity mainly against Gram (+) bacteria. Fifty eight compounds, representing 94.4% of the oil, were identified. Isomers of the cyclobutane ethanol, 1-methyl-2-(1-methylethenyl), grandisol and fraganol were the main components of the oil. Also abundant were artemisia ketone, terpinen-4-ol and 1,8-cineole, Fig. **(2)**. Volatile oil from populations of *Achillea clypeolata* Sibth. et Sm. growing in the wild was also studied for yield, composition and antibacterial activity [70]. Eighteen identified components constituted 89.8% of the oil. The major components in the oil were (*E*)-γ-bisabolene, 1,8-cineole, Fig. **(2)**, borneol, Fig. **(4)** and caryophyllene-oxide, together making 57.3% of the total oil content. In an antibacterial diffusion assay, the oil showed activity against all tested Gram (-) bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and Gram (+) *Staphylococcus aureus*.

Sokmen *et al.* [71] reported the antimicrobial activity *in vitro* of the essential oil from *Achillea biebersterini* Afan. The analysis of the oil resulted in the identification of twenty-three components. Piperitone, eucalyptol, camphor, Fig. **(1)**, chrysanthenone and borneol, Fig. **(4)** were the main components. Antimicrobial activity tests carried out with the fractions of the oil showed that the activity was mainly observed in those containing eucalyptol and camphor, Fig. **(1)** in particular, followed by borneol, Fig. **(4)** and piperitone. Examples of other antimicrobial essential oils from the *Achillea* genus also included those obtained by steam distillation from the flower, leaf and stem of *Achillea santolina* L., a species from Egypt [72], and the essential oil of *Achillea atrata* L. and its major component 1,8-cineole, Fig. **(2)**, which exhibited strong inhibitory action against 18 micomycetes with MIC values of 2-8 mg/ml [73].

In recent years, a large number of studies have also been conducted on the antimicrobial activity and chemical composition of essential oils from the genus *Artemisia*. For example, the essential oil of *Artemisia annua* L. aerial parts, consisting of camphor, Fig. **(1)**, germacrene D, Fig. **(7)**, *trans*-pinocarveol, β-selinene, β-caryophyllene, Fig. **(3)** and artemisia ketone, was screened for its antimicrobial activity [74]. The essential oil notably inhibited the growth of tested Gram (+) bacteria *Enterococcus hirae* and both tested fungi *Candida albicans* and *Saccharomyces cerevisiae*. More recently, Rasooli *et al.* [75] investigated the antimicrobial effects of essential oil from *Artemisia annua* against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*,

Pseudomonas aeruginosa, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Bacillus licheniformis*, *Candida albicans* and *Saccharomyces cerevisiae*. The oil was found to have antimicrobial and particularly antibacterial effects. The high content in monoterpene hydrocarbons seem to contribute to the strong antimicrobial activity of *Artemisia annua*.

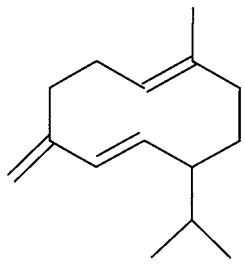

Fig. (7). Structure of germacrene D

Kalemba *et al.* [76] investigated the antibacterial and antifungal activity of the essential oil of *Artemisia asiatica* Nakai, its main constituents 1,8-cineole, Fig. **(2)** and selin-11-en-4α-ol, and the monoterpene alcohol fraction. The essential oil exhibited good inhibitory activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Rhodotorula rubra* and *Aspergillus fumigatus*. The monoterpene alcohol fraction showed the highest antibacterial potency. The essential oils obtained by steam distillation of the aerial parts of two populations of *Artemisia absinthium* L. from France and Croatia were investigated for antimicrobial activity [77]. The results showed that *Artemisia absinthium* essential oil inhibited the growth of both tested yeasts (*Candida albicans* and *Saccharomyces cerevisiae* var. *chevalieri*). The oils of French *Artemisia absinthium* contain (*Z*)-epoxycimene and chrysanthenyl acetate as major components, while those of Croatian origin contain mainly (*Z*)-epoxycimene and β-thujone, Fig. **(5)**. The essential oil of *Artemisia absinthium* also inhibited the growth of *Listeria monocytogenes* type 4A [78].

Masotti *et al.* [79] analyzed the volatile components of another *Artemisia* species, *Artemisia molinieri* Quezel, an endemic wormwood of Southern France. Among the sixty nine compounds identified, major components were ascaridole, α-terpinene, *p*-cymene, 1,8-cineole, Fig. **(2)**

and germacrene D, Fig. **(7)**. Bioassays were performed on a sample of essential oil which showed a strong inhibition of the growth of both tested yeasts (*Candida albicans* and *Saccharomyces cerevisiae* var. *chevalieri*) and minor activity on both tested Gram (-) bacteria (*Escherichia coli* and *Enterococcus hirae*).

The essential oils of the leaves of *Artemisia nilagirica* (Clarke) Pamp from South India was also investigated, and led to the identification of fifty-nine compounds including α- and β-thujone, Fig. **(5)** and borneol, Fig. **(4)** [80]. The leaf oil inhibited the growth of the plant pathogen *Phytophthora capsici*. This property has been attributed to the presence of thujone in the oil. During screening of essential oils from the leaves of ten angiospermic taxa against the dermatophytes *Epidermophyton floccosum* and *Trichophyton violaceum*, the essential oil of *Artemisia nilagirica* was also found to possess complete antidermatophytic activity [81]. It was fungistatic in nature and had a broad fungitoxic spectrum.

More recently, Cha *et al.* [82] investigated the chemical composition and antimicrobial activity of the essential oils of *Artemisia scoparia* and *Artemisia capillaris* Thunb. The essential oil from *Artemisia scoparia* was rich in camphor, Fig. **(1)**, 1,8-cineole, Fig. **(2)** and β-caryophyllene, Fig. **(3)** as the major compounds, whereas *Artemisia capillaris* oil was rich in β-pinene, Fig. **(6)**, β-caryophyllene, Fig. **(3)** and capillene. The essential oils and some of their major compounds were tested for their antimicrobial activity against 15 different genera of oral bacteria. The essential oils exhibited considerable inhibitory effect against all the oral bacteria tested, while the major components demonstrated various degrees of growth inhibition.

Hifnawy *et al.* [83] reported the antibacterial activity of the essential oils of *Artemisia monosperma* Del, *Artemisia judica* L. and *Artemisia herba-alba* Asso against *Bacillus subtilis* and *Escherichia coli*. The effect of one of these species, *Artemisia herba-alba* (mugwort) oil on spore germination, mycelial elongation and sporulation was studied in 3 fungi [84]. All three stages of fungal asexual reproduction were affected, but mycelium growth was the most sensitive. Of the 3 fungi studied, *Zygorrhynchus* spp. was found to be the most sensitive, followed by *Aspergillus niger* and then *Penicillium italicum*. The essential oil of *Artemisia herba-alba* also showed a very strong action on *Staphylococcus*, *Candida* and *Microsporum* [85].

Other antimicrobial species from the *Artemisia* genus are *Artemisia afra* Jacg. and *Artemisia princeps* var. *orientalis* Pamp. *Artemisia afra* is indigenous to the Eastern highlands of Zimbabwe, where it is used in folk medicine. Hydro-distilled volatile oil from the aerial parts of the plant was tested for antifungal activity against 10 fungal species using the dry weight method [86]. The results obtained showed that the essential oil exhibited significant activity against *Aspergillus ochraceus*, *Candida albicans*, *Alternaria alternata*, *Geotrichum candidum*, *Aspergillus niger*, *Penicillium citrium* and *Aspergillus parasiticus*. The antimicrobial activity of this essential oil was also tested against 14 microbial strains [87]. The test organisms were selected on the basis of their significance as food spoilage and/or poisonous common human and plant pathogens. More recently, the volatile oils of five plants widely used in African traditional healing, including *Artemisia afra*, were tested against 3 strains of *Staphylococcus aureus* (2 of which were methicillin-resistant), one strain of *Pseudomonas aeruginosa*, 2 strains of *Candida albicans* and one strain of *Cryptococcus neoformans* [88]. All microorganisms were inhibited by <1% of the oil, with the exception of *Staphylococcus aureus*, *Staphylococcus aureus* methicillin-resistant and *Pseudomonas aeruginosa*.

The volatile constituents of *Artemisia princeps* var. *orientalis* (wormwood) were investigated for phytotoxic and antimicrobial activities [89]. *Escherichia coli* was not susceptible to the wormwood essential oil, but the growth of *Bacillus subtilis*, *Aspergillus nidulans*, *Fusarium solani* and *Pleurotus ostreatus* was severely inhibited. Lee and Lee [90] evaluated the antimicrobial activity of the essential oil from *Artemisia princeps* var. *orientalis* against selected pathogenic bacteria and fungi from fish. At concentrations of over 500 ppm, the inhibitory effect of the oil was at its peak against *Aeromonas hydrophyla* and *Aeromonas salmonicida*, but the bacteria *Edwardsiella tarda*, *Vibrio anguillarum*, *Vibrio ordalli* and *Streptococcus* spp. were insensitive. The essential oil of this species could be also valuable for the development of a new natural fungicide to control soilborne take-all disease of wheat by seed treatment. Mycelial growth of *Gaemannomyces gramini* var. *tritici* was inhibited 100% at 50 mg/l of essential oil. Liu *et al.* [91] analyzed the chemical composition of this essential oil, and twenty compounds, constituting 82.82% of the total oil, were characterized. Bornane,

chamazulene and 1α,2β,3β,6α-3-methyl-6-(1-methylethyl)-1,2-cyclo hexanediol were the major components.

Reports on the chemical composition and antimicrobial activity of other *Artemisia* species have also been found in the literature. Haggag *et al.* [92] reported the chemical analysis of the essential oil from the fresh aerial parts of *Artemisia vulgaris* L. One hundred and twelve components (94.27% of total oil components) were identified. α-thujone was found to be the major component. The oil showed good antimicrobial activity *in vitro* against certain strains of bacteria and fungi. The essential oil from the wormwood species *Artemisia glabella* Kar. et Kir. was analyzed for its chemical composition and antimicrobial activity [93]. The antibacterial, antifungal and antiviral properties of this essential oil indicate its potential for the treatment of upper airway pathologies. The main chemical components are 1,8-cineole, Fig. **(2)**, linalol, 4-terpineol, α-terpineol and sabinol derivatives. The essential oil also contains a series of genetically related compounds including *p*-cymene, cuminaldehyde, α-thujene, sabinene and some related esters.

The composition of the essential oil of the aerial parts of *Artemisia lobelia* All, which was found to show strong activity against *Pseudomonas aeruginosa* was studied [94]. The oil was characterized by the high content of monoterpenes and oxidized terpenes. The major constituents were camphor, Fig. **(1)**, 1,8-cineole, Fig. **(2)** and artemisia ketone. Essential volatile oils of *Artemisia lerchiana* Web. ex Stechm. and *Artemisia glauca* Pall growing in the territory of Kalmykia, Russia, had secondary antibacterial effects towards *Staphylococcus aureus* and *Corynebacterium diphtheriae* [95,96]. A basteriostatic effect was more strongly demonstrated against these last microorganisms.

Other essential oils from the *Artemisia* genus also showed potent antifungal activity. The essential oil from *Artemisia nelagrica* L. showed strong activity against the dermatophytes *Trichophyton rubrum*, *Microsporum gypseum*, *Aspergillus fumigatus* and *Cladosporium trichoides* [97]. The antifungal effects of some Chinese medicines, including *Artemisia argyi* Leu & Van, and their volatile components were analyzed [98]. *Artemisia argyi* and its essential components showed activity, and the cinnamoaldehyde isolated from it had the strongest antifungal effect.

Examples of other antimicrobial essential oils from the *Artemisia* genus also included the essential oil of *Artemisia iwayomogi* Kitamura

[99], the essential oil of *Artemisia khorasanica* Podl., a common perennial herb growing wild in northeastern parts of Iran [100], the essential oils isolated from the aerial parts of *Artemisia absinthium*, *Artemisia santonicum* L. and *Artemisia spicigera* C. Koch, three Turkish *Artemisia* species [101], and the essential oil fraction of *Artemisia dracunculus* L. var. *dracunculus*, which showed antifungal activity against *Colletrotichum fragariae*, *Colletrotichum gloeosporioides* and *Colletrotichum acutatum* [102]. From this essential oil, methyleugenol was isolated and identified as an antifungal constituent. Steam distillation of the essential oil from *Artemisia dracunculus* var. *dracunculus*, known as French tarragon, yields a mixture of almost fifteen different components [103]. Ten bacterial species were exposed to the oil and to several of its constituents, and the level of bacterial inhibition was determined. Among the bacteria tested were several of significance for public health, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Yersinia enterocolitica*. The most inhibitory compound in the essential oil from French tarragon were anysaldehyde, *p*-cymene, eugenol, limonene, linalool, menthol, *cis*-ocimene, α-phellandrene, and α- and β-pinene, Fig. **(6)**.

Other antimicrobial genera from the Anthemideae tribe are the genera *Tanacetum* (= *Chrysanthemum*) and *Helichrysum*. El Shazly *et al.* [104] investigated the chemical composition and antimicrobial activity of the essential oil of *Tanacetum santolinoides* (DC.) Feinbr. and Fertig. The essential oil showed strong *in vitro* activity against *Escherichia coli*, *Bacilus subtilis* and *Candida albicans*. The main constituents were thymol, *trans*-thujone, *trans*- and *cis*-chrysanthenyl acetate, umbellulone and 1,8-cineole, Fig. **(2)**. The antimicrobial activity of the essential oil from the air-dried and processed flowers of *Chrysanthemum indicum* L. was evaluated against 15 microorganisms, including 3 yeasts and 7 clinical isolated strains [105]. These results show that both essential oils possessed significant antimicrobial effect. However, some difference in antimicrobial activity between two oils was observed for several microorganisms, which was attributed to the variation in percentage of the components. With a higher percentage of camphor, Fig. **(1)**, the oils of the processed flowers exhibited greater basteriostatic activity than that of the air-dried ones. The essential oil of *Chrysanthemum indicum* also

exhibited antibacterial activities against both *Staphylococcus aureus* and *Escherichia coli* [106].

The essential oil of *Chrysanthemum boreale* Makino exhibited antibacterial activity (MIC more than 800 μg/ml) after it was tested against 6 Gram (+) and 8 Gram (-) bacteria [107]. Eighty seven constituents were identified, representing 94.13% of the total oil. The major components were camphor, Fig. **(1)**, α-thujone, *cis*-chrysanthenol, 1,8-cineole, Fig. **(2)**, α-pinene and β-caryophyllene, Fig. **(3)**. More recently, the fungitoxic activity against *Botrytis cinerea* and *Phytophthora citrophthora* of the essential oil of *Chrysanthemum viscidehirtum* (Schott) Thell., a Moroccan medicinal plant, has been reported [108]. The oil completely inhibited the growth of both fungi at a concentration of 150 μg/ml. Analysis of the oil from *Chrysanthemum viscidehirtum* showed that it consisted mainly of β-farnesene, limonene and oxygenated sesquiterpenes. Antifungal activity against *Penicillum digitatum* and *Geotrichum citri-aurantii* has also been reported [109]. Essential oil of *Chrysanthemum viscidehirtum* at a concentration of 150 ppm strongly inhibited *in vitro* growth of all fungi. This essential oil also exhibited activity against 21 microbial strains, particularly *Salmonella typhi* and *Proteus mirabilis* [110].

Other antimicrobial species from the *Tanacetum* genus are *Tanacetum parthenium* Schultz-Bip and *Tanacetum vulgare*. The essential oil of *Tanacetum vulgare* in 100% inhibited 13 Gram (+) and 15 Gram (-) bacterium strains as well as 7 thread-granulating and yeast fungus strains [111]. *Tanacetum vulgare* oil was composed of terpenes such as artemisia ketone, umbellulone, piperitone, thujone, artemisia alcohol, borneol, Fig. **(4)**, camphor, Fig. **(1)**, chrysanthemyl acetate, terpinyl acetate, carveyl acetate and davanone [112]. Kalodera *et al.* [113] investigated the chemical composition and antimicrobial activity of *Tanacetum parthenium* essential oil. Among monoterpenes, the oxidized monoterpenes were well represented, especially camphor, Fig. **(1)**, *trans*-chrysanthenyl acetate, linalol, linalyl acetate and bornyl acetate. Among the sesquiterpenic components, β-caryophyllene, Fig. **(3)**, *trans*-β-farnesene, germacrene D, Fig. **(7)** and δ-cadinene were found. Tests for antimicrobial activity of the essential oil included bacteria, fungi, molds and dermatophytes. Gram (+) species had lower sensitivity than the Gram (-) ones, molds, dermatophytes and some fungi.

The antifungal activity of another *Tanacetum* species, *Chrysanthemum coronarium* L. was evaluated against 12 agricultural pathogens [114]. Flowerhead oil was active both in contact and headspace *in vitro* assays producing hyphal growth inhibition, although it was less active on faster growing fungi. The main compounds identified in the oil were camphor, Fig. **(1)**, α- and β-pinene, Fig. **(6)** and lyratyl acetate.

In recent years, a large number of studies have also been performed concerning the antimicrobial activity and chemical composition of essential oils from the genus *Helichrysum*. Roussis *et al.* [115] examined the chemical composition of the essential oils obtained from the aerial parts of *Helichrysum stoechas* (L.) Moench ssp. *barrelieri* (Teno) Nyman var. *scandens* Guss. and *Helichrysum stoechas* ssp. *barrelieri* var. *spathulatum* Raulin growing in Crete. The essential oils were active against 2 Gram (+) and 4 Gram (-) bacteria and 3 pathogenic fungi. From the thirty-nine identified constituents, representing 89.8% and 87% of the two oils respectively, β-caryophyllene, Fig. **(3)**, α-humulene, α-pinene and limonene were found to be the major components.

The chemical composition of the essential oils obtained from the aerial parts of *Helichrysum kraussii* Sch. Bip. and *Helichrysum rugulosum* Less., two *Helichrysum* species from South Africa were also analyzed [116]. From the thirty-nine identified constituents, representing 85.1% and 92.9% of the two oils respectively, β-caryophyllene, Fig. **(3)**, α-pinene, β-caryophyllene oxide, *cis*-α-bisabolene, β-bisabolene and α-humulene were found to be the main components. Furthermore, the oils were tested against 6 Gram (+) or (-) bacteria and 3 pathogenic fungi. It was found that oils from both plants, and especially that of *Helichrysum rugulosum* exhibited interesting antibacterial activity. Examples of other antimicrobial essential oils from the *Helichrysum* genus also included the essential oil of *Helichrysum italicum* G. Don ssp. *microphyllum* (Willd) Nym, the Sardinian dwarf curry plant [117] and the essential oil obtained from the aerial parts of *Helichrysum amorginum* Boiss. & Orph. cultivated in Greece, which was found to exhibit a moderate antimicrobial activity against 4 Gram (-) bacteria, 2 Gram (+) and 3 pathogenic fungi [118].

Other genera from the Anthemideae tribe also showed a broad spectrum of antibacterial and antifungal activity. Govinden-Soulange *et al.* [119] investigated the chemical composition and *in vitro* antimicrobial

activities of the essential oils from endemic *Psiadia* species growing in Mauritius. *In vitro* antimicrobial assays, using the agar-well diffusion method, revealed that most of the oils were not very active against the tested microorganisms, except for that of *Psiadia lithospermifolia* (Lam.) Cordem., which significantly inhibited the growth of *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aureofaciens*. This activity has been attributed to the presence of δ-elemene, (*E*)-farnesene, α-curcumene, selina-4,7(11)-diene, (*E,Z*)-α-farnesene and β-bisabolene, some of which have established antimicrobial profiles. Likewise, the fungitoxic action of the oil of *Psiadia arguta* (Pers.) Voigt against *Aspergillus ochraceus*, *Candida pseudotropicalis* and *Fusarium manoliforme* may be attributed to the presence of isoeugenol. Volatile constituents obtained by hydrodistillation of the leaves of another *Psiadia* species, the Malagasy endemic plant *Psiadia lucida* (Cass.) Drake, was also found to possess interesting antimicrobial activities [120].

Uzel *et al.* [121] investigated the chemical composition and antimicrobial activity of the essential oils of *Anthemis xylopoda* O. Schwarz, an endemic taxon of Turkey. Borneol, Fig. **(4)** was the major constituent of both the oils studied. The essential oil from the flowers exhibited moderate activity against *Bacillus subtilis*, *Escherichia coli* and 3 *Candida* species, but strong activity against the rest of bacteria. The essential oil from the leaves exhibited moderate activity against *Bacillus cereus*, *Staphylococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and 3 *Candida* species, but strong activity against the other test organisms. Reports on the chemical composition and antimicrobial activity of other *Anthemis* species have also been found in the literature. The antimicrobial activity of the essential oil from medicinal plants which are recommended for the treatment of microbial infections in aromatherapy and complementary medicines were tested against *Candida* spp. [122]. The essential oil from *Anthemis nobilis* (Anth) inhibited growth of *Candida albicans*, *Candida utilis* and *Candida tropicalis*. The oil of *Anthemis nobilis* also gave over 80% control value against the phytopathogenic fungi *Pyricularia grisea* [123]. The essential oil of another species of *Anthemis* genus, *Anthemis cretica* L. subsp. *leucanthemoides* (Boiss.) Grierson was analyzed and its antimicrobial activity was also investigated [124]. Nineteen compounds were characterized, representing 87.7% of the oil, with camphor, Fig. **(1)** the main constituent.

The essential oil composition and antimicrobial activity of *Osmitopsis asteriscoides* (Berg) Less, a medicinal plant used in traditional preparations in South Africa has been investigated [125]. Three different antimicrobial methods were comparatively evaluated against *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* The two major essential oil components, camphor, Fig. **(1)** and 1,8-cineole, Fig. **(2)** were investigated, indicating the positive antimicrobial efficacy of 1,8-cineole, Fig. **(2)**, independently and in combination with camphor, Fig. **(1)**.

The genus *Eriocephalus* of the Anthemideae tribe is endemic to Southern Africa and is comprised of 32 species, of which several are economically important as traditional herbal remedies. Njenga *et al.* [126] initiated an investigation into the antimicrobial activity of the genus *Eriocephalus*, using the disc diffusion assay against a range of Gram (+) and (-) bacteria as well as a few selected fungi. The study included fifteen *Eriocephalus* species with one hundred and twenty-three essential oil samples. The essential oil of *Eriocephalus aromaticus* L. produced the most promising activity of all species studied, with MIC values of 400 μg/ml and 200 μg/ml for *Bacillus cereus* and *Staphylococcus aureus*, respectively.

Another member of the Anthemideae tribe *Inula viscosa*, a plant growing spontaneously in the Mediterranean area, is currently used in popular medicine for its therapeutic effects. Cafarchia *et al.* [127] examined the chemical composition and antifungal activity of the four essential oils obtained by steam distillation of the leaves, flowers, whole plant and whole plant without flowers of *Inula viscosa*. All the essential oils proved to have a significant antifungal activity against dermatophytes even at low concentrations (0.01 mg/ml). The essential oil of the leaves exhibited the greatest antifungal efficacy. The high concentration of the sesquiterpene carboxyeudesmadiene occurring in this essential oil may explain its greater antifungal activity. The essential oil of *Inula viscosa*, growing wild in Southern Turkey was investigated and twenty components were identified [128,129]. The main ones were γ-terpinene, *p*-cymene, thymol and carvacrol, as well as 1,8-cineole, Fig. **(2)**, pulegone and anethole. Biological assays showed that fungitoxicity against the soil-borne plant-disease-causing fungi *Fusarium moniliforme*, *Rhizoctonia solani*, *Sclerotinia sclerotiourum* and *Rhizoctonia capsici* was due to

different concentrations of the phenolic fraction (specially thymol and/or carvacrol) in the essential oil.

Other antimicrobial species from the *Inula* genus are *Inula helenium* L. and *Inula cuspidata* C B Clarke. The chemical composition of *Inula helenium* oil was analyzed by Bourrel *et al.* [130]. Besides the already known alantolactones (alantolactone and isoalantolactone), fifteen minor constituents were also identified in the oil. The essential oil sample was subjected to microbial screening against 5 bacteria and 7 fungi and the results were comparable to natural compounds known for their antimicrobial activities. The essential oil obtained from the leaves of *Inula cuspidata* showed remarkably good antifungal activity against 5 plant and human pathogenic fungi: *Aspergillus nidularis*, *Aspergillus glaucus*, *Aspergillus tereos*, *Aspergillus flavus* and *Aspergillus fumigatus* [131].

In several species, some differences in antimicrobial activity were observed, which were attributed to the variation in the chemical composition according to their geographic origin. The essential oils from aerial parts of *Tagetes minuta* L. grown in Egypt, South Africa and the United Kingdom were obtained by hydrodistillation. In an antimicrobial screening, the activity was greater in oils from the United Kingdom than in oils from Egypt or South Africa [132]. Although they had similar constituents, the oils had different percentage compositions. Dehydrotagetone was the most abundant component of the oil from the United Kingdom, while the oils from South African and Egypt were characterized by a high content of *cis*-β-ocimene. Romagnoli *et al.* [133] reported the chemical characterization and antifungal activity of another *Tagetes* species, the capitula of *Tagetes patula* L. The oil exerted good antifungal activity against 2 pathogenic fungi, *Botrytis cinerea* and *Penicillium digitatum*, providing complete growth inhibition at 10 μg/ml and 1.25 μg/ml, respectively. Thirty compounds were identified, representing 89.1% of the total detected. The main components were piperitone, piperitenone, terpinolene, dihydrotagetone, *cis*-tagetone, limonene and allo-ocimene. The contribution of the two main compounds, piperitone and piperitenone, to the antifungal efficacy was suggested.

Other antimicrobial genera from the Anthemideae tribe are the genera *Santolina* and *Senecio*. Haggag *et al.* [92] investigated the chemical composition and antimicrobial activity of the essential oil of *Santolina chamaecyparissus* L. cultivated in Egypt. In the essential oil,

seventy-four components (85.88% of total oil components) were identified. Artemisia ketone was found to be the major component. The oil showed a good antimicrobial activity *in vitro* against certain strains of bacteria and fungi. Other studies revealed that *Santolina chamaecyparissus* oil was effective in controlling candidiasis *in vitro* and *in vivo* [134,135]. It had a synergistic effect on clotrimazole in controlling *Candida albicans in vitro*. It significantly controlled vaginal and systemic candidiasis and was able to control superficial cutaneous mycoses.

The antimicrobial activity of the essential oil of *Santolina insularis* (Genn. ex Fiori) Arrig., an aromatic plant endemic to the island of Sardinia, Italy, was evaluated [136,137]. The results revealed significant antibacterial activity against Gram (+) and Gram (-) bacteria. The antiviral activity of this species, *Santolina insularis*, was also reported [138]. The antiviral activity was studied against HSV-1 and herpes simplex virus type II (HSV-2) by plaque reduction and yield reduction assays. *Santolina insularis* essential oil was effective in inactivating HSV-1 and HSV-2 and this activity was principally due to direct virucidal effects. Antiviral activity against HSV-1 and HSV-2 was not observed in a post-attachment assay, and attachment assays indicated that virus adsorption was not inhibited. Furthermore, reduction of plaque formation assays also showed that *Santolina insularis* essential oil inhibits cell-to-cell transmission of both HSV-1 and HSV-2 [139].

From the genus *Senecio*, El Shazly *et al.* [140] investigated the chemical composition and biological activity of the essential oils of *Senecio aegyptus* var. *discoideas* Boiss. Volatile oils from the flowers and leaves showed significant levels of antifungal activity against *Candida albicans*, and moderate effects against Gram (+) bacteria. However, they had weak activity against Gram (-) bacteria. Analysis of the oils was perfomed and thirty-four and thirty-seven compounds were identified. The main component was isolated and characterized as the sesquiterpene 1,10-epoxyfurano-eremophilane. The oils from the flowers, leaves and stems were rich in monoterpene hydrocarbons while the root oil mainly contained furanoeremophilanes. Another *Senecio* species cultivated in Egypt, *Senecio mikanioides* Otto, yielded forty-five and twenty-one components, representing 88.28% and 80.44% of the total composition of the oils of fresh aerial parts and underground organs, respectively [141]. The two investigated oils are rich in hydrocarbons. α-pinene and β-myrcene are the main compounds in the oil from the plant aerial parts,

while dehydroarmadendrene and camphene are the major ones in the essential oil from the underground organs. The oils showed significant antimicrobial activity against some tested microorganisms.

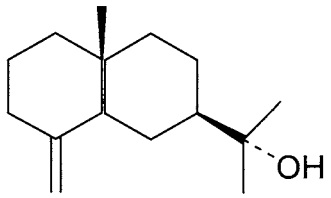

Fig. (8). Structure of β-eudesmol

Other antimicrobial species from the *Senecio* genus are *Senecio graveolens* Weed. and *Senecio desfontainei* Druce. The chemical composition of the oil obtained from *Senecio graveolens* was analyzed [142]. The components identified were: α-pinene, α-phellandrene, α-terpinene, *p*-cymene, sabinene, γ-terpinene, terpinolene, terpinen-4-ol and β-eudesmol, Fig. **(8)**. The investigation by the agar-well diffusion method of the antimicrobial activity showed that it has antibacterial effects on *Micrococcus luteus* oxacillin-sensitive and oxacillin-resistant, *Staphylococcus aureus* as well as antifungal effects on clinically-isolated *Candida albicans*. The essential oil of *Senecio desfontainei* was analyzed, and a total of forty one compounds were identified [143]. Oil profiles and content of the flowers, leaves, stems and roots were determined. In the flowers, β-myrcene, cymene, phellandrene and dehydrofukinone are the major constituents. In the leaves and stems, dehydrofukinone was the major component, constituting 42.8% and 77.6% of the oils, respectively. The major components of the root oil were eremophilane, β-caryophyllene, Fig. **(3)** and (*Z*)-β-farnesene. The antimicrobial activity of the oils was evaluated and showed broad and powerful activity against 2 Gram (+), 2 Gram (-) bacteria and 2 fungi.

Reports on the chemical composition and antimicrobial activity of other genera from the Anthemideae tribe have also been found in the literature. The essential oil from aerial parts of *Eupatorium cannabinum* L. ssp. *cannabinum*, which shows interesting antibacterial activity mostly

against Gram (+) bacteria, were chemically analyzed [144]. Fifty-nine compounds were identified accounting for 94.1% of the oil. Germacrene D, Fig. **(7)** was the most abundant component, with appreciable amounts of α-farnesene and δ-2-carene. Of the oxygen-containing components, elemol and α-cadinol were the most abundant. In the essential oil of another *Eupatorium* species, *Eupatorium patens* Hook, the main components were β-caryophyllene, Fig. **(3)**, γ-murolene and α-pinene [145]. Analysis to determine their antimicrobial action showed potent activity against *Bacillus subtilis*. Examples of another antimicrobial essential oil from the genus *Eupatorium* also included *Eupatorium triplinerve* Vahl [146].

Belem *et al.* [147] investigated the antifungal activity of essential oil obtained from *Matricaria chamomila* L. The oil was tested *in vitro* over 20 strains of *Malassezia furfur* isolated from patients with versicolor phthiriasis. According to the test results, it was verified that the essential oil produced significant inhibiting activity. This oil also inhibited the growth of *Listeria monocytogenes* type 4A [78]. The essential oil of another *Matricaria* species, *Matricaria recutita* has a high content of farnesene, patchoulene, bisabolol and bisabolol oxide, which contributes to the oils fungicidal and bactericidal properties [148].

Soliman and Badena [149] tested the essential oils from twelve medicinal plants for inhibitory activity against *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus* and *Fusarium moniliforme* The essential oil of *Chamomille recutita* (L.) Rausch. were partially effective against the test toxigenic fungi. The oil also showed antimicrobial activity against *Staphylococcus aureus*, *Corynebacterium amycolatum*, *Escheria coli* and *Candida albicans* [150]. The antimicrobial activity of the essential oil of another *Chamomille* species, *Chamomille suaveolens* L., equalled or approached the effectiveness of those of *Chamomille recutita* [151]. Activity was found against *Staphylococcus aureus*, *Corynebacterium michiganense*, *Candida albicans*, *Helminthosporium gramineum*, *Trichothecium roseum* and *Bacillus cereus*.

Chemical analysis of the essential oil of anther Anthemideae species, *Asteriscus maritimus* (L.) Less. allowed the identification of fourteen components, among which the most abundant was myrtenyl acetate [152]. The presence of terpinen-4-ol was responsible for the antimicrobial activity of the oil.

The volatile constituents of *Conyza dioscorides* L. (Desf.) growing in Egypt showed promising antimicrobial activities against some tested microorganisms [153]. The volatile constituents consisted mainly of sesquiterpene hydrocarbons and oxygenated sesquiterpenes. The major sesquiterpene hydrocarbons are β-maaliene and α-elemene, while the major oxygenated sesquiterpene compounds are α-cadinol, muurolol, caryophyllene oxide isomers and a sesquiterpene alcohol.

The essential oils of two *Jasonia* species, *Jasonia candidans* L. and *Jasonia montana* Vahl Botseh. were also analyzed [154]. Intermediol was the main constituent in the volatile oil of *Jasonia candidans*, while camphor, Fig. **(1)**, borneol, Fig. **(4)**, bornyl acetate, chrysanthemol, intermediol and 1,8-cineole, Fig. **(2)** in the essential oil of *Jasonia montana*. The two oils showed antibacterial activity against *Bacillus subtilis*, and a marked antifungal activity against *Trichophyton mentagrophytes*, *Cryptococcus neoformans* and *Candida albicans*.

Examples of other antimicrobial essential oils from the Anthemideae tribe also included Roman chamomile (*Chamaemelum nobile* L.) [155], the essential oil of the aerial parts of *Baccharis notosergila* Griseb. [156], the essential oil of the herb *Kleinia odora* (Forssk) DC. [157], the essential oil isolated by steam distillation from *Ambrosia artemisifolia* L. dried flowering herb [158], and the essential oils from *Centaurea sessilis* Willd and *Centaurea armena* Boiss. which showed moderate antibacterial activity against Gram (+) and (-) bacteria, but no antifungal effect against 2 yeastlike fungi [159]. The main component of these oils was found to be β-eudesmol, Fig. **(8)**, while methyleugenol contained in the antimicrobial essential oil of *Echinophora sibthorpiana* Guss. (Cortuk) showed some inhibitory activity against bacteria and fungi [160].

TERPENOIDS

The diverse, widespread and exceedingly numerous class of natural products that are derived from a common biosynthetic pathway based on mevalonate as parent, are synonymously named terpenoids, terpenes or isoprenoids. Essentially, they are derived from the basic 5-carbon isoprene unit, biosynthetically as isopentenyl pyrophosphate, which is itself derived from acetate via mevalonic acid. They may be classified into diverse groups according to the number of isoprene units, e.g.,

monoterpenes (C_{10}), typical constituents of the essential oils, sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes and steroids (C_{30}),... Monoterpenes, sesquiterpenes, diterpenes and triterpenes are ubiquitous in terrestrial plants and play an essential role in life, carrying out many different kinds of functions.

During the last years, a large number of studies have been performed concerning the antimicrobial activity of terpenoids from the Anthemideae tribe. These reports concerned mainly sesquiterpenes and sesquiterpene lactones. Some of these compounds were isolated by bioassay-guided fractionation, after previously detecting antimicrobial activity on the part of the plant.

The antimicrobial activity of dichloromethane extracts (leaves, flowers and underground parts) and some bioassay-guided compounds isolated from *Calea platylepis* Sch. Bip. were evaluated by the well diffusion method [161]. Among other, the compound extracted was the sesquiterpene (+)-4α-7β-aromadendranediol, which demonstrated a broader spectrum of action inhibiting the growth of various strains of microorganisms (bacteria and fungi), with an MIC of 500 μg/ml.

The bioassay-guided fractionation of the antifungal dichloromethane extract from the roots of *Vernonanthora tweedieana* (Baker) H. Rob. allowed the isolation of one active sesquiterpene, identified as 6-cinnamoyloxy-1-hydroxyeudesm-4-en-3-one, Fig. **(9)** [162]. MIC values of this compound showed *Trichophyton mentagrophytes* as the most sensitive strain.

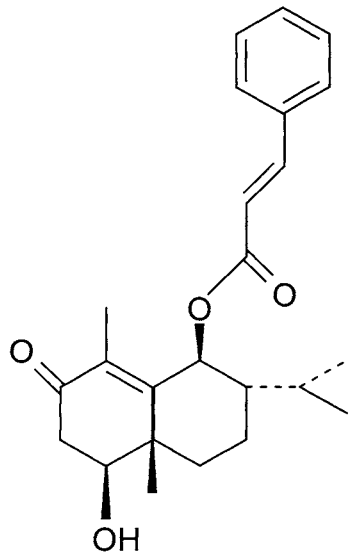

Fig. (9). Structure of 6-cinnamoyloxy-1-hydroxyeudesm-4-en-3-one isolated from *Vernonanthora tweedieana*

Al Dabbas *et al.* [163] reported the antibacterial activity of the ethyl acetate extract of the whole aerial part of *Varthemia iphinoides* L. The bioassay-guided fractionation led to the isolation and identification of an antibacterial eudesmane sesquiterpene, selina-4,11(13)-dien-3-on-12 oic acid, Fig. **(10)**. This compound exhibited potent antimicrobial activity against 6 bacterial species (*Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Bacillus cereus* and *Salmonella enteritides*). The MIC values of this sesquiterpene, which was determined by the agar dilution method, ranged between 250 and 500 μg/ml.

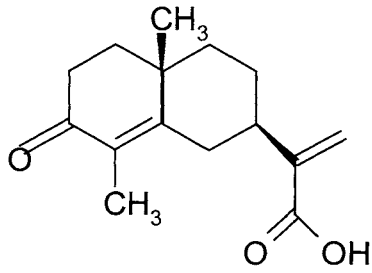

Fig. (10). Structure of selina-4,11(13)-dien-3-on-12 oic acid isolated from *Varthemia iphinoides*

Investigations of an antibacterial dichloromethane extract of the aerial parts of *Senecio aegyptus* var. *discoideus* afforded nine eremophylane-type sesquiterpenes, Fig. **(11)**, of which six are new [164]. The antibacterial activity of the isolated compounds was tested against *Bacillus cereus* and a *Serratia* spp. The growth of both microorganisms was inhibited by some of the compounds. From the whole plant of *Ligulariopsis shichuana* Y L Chen, Wang *et al.* [165] isolated other new eremophilenolides. All of these sesquiterpenes have eremophylane skeletons with 7(11)-en-8(12) lactone units. These compounds showed moderate antibacterial activities towards *Escherichia coli* and *Bacillus subtilis*.

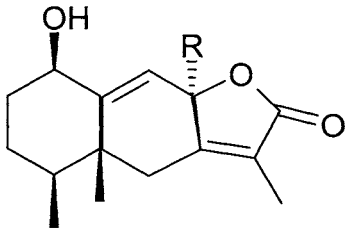

Fig. (11). Structure of eremophylane-type sesquiterpenes isolated from *Senecio aegyptus* var. *discoideus*

Meepagala *et al.* [166] detected antifungal activity in *Artemisia douglasiana* Besser against 3 *Colletotrichum* species. The active principle was isolated by bioassay-directed fractionation and was characterized as the sesquiterpene vulgarone B. Antifungal activity of vulgarone B was further evaluated using 96-well microtiter assay against *Colletotrichum*

acatatum, *Colletotrichum fragariae*, *Colletotrichum gloeosporoides* and *Botrytis cinerea*. Structure-activity studies revealed that the α,β-unsaturated carbonyl functionality is a prerequisite for the antifungal activity of this sesquiterpene ketone. Antifungal fractions derived from the chloroform extract of another *Artemisia* species, *Artemisia annua*, afforded two cadinane derivatives, arteannuin B, Fig. **(12)** and artemisinin [167]. Arteannuin B, Fig. **(12)**, the main sesquiterpenoid in *Artemisia annua*, showed antifungal activity against one human (*Candida albicans*) and 4 plant pathogenic fungi (*Gaeumanomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Gerlachia nivalis* and *Verticilum dohliae*).

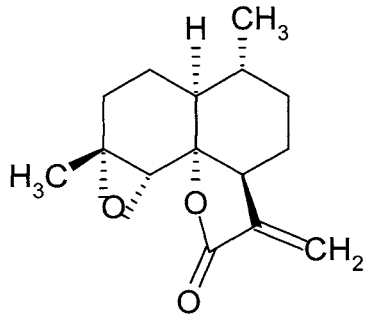

Fig. (12). Structure of arteannuin B

Cernuole is a sesquiterpene isolated from *Bidens cernua* [168,169]. This compound suppresses *in vitro* the growth of Gram (+) bacteria and microdermatophytes in concentration of 5-20 mg/l, feebly active as regard to Gram (-) bacteria.

The methanol extract of the campsite plant *Xanthium strumarium* (Cocklebur) displayed antimicrobial activity against *Proteus vulgaris*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Candida pseudotropicalis* [170]. This activity was attributable to the presence of the sesquiterpene xanthanol.

Antibacterial bisabolane sesquiterpenes and tricyclic sesquiterpenes, the latter consisting of an irregularly built C_{15} backbone, were also isolated from *Leontopodium alpinum* [36]. These compounds showed significant antibacterial activity towards *Enterococcus faecium*,

Staphylococcus aureus, *Streptococcus pneumoniae* and *Streptococcus pyogenes* strains.

Examples of other antimicrobial sesquiterpenes from the Anthemideae tribe also included four new oxygenated bisabolane fucosides from *Carthamus lanatus*, which exhibited noticeable antibacterial activity [171], and trichothecenes. Trichothecenes are a familly of sesquiterpenoid compounds produced by various genera of fungi. However, a Brazilian shrub from the Anthemideae tribe, *Baccharis megapotamica* Spreng., was reported to contain a series of potent antimicrobial agents belonging to the trichothecene complex [172]. From another *Baccharis* species, *Baccharis coridifolia* DC., four macrocyclic trichothecenes, roridin A and E and verrucanin A and J, were isolated. Garcia *et al.* [173] investigated their inhibitory activity against the arenavirus Junin, the ethiological agent of Argentinian hemorrhagic fever. The most active compound was verrucanin J. From time-of-addition and removal experiments, it can be concluded that verrucanin J inhibited a later stage in the replicative cycle of this virus after 5 hours of adsorption.

Antimicrobial substances from the rhizomes of chicory (*Cichorium intybus*) were studied, in connection with a proposed defensive mechanism [174]. A new antimicrobial sesquiterpenoid, 8α-angeloyloxycichoralexin, in addition to guaianolides such as cichoralexin and 10α-hydroxyciclopumilide was isolated. These sesquiterpenes exhibited antifungal activities against *Pericularia oryzae*, *Pellicularia sasakii* and *Alternaria kikuchiana.*

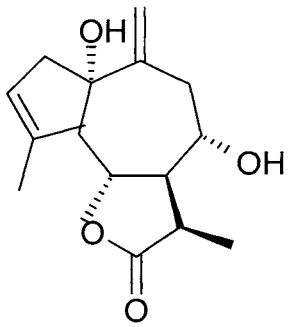

Fig. (13). Structure of rupicolin A

Examples of other antimicrobial sesquiterpenes from the Anthemideae tribe also included new sesquiterpenes from the hexane extract of aerial parts of *Santolina rosmarinifolia* subsp. *canescens* (Lagasca) Nyman [175], and the guaiane sesquiterpenes rupicolin A, Fig. **(13)** and B, 1-deoxy-1α-peroxy-rupicolin A and B isolated from *Achillea clavennae* [25].

Studies into other antimicrobial sesquiterpenes from the Anthemideae tribe also included sesquiterpene lactones. These reports concerned mainly the genus *Centaurea*. In the search for new antifungal sources of sesquiterpene lactones, Barrero *et al.* [176] investigated six *Centaurea* species from Spain and Morocco, *Centaurea bombycina* Boiss. ex DC., *Centaurea granatensis* Boiss., *Centaurea monticola* Boiss. ex DC., *Centaurea incana* Desf., *Centaurea maroccana* Ball and *Centaurea sulphurea* Willd. The activity against the fungus *Cunnighamella echinulata* of the sesquiterpene lactones isolated from the *Centaurea* plants, cnicin, salonitenolide, costunolide, Fig. **(14)**, dehydrocostunolide, Fig. **(15)**, lychnopholide and eremantholide has been evaluated. Costunolide, Fig. **(14)** and dehydrocostunolide, Fig. **(15)** showed noticeable MIC values, while more polar lactones were inactive. These results suggest that a relatively low polarity is one of the molecular requirements for the antifungal activity of sesquiterpene lactones.

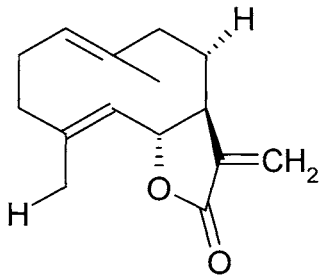

Fig. (14). Structure of costunolide

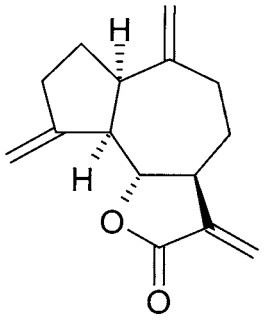

Fig. (15). Structure of dehydrocostunolide

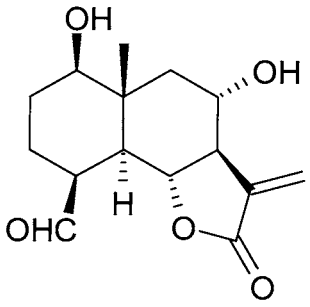

Fig. (16). Structure of 4-*epi*-sonchucarpolide

Three new eudesmanolides, 4-*epi*-sonchucarpolide, Fig. **(16)**, its 8-(3-hydroxy-4-acetoxy-2-methylene-butanoyloxide) derivative and atticin, Fig. **(17)** have been isolated from other *Centaurea* species from Greece, *Centaurea thessala* Hausskn. var. *drakiensis* and *Centaurea attica* Nym. var. *attica* [177]. The *in vitro* antifungal activity of most compounds was tested against 9 fungal species using the microdilution method. All the compounds tested showed great antifungal activity. Examples of other antimicrobial sesquiterpene lactones from the *Centaurea* genus also included new eudesmanolides and one new elemane derivative from *Centaurea deusta* Ten., which showed high antifungal activity [178] and malacitanolide and the germacranolide cnicin isolated from *Centaurea spinosa* L., which were active against 3 Gram (+) and 3 Gram (-) bacteria [179].

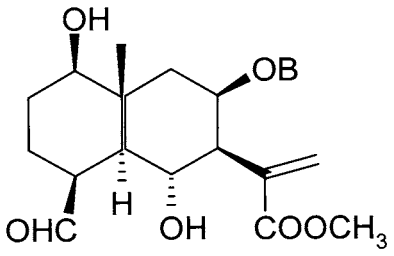

Fig. (17). Structure of atticin

Other genera from the Anthemideae tribe also yielded sesquiterpene lactones with a broad spectrum of antibacterial activity. *Vernonia colorata* (Will) Drake is used throughout Africa for a variety of ailments, including infectious diseases. Through conventional chromatographic techniques and bioassay-guided fractionation, Rabe *et al.* [180] isolated the sesquiterpene lactones vernolide, 11β,13-dihydrovernolide and vernodalin, Fig. **(18)**. Only 11β,13-dihydrovernolide is a novel compound, although its antibacterial activity is low compared to the other compounds, which had MIC values of 0.1-0.5 mg/ml against Gram (+) bacteria. From another *Vernonia* species, *Vernonia arborea* HK, zaluzanin D, Fig. **(19)**, an antifungal sesquiterpene lactone has been isolated as a major constituent [181].

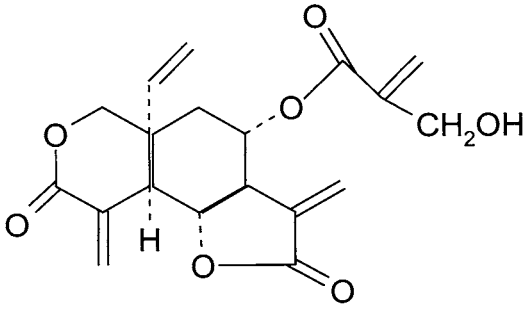

Fig. (18). Structure of vernodalin

Cho *et al.* [182] initiated studies for the isolation of the antibacterial constituents from the whole plant of *Artemisia princeps* var. *orientalis*, which were active towards 9 human intestinal bacteria. The biologically active constituents of the *Artemisia* whole plant were characterized as the sesquiterpene lactones tanapartholide A and B, Fig. **(20)**, which produced a clear inhibitory effect against *Clostridium perfringens*, *Bacteroides fragilis* and *Staphylococcus aureus*. These compounds did not affect the growth of the lactic acid-producing bacteria (*Bifidobacterium adolescentis*, *Bifidobacterium breve*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and *Escherichia coli*, whereas weak growth inhibition towards *Bifidobacterium bifidum* was observed. These naturally occurring *Artemisia* whole-plant-derived materials could be useful as a new preventive agent against various diseases caused by harmful intestinal bacteria such as clostridia.

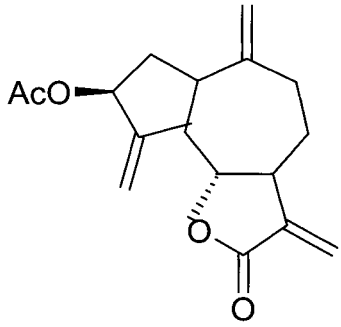

Fig. (19). Structure of zaluzanin D

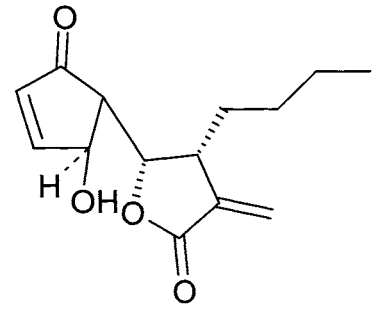

Fig. (20). Structure of tanapartholide

Artemisinic acid, a biogenetic precursor of artemisinin, is an important sesquiterpene lactone produced by the herb *Artemisia annua*, which was active against different bacteria and certain fungal species [183]. Examples of other antimicrobial sesquiterpene lactones from the *Artemisia* genus also included tauremisin, artemin and taurin from the above-ground part of *Artemisia taurica* Willd. [184], the eudesmanolide 8α-hydroxytaurin from Turkish *Artemisia santonicum* [185] and santonin, Fig. **(21)** from *Artemisia cina* Berg, which exhibited significant antimicrobial activity against *Bacillus subtilis* and *Bacillus cereus* [186].

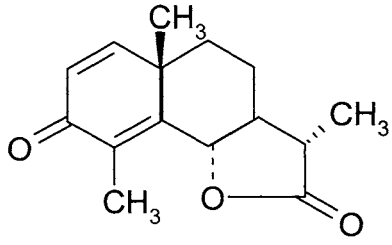

Fig. (21). Structure of santonin

Other genera from the Anthemideae tribe such as *Inula*, *Tanacetum* and *Xanthium* also yielded sesquiterpene lactones with a broad spectrum of antibacterial and antifungal activity. Isoalantolactone, a major constituent of *Inula racemosa* Hook. f., was tested for its

antimicrobial activity against 5 bacteria, 6 human and 6 plant pathogenic fungi [187]. The sesquiterpene lactone showed toxicities at 500 μg/ml against 3 soil borne phytopathogenic fungi (*Gaeumannomyas graminis* var. *tritici*, *Rhizoctonia cerealis* and *Phytophthora capsici*). Moreover, isoalantolactone displayed weaker antibacterial activities towards *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Sarcina lentus* and *Staphylococcus aureus*. The investigation disclosed the strong inhibition of isoalantolactone to phytopathogenic fungi, raising a possibility that the lactone could be considered as a starting point for projects aimed at the development of new fungicides. Another sesquiterpene lactone, tomentosin, has been isolated and identified from *Inula viscosa* flowers from the Puglia region of Italy [188]. The molecule at 1 mg/ml concentration proved to be active *in vitro* against *Microsporum canis*, *Microsporum gypseum* and *Trichophyton mentagrophytes*. This plant also yielded a new sesquiterpene lactone, tayunin, which inhibits the growth of *Microsporum canis* at a concentration of 10 μg/ml and of *Trichophyton rubrum* at 50 μg/ml [189]. Examples of other antimicrobial sesquiterpene lactones from *Inula* genus also included the guaianolides- and eudesmanolides-type from *Inula montana* L. [190].

Jang *et al.* [191] investigated the antimicrobial activities of sesquiterpene lactones isolated from two species from the *Chrysanthemum* genus, *Chrysanthemum zawadskii* Herbich and *Chrysanthemum boreale*. Tulipinolide exhibited strong antibacterial activity, while angeloylcumambrin B, tigloylcumambrin B and costunolide, Fig. **(14)** showed a broad antifungal activity. The species *Chrysanthemum boreale* also yielded the antimicrobial sesquiterpene lactone cumambrin B [192]. Stefanovic *et al.* [193] reported the antimicrobial activity of Yugoslav plant species of the genus *Tanacetum*. Antimicrobial activities of sesquiterpene lactones from *Tanacetum parthenium*, *Tanacetum macrophyllum* (Waldst. & Kit.) Schultz., *Tanacetum vulgare* and *Tanacetum serotinum* (L.) Schultz-Bip were proved *in vitro* with following microorganisms: *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* spp.

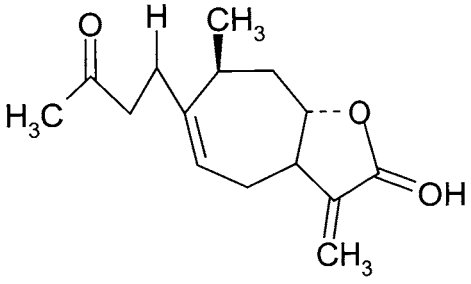

Fig. (22). Structure of xanthatin

Bioassay-guided fractionation of the antimicrobial dichloromethane extract from *Xanthium spinosum* L. yielded xanthatin, Fig. **(22)** [194]. This compound was active against *Colletotrichum gloesporoides*, *Trichotecium roseum*, *Bacillus cereus* and *Staphylococcus aureus*. These compounds together with another sesquiterpene lactone xanthinin, Fig. **(23)** were also isolated from *Xanthium italicum* Moretti [195].

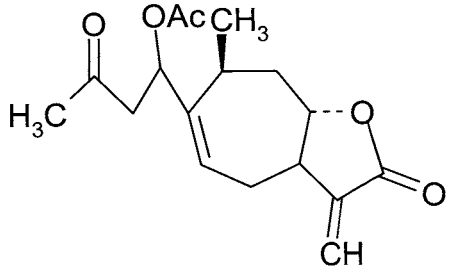

Fig. (23). Structure of xanthinin

Examples of other antimicrobial sesquiterpene lactones from the Anthemideae tribe also included those isolated from *Anaphalis margaritacea* (L.) Benth & Hook., Fig. **(24)** [196], two new antibacterial melampolide-type sesquiterpene lactones from *Smallanthus sanchifolius* (Poepp. and Endl.) H. Robinson, which exhibited potent antimicrobial activity against *Bacillus subtilis* and *Pyricularin oryzae* [197], and six

matricaria esters and two matricaria lactones isolated from *Matricaria chamomilla* [198].

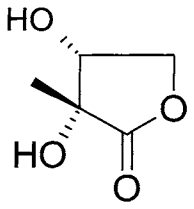

Fig. (24). Structure of hydroxylactones isolated from *Anaphalis margaritacea*

In the diterpenoid and triterpenoid groups, there are only a few reports concerning antimicrobial activity in the Anthemideae tribe. The resinous exudates of *Baccharis grisebachii*, which is used to treat ulcers, burns and skin sores in Argentina, showed activity towards dermatophytes and bacteria [199]. Two diterpenes were isolated by guided bioassay from the exudate. One of these, the diterpene labda-7,13*E*-dien-2β-15-diol, Fig. **(25)** was active towards *Epidermophyton floccosum* and *Trichophyton rubrum* with MIC values of 12.5 μg/ml, while the MIC against *Microsporum canis* and *Trichophyton mentagrophytes* was 25 μg/ml. The diterpene was also active towards *Microsporum gypseum* and showed inhibition in both *Staphylococcus aureus* tested (methicillin-resistant and methicillin-sensitive strains).

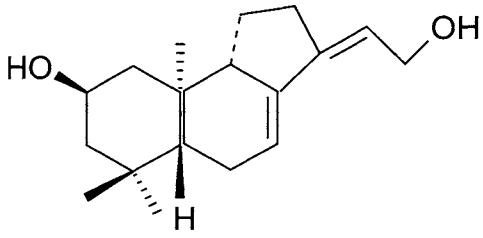

Fig. (25). Structure of labda-7,13*E*-dien-2β-15-diol isolated from *Baccharis grisebachii*

The antimicrobial properties of the resinous exudates from twigs and leaves of *Eupatorium salvia* Colla were tested against 5 Gram (-) and 5 Gram (+) bacteria [200]. Comparison of the antimicrobial activities of 7-hydroxy-8(17)-labden-15 oic acid (salvic acid) and its acetate, both compounds isolated from the plant, with that of the crude extract suggested that the latter ester derivative was the major active component in the exudates. These results validate the vernacular medicinal use of *Eupatorium salvia* as an antiseptic agent.

Mendoza *et al.* [201] investigated the antibacterial activity of 13-*epi*-sclareol, a labdane type diterpene isolated from the resinous exudates of *Pseudognaphalium heterotrichium* (Phil.) A. Anderb. and *Pseudognaphalium cheiranthifolium* (Lam.) Hilliard et Burtt. Gram (+) bacteria were selectively affected by 13-*epi*-sclareol. At concentrations of 30 μg/ml, the compound produced the lysis of *Bacillus cereus* and *Bacillus subtilis*. More recently, the interaction of 13-*epi*-sclareol with the bacterial respiratory chain was analyzed [202]. The compound inhibited oxygen consumption of intact Gram (+) cells, but not Gram (-) bacteria. These results suggest that the target site of 13-*epi*-sclareol is located between coenzyme Q and cytochrome C. Using cytoplasmic membrane fractions, the results of the analysis of the enzyme activities associated with the respiratory chain complexes were the same for both Gram (+) and Gram (-) bacteria, indicating that the compound has no access to the cytoplasmic membrane of intact Gram (-) bacteria. Thus, the Gram (-) envelope may act as a physical barrier that prevents the access of this compound to the site of action. From a related genus, the Colombian species of *Gnaphalium*, Ruben and Torrenegra [203] isolated several diterpenes which show antimicrobial activity.

Other antimicrobial terpenes from the Anthemideae tribe are pyrethrins. Bioassay-directed fractionation of the organic extract of Kenyan pyrethrum flowers (*Chrysanthemum cinerariifolium* Vissiani) resulted in the isolation of two natural pyrethrin esters, pyrethrins I and II, as the major constituents [204]. These esters elicited inhibition of the multiple drug resistant *Mycobacterium tuberculosis*.

Wedelia paludosa DC., a traditionally-used native Brazilian medicinal plant, showed antifungal activity against dermatophytes in dilution tests [205]. The hexane, dichloromethane and butanol extracts displayed activity against *Epidermophyton floccosum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes* with MIC values between 250

and 1000 μg/ml. Bioassay-guided fractionation led to the isolation of the diterpene kaurenoic acid, which also showed activity against these dermatophytes.

Examples of other antimicrobial diterpenoids from the Anthemideae tribe also included a nor-ent-kaurane diterpene, Fig. **(26)** from the aerial parts of *Antennaria geyeri* L. [196], three new diterpenes jasonin A-C isolated from the aerial parts of *Jasonia montana* [206] and the terpenes 15-hydroxy-7-labden-17-oic acid, Fig. **(27)** and their acetate from *Eupatorium glutinosum* Matico, which were active against 2 Gram (-) and 2 Gram (+) bacteria [207,208].

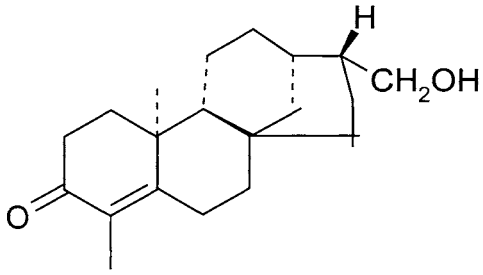

Fig. (26). Structure of nor-ent-kaurane diterpene isolated from *Antennaria geyeri*

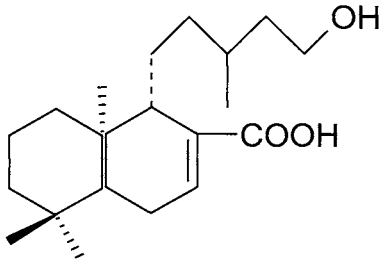

Fig. (27). Structure of 15-hydroxy-7-labden-17-oic acid isolated from *Eupatorium glutinosum*

In the triterpenoid group, Almanza *et al.* [209] isolated the triterpenoid oleanolic acid, Fig. **(28)** from *Baccharis leptophylla* DC. Data is reported on the activity of this compound against *Candida albicans*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Microsporum canum*. This compound together with its methyl ester as

well as erythrodiol were also isolated from the species from the Mexican state of Veracruz, *Baccharis conferta* H.B.K. [210]. Oleanolic acid, Fig. **(28)** shows weak antibacterial activity against *Micrococcus luteus* and *Escherichia coli.*

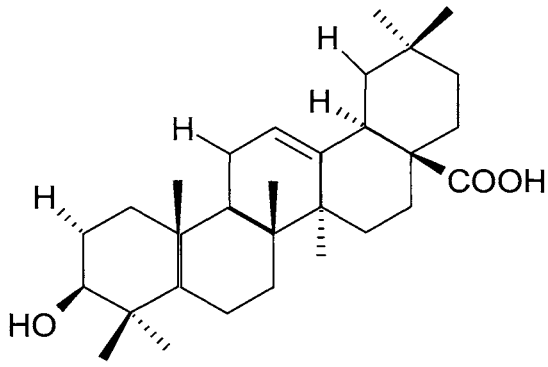

Fig. (28). Structure of oleanolic acid

The detection of triterpenoid compounds in extracts from *Bidens pilosa* may rationalize the use of this plant in traditional Egyptian medicine in the treatment of wounds and against bacterial infections of the gastrointestinal tract [211,212]. The chloroform extract gave β-amyrin, Fig. **(29)**, phytosterin B and β-sitosterol glucoside, while the petroleum ether extract afforded β-amyrin, Fig. **(29)**, phytosterin B, lupeol, Fig. **(30)**, lupeol acetate, linolic acid and linolenic acid. The antimicrobial test was carried out to indicate an evident activity.

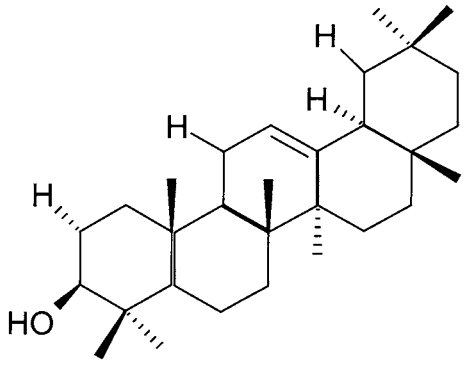

Fig. (29). Structure of β-amyrin

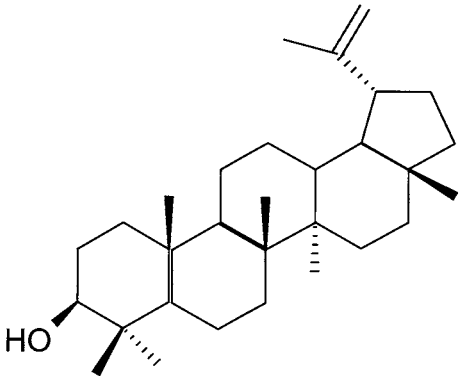

Fig. (30). Structure of lupeol

Examples of other antimicrobial triterpenoids from the Anthemideae tribe also included two new pentacyclic triterpenoids from *Aster ageratoides* Turcz. var. *pilosus* Willd [213] and β-sitosterol from *Senecio lyratus* Michaux with *in vitro* antifungal and antibacterial activities [214].

Compounds chemically related with the triterpenoid group such as triterpene saponins were also isolated as antimicrobial constituents in the Anthemideae tribe. Seven saponins were bioassay-guided isolated from the flowers of *Bellis perennis* L. [215]. Compounds had an antimicrobial

effect on the microorganisms *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Candida monosa* (nystatin-sensitive). Ethanol extracts of whole plants of this species also yielded a fraction which was shown to contain the saponin polygalacic acid [216]. *In vitro* and *in vivo* testing demonstrated activity against *Ceratocystis ulmi*, the fungus responsible for Dutch elm disease. The triterpenoid polygalacic acid bisdesmoside bellisaponin 1, and the corresponding monodesmoside (prosapogenin) isolated from *Bellis perennis* inhibited the growth of *Candida* and *Cryptococcus* spp. *in vitro* [217]. The bisdesmoside was more active than the prosapogenin.

PHENOLIC COMPOUNDS

Phenols probably constitute the largest group of plant secondary metabolites. Widespread in nature, and found in most classes of natural compounds having aromatic moieties, they range from simple structures with one aromatic ring to highly complex polymeric substances. Phenols are important constituents of some medicinal plants and they are used in the food industry as colouring agents, flavourings, aromatizers and antioxidants. In recent years, a large number of studies have been performed concerning the antimicrobial activity of phenolic compounds of the Anthemideae tribe. These reports concerned mainly flavonoids and coumarins.

Flavonoids are natural polyphenolic substances widely distributed in the different parts of plants such as fruits, bark, stems, roots, leaves and flowers. Structurally they are characterized by a pyran ring or a similar structure of three carbons. These polyphenolic compounds are well known for displaying a remarkable spectrum of biological activities, including antibacterial and antifungal properties. In the Anthemideae tribe, some of these compounds were isolated by bioassay-guided fractionation, after previously detecting antimicrobial activity on the part of the plant.

Bioassay-guided fractionation of an extract of *Artemisia annua* was conducted in order to assess the possible presence in the plant material of inhibitors of bacterial multidrug resistance pumps [218]. Fractions were tested for *Staphylococcus aureus* growth inhibition in the presence of a subinhibitory dose of weak antibacterial alkaloid berberine. Active fractions yielded the flavones chrysoplenol D and chrysoplenetin,

which themselves had very weak growth inhibitory action, but which made a potent combination with berberine.

Other species from the *Artemisia* genus also yielded antimicrobial flavonoids. Flavonoids isolated from the Polish medicinal herbs *Artemisia molinieri*, *Artemisia selengensis* Turcz. ex Bess. and *Artemisia stolonifera* (Maxim.) Kom. showed antifungal activity against some species of phytopathogenic fungi [219].

Two new flavones, 4',6,7-trihydroxy-3',5'-dimethoxyflavone and 5',5-dihydroxy-3',4',8-trimethoxyflavone, were isolated from *Artemisia giraldii* Pamp. [220]. These two new flavones showed antimicrobial activity towards *Staphylococcus aureus*, *Sarcinia lutea*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* spp., *Aspergillus flavus* and *Trichoderma viride*.

Aerial parts of *Artemisia sublessingiana* Kell. Krasch et Poljak afforded isorhamnetin-3-*O*-rutinoside, hispidulin, 5,7,4'-trihydroxy-6,3'-dimethoxyflavone and chrysoeriol [221]. The individual compounds were tested *in vitro* against Gram (-) and Gram (+) bacteria, phytopathogenic and dermatophytic fungi, and other microorganisms. All suppressed growth of *Trichophyton gypseum* (MIC of 6.1 μg/ml), and some of them exhibited good activity against *Fusarium solani*, *Botrytis cinerea* and *Verticilium dahliae*.

Zhu *et al.* [222] examined the antimicrobial activities of four flavonoids, luteolin-7-rutinoside, cynaroside, Fig. **(31)**, apigenin-7-rutinoside and apigenin-7-*O*-β-*D*-glucopyranoside, isolated from the n-butanol soluble fraction of artichoke leaf extracts (*Cynara scolymus*). The compounds showed activity against most of the tested organisms, and were more effective against fungi than bacteria. The MIC values of these compounds were between 50 and 200 μg/ml.

The bioassay-guided fractionation of the ethanolic and etheric leaf and inflorescence extracts from *Centaurea ruprestis* yielded the flavonoid quercetagetin T-methyl ether 7-*O*-β-*D*-glucopyranoside [27]. The antimicrobial spectrum of quercetagetin was broad, but weak for Gram (-) bacteria and moderate for dermatophytes. Specifically, it showed antibacterial activity against *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella java* and *Serratia* spp.

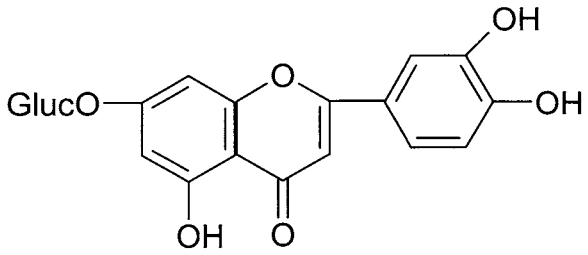

Fig. (31). Structure of cynaroside

Three flavonoids, 5,7-dihydroxy-4'-methoxyflavone, 3,5,7-trihydroxy-4'-methoxyflavone and 3,5,7-trihydroxy-3'-methoxyflavone, were isolated in a bioguided study from the dichloromethane extract of *Baccharis leptophylla* [209]. Data on the activity of these compounds against *Candida albicans*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Microsporum canum* is reported. From another *Baccharis* species, *Baccharis peduculata* (Mill.) Cabrera., a flavone ether was isolated as an antifungal principle [223].

Systematic bioguided chemical investigation of *Echinops echinatus* Roxb. yielded apigenin-7-*O*-glucoside, echinacin, Fig. **(32)** and echinacitin [224,225]. Antifungal activity was assayed in a spore germination test with *Alternaria tenuissima*, which incites leaf blight disease in pigeon pea. All compounds showed high efficacy towards the pathogen at 25-150 μg/ml.

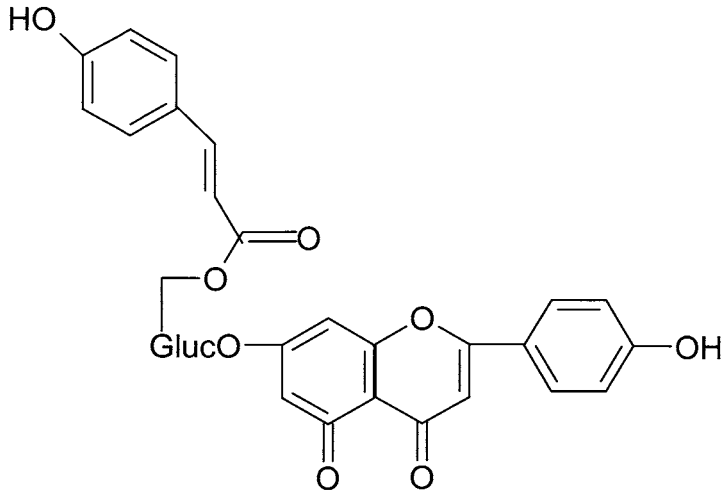

Fig. (32). Structure of echinacin

From the drug flos Calendulae (*Calendula officinalis* L.), two flavonoids were isolated by bioassay-guided fractionation [226]. They showed potent antimicrobial activity against the following microorganisms: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Candida monosa* and *Sarcinia lutea*.

The antimicrobial activity of another flavonoid, apigenin, Fig. **(33)** isolated from *Moquinia kingii* Gamerro, was also screened using 22 strains including Gram (+) and (-) bacteria and the yeasts *Candida albicans* and *Candida tropicalis* [227]. The compound was active for both activities.

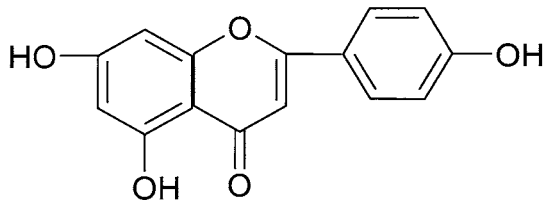

Fig. (33). Structure of apigenin

Other flavonoids from the Anthemideae tribe also showed a broad spectrum of antimicrobial activity. Crude extracts of *Haplopappus sonorensis* (A. Gray) S.F. Blake showed activity against *Mycobacterium tuberculosis* [228]. 5-hydroxy-3,7,4'-trimethoxyflavone, 5,7-dihydroxy-3,4'-dimethoxyflavone (ermanin), Fig. **(34)** and 5,4'-dihydroxy-3,7-dimethoxyflavone were identified by assay-guided fractionation, as the antimycobacterial principles. The flavonoid ermanin, Fig. **(34)** was the most active compound.

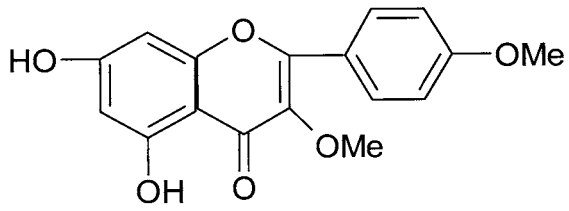

Fig. (34). Structure of ermanin

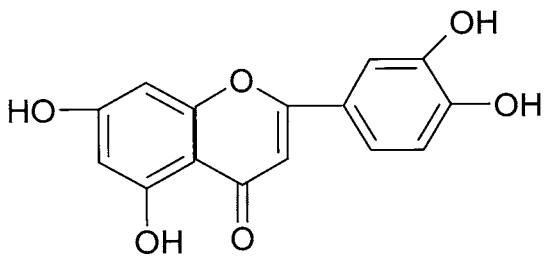

Fig. (35). Structure of luteolin

The antimicrobial activity of luteolin, Fig. **(35)**, a flavonoid isolated from *Wedelia paludosa* was also evaluated [205]. The compound showed activity towards the dermatophytes *Epidermophyton floccosum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

The antimicrobial polymethylated flavones, 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone, 5-hydroxy-3,6,7,8-tetramethoxyflavone and 5,6-dihydroxy-3,7-dimethoxyflavone, have been isolated from *Gnaphalium affine* D Don. [229,230]. These flavonoids showed antimicrobial activity against *Spodoptera litura*. Structure-activity studies suggested the importance of the 6-position substitution of the flavonoid; however, hydrophilic substituents decreased the activity. The flavonoid 8-*O*-(2-methyl-2-butenoyl)-5,7-dihydroxy-3-methoxyflavone isolated from *Gnaphalium robustum* L. also inhibited the growth of *Escherichia coli* [231].

Dai *et al.* [232] isolated two new flavone glycosides, 3,5,6-trihydroxy-4'-methoxyflavone-7-β-*D*-glucoside, Fig. **(36)** and 3,5,6-trihydroxy-4'-methoxyflavone-7-β-*D*-galactoside, Fig. **(37)** from *Serratula strangulata* Iljin. Both compounds exhibited *in vitro* antibacterial activity against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*.

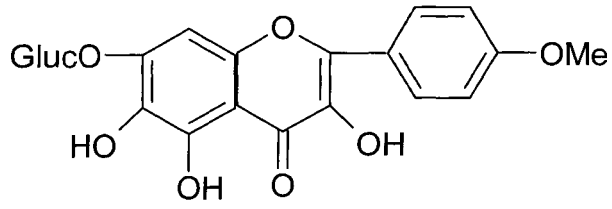

Fig. (36). Structure of 3,5,6-trihydroxy-4'-methoxyflavone-7-β-*D*-glucoside isolated from *Serratula strangulata*

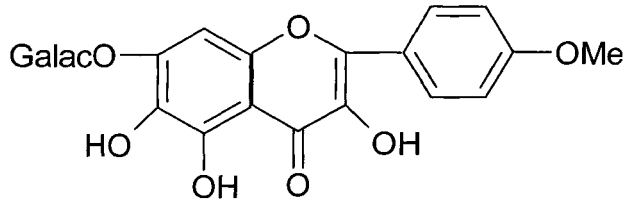

Fig. (37). Structure of 3,5,6-trihydroxy-4'-methoxyflavone-7-β-*D*-galactoside isolated from *Serratula strangulata*

Silybin is a flavonoid derived from *Silybum marianum* L. with potent antibacterial activity towards Gram (+) bacteria, although it has no antimicrobial activity against Gram(-) bacteria or fungi. Lee *et al.* [233] investigated the mode of action of silybin against the Gram (+) bacteria cell. The results showed that silybin inhibited RNA and protein synthesis in Gram (+) bacteria.

Other flavonoids from the Anthemideae tribe also showed a broad spectrum of antifungal activity, such as those isolated from the genus *Eupatorium*. Methylpariochromene A, a root constituent of *Eupatorium riparium*, displayed antifungal activity against 5 of the 7 fungal species tested [234]. The flavonoid showed toxicity to the fungus *Colletotrichum gloeosprioides*, a tropical pathogen, comparable to that of a reference compound fungicide, although the compound appeared to be fungistatic. Two flavonoids isolated from another *Eupatorium* species, *Eupatorium toppingianum* Elmer (IK), 6-hydroxy-7,8-dimethoxy-2,2-dimethylchromene and 6-(1-hydroxyethyl)-7,8-dimethoxy-2,2-dimethylchromene, were active against *Trichophyton mentagrophytes* [235].

Examples of other antimicrobial flavonoids from the Anthemideae tribe also included those from the Argentinian *Tagetes* [236], from *Anthemis cotula* L. [237], six flavonols isolated from *Helichrysum conglobatum* (Viv.) Steud. growing in Egypt [238] and another *Helichrysum* species, *Helichrysum compactum* Boiss., which yielded several flavonoids with antibacterial and antifungal activity [239].

Other antimicrobial phenolic compounds from the Anthemideae tribe are coumarins, which are derivatives of benzo-α-pyrone. Feresin *et al.* [199] isolated eight *p*-coumaric acid derivatives from the Argentinian medicinal plant *Baccharis grisebachii*. Two of them, 3-prenyl-*p*-coumaric acid, Fig. **(38)** and 3,5-diprenyl-*p*-coumaric acid were active towards *Epidermophyton floccosum* and *Trichophyton rubrum*, with MIC values of 50 and 100-125 μg/ml, respectively.

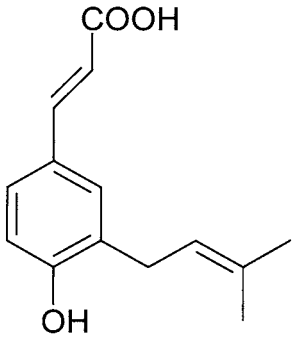

Fig. (38). Structure of 3-prenyl-*p*-coumaric acid isolated from *Baccharis grisebachii*

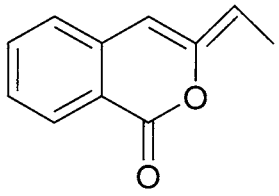

Fig. (39). Structure of corfin

From the roots of *Chaptalia nutans* (L.) Polak., a plant traditionally used in Brazilian folk medicine, Truiti *et al.* [240] isolated the pure compound 7-*O*-β-*D*-glucopyranosyl-nutanocoumarin. The compound inhibited *Bacillus subtilis* and *Staphylococcus aureus* at concentrations of 62.5 g/ml and 125 g/ml, respectively. The antibacterial property of *Chaptalia nutans* appears to justify its use for the treatment of wounds which are contaminated through bacterial infections.

Seven new naturally occurring 3-butylisocoumarins were also isolated and identified from *Chamaemelum mixtum* (L.) All. and *Artemisia dracunculus* L. [241]. Some of these compounds, corfin, Fig. **(39)** and artemidinol, Fig. **(40)** were active against a susceptible strain of the rice blast fungus *Pyricularia grisea.* The 3-butyl side-chain is a prerequisite for high activity.

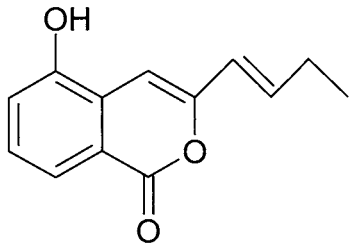

Fig. (40). Structure of artemidinol

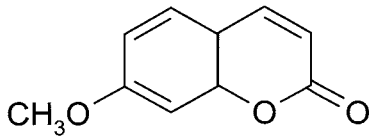

Fig. (41). Structure of herniarin

Fungal cultures of *Microsporum cookie* were incubated with the aqueous extract of German chamomile (*Chamomila recutita*) and one coumarin herniarin, Fig. **(41)**, isolated from this plant [242]. Both herniarin, Fig. **(41)** and *Chamomila recutita* extract show a clear antifungal activity at 100 and 23 μg/ml, respectively. The main changes were due to inhibition of hyphal outgrowth, apical furcation and thickening of the cell wall.

Examples of other antimicrobial coumarins from the Anthemideae tribe also included the coumarin derivatives isolated from the seeds of *Centaurea nigra* L. [243] and one coumarin isolated from *Leontopodium alpinum*, which selectively inhibited the growth of *Streptococcus pyogenes* and *Streptococcus pneumoniae* [36].

From simple phenolic derivatives, there are only a few reports concerning antimicrobial activity from the Anthemideae tribe. Examples included four caffeoylquinic acid derivatives, chlorogenic acid, Fig. **(42)**, cynarin, Fig. **(43)**, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid isolated from *Cynara scolymus* [222] and cynaropicrin, Fig. **(44)** isolated from *Moquinia kingii* [227].

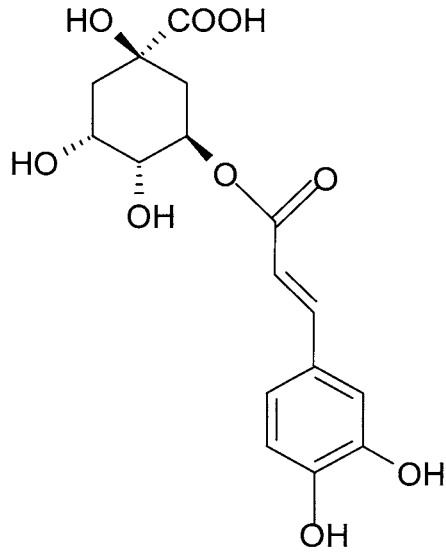

Fig. (42). Structure of chlorogenic acid

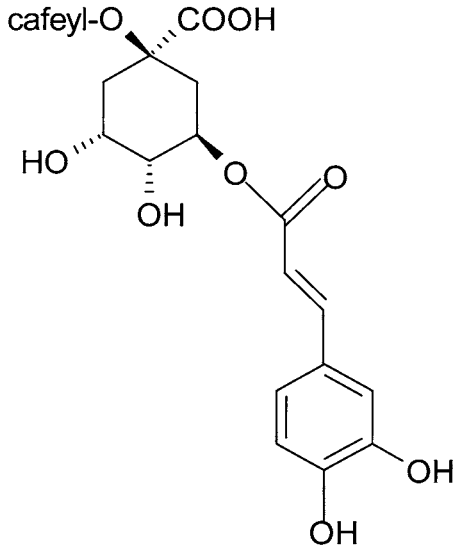

Fig. (43). Structure of cynarin

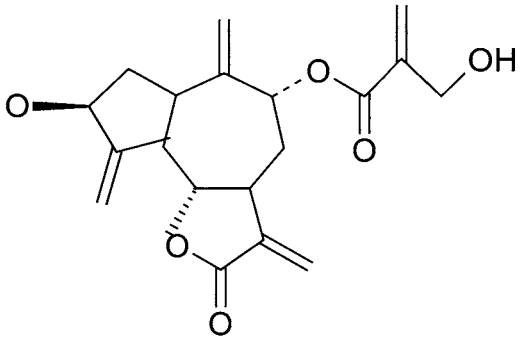

Fig. (44). Structure of cynaropicrin

OTHER COMPOUNDS

Reports on antimicrobial constituents from the Anthemideae tribe belonging to other structural types were also found in the literature. The benzofuran euparin, Fig. **(45)** isolated from *Calea platylepis*, demonstrated a broader spectrum of action inhibiting the growth of various strains of microorganisms (bacteria and fungi) [161]. Another benzofuran, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone, was identified from an antimicrobial extract of mugwort (*Artemisia asiatica*) [244]. The compound showed inhibitory effect on the growth of microorganisms such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Lactobacillus plantarum*.

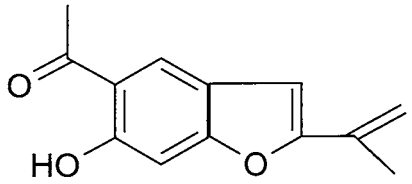

Fig. (45). Structure of euparin

A novel acetophenone-4-*O*-glucoside named nauplathizine was isolated as an active constituent from the antibacterial acetone extract of the aerial parts of *Nauplius aquaticus* (L.) [245]. The dichloromethane extract of underground parts of *Calea uniflora* Less. exhibited antifungal activities [246]. Four *p*-hydroxyacetophenone derivatives were isolated as the main compounds. Some of them showed antifungal activity, with MIC values between 500 and 1000 μg/ml against pathogenic *Candida* spp. and dermatophytes.

The thiophene polyine (*E*)-2[5-hept-5-en-1,3-diynyl)-thien-2-yl]-ethan-1,2-diol isolated from an ethanolic extract of the underground parts of *Leuzea carthamoides* DC. demonstrated significant antifungal activity [247], while the dichloromethane extract of the air-dried leaves of *Chrysanthemum coronarium* afforded N-isobutyl-6-(2-thiophenyl)-2,4-hexadienamide, Fig. **(46)** [248]. Antimicrobial tests by the agar well diffusion method indicated that it has low activity against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*, and is inactive against *Staphylococcus aureus* and *Escherichia coli* at a concentration of 20 μg/ml.

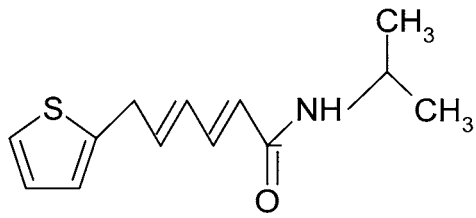

Fig. (46). Structure of N-isobutyl-6-(2-thiophenyl)-2,4-hexadienamide isolated from *Chrysanthemum coronarium*

Significant activity of the total lipid fraction of *Carthamus lanatus* was found against *Staphylococcus aureus* [249]. The lipid composition was examined and almost equal amounts of triacylglycerols, digalactosyl diacylglycerols and phospholipids were determined. Examples of other antimicrobial lipids from the Anthemideae tribe also included *Xanthium strumarium* lipids [250], and glyceroglycolipids from *Serratula*

strangulata, Fig. **(47)** which exhibited significant antibacterial activity [251].

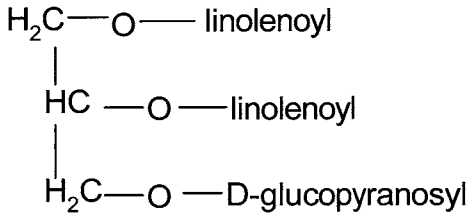

Fig. (47). Structure of glyceroglycolipids isolated from *Serratula strangulata*

Other antimicrobial compounds from the Anthemideae tribe are polyacetylenes, lignans, proteins and alkaloids. From a dichloromethane extract of *Artemisia borealis* Pallas, two polyacetylenes were isolated through activity-guided fractionation [252]. The genuine polyacetylenes and their acetates exhibited antifungal activity in the brine shrimp assay. Some of these compounds such as capillin, were also isolated from *Artemisia capillaris* [253]. Another polyacetylene, dehydrofalcarindiol, Fig. **(48)** from *Artemisia pacifica* Nutt inhibited the growth of *Bacillus subtilis* at 25 μg/ml, *Staphylococcus aureus* at 50 μg/ml, *Klebsiella pneumoniae* at 100 μg/ml and *Candida albicans* at 25 μg/ml [254]. The chemical and bio-guided investigation of the dichloromethane extract from the aerial organs of *Bellis perennis*, the common daisy, showed that polyacetylenes were one of the dominant classes of compounds [255]. Of these, only deca-4,6-diynoic acid and deca-4,6-diyne-1,10-diocic acid showed antimicrobial activity, both compounds being mainly effective against Gram (+) and Gram (-) bacteria, respectively. The polyacetylene antibiotic 1-phenylhepta-1,3,5,-triyne isolated from plant *Bidens pilosa*, displayed marked antimicrobial activity against a wide variety of microorganisms, including bacteria, yeasts and molds [256].

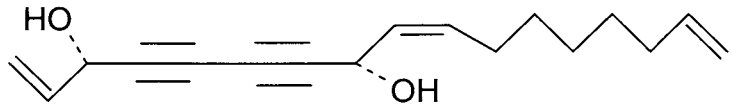

Fig. (48). Structure of dehydrofalcarindiol

A bioactivity-guided fractionation of an extract of *Terminalia bellerica* Roxb. fruit led to the isolation of two new lignans, named termilignan and thamilignan [257]. The compounds showed demonstrable anti-HIV and antifungal activity *in vitro*. Two lignan compounds were isolated from the herbal drug fructus bardanae (*Arctium lappa*) [258]. The compounds isolated were active against the following microrganisms: *Candida monosa* (nistatin-sensitive), *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Bacillus subtilis*.

A novel antifungal protein, designated chrysancorin, was isolated from the seeds of *Chrysanthemum coronarium* var. *spatiosum* LH Bailey [259]. It inhibits the activity of human immunodeficiency virus-reverse transcriptase (HIV-RT). The protein also possesses antifungal activity towards *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Physalospora piricola*, but not against *Rhizoctonum solani*, *Fusarium oxysporum*, *Coprinus comatus* and a variety of bacteria tested.

Hol and Van Veen [260] investigated the growth-reducing effects of pyrrolizidine alkaloids from *Senecio jacobaea* L. on 9 plant-associated fungi (5 strains of *Fusarium oxysporum*, 2 of *Fusarium sambucinum* and 2 of *Trichoderma* spp.). The growth rate of 6 strains was inhibited by alkaloids at the highest test concentration (3.33 mg/ml), with the magnitude of the inhibition (7-35%) being dependent upon the specific fungus-alkaloid interaction.

ABBREVIATIONS

HSV-1 = Herpes Simplex Virus Type I
CPE = Cytopathic Effect
MIC = Minimum Inhibitory Concentration
VSV = Vesicular Stomatitis Virus
HIV = Human Immunodeficiency Virus

HSV-2 = Herpes Simplex Virus Type II
HIV-RT = Human Immunodeficiency Virus- Reverse Transcriptase

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ANTIOSTEOPOROTIC AGENTS FROM NATURAL SOURCES

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ABSTRACT: This review covers the structures of compounds with antiosteoporotic activity isolated from plants and are organized according to chemical classes. A total of 104 structures have been reviewed from 79 references found in the literature up to December 2005.

INTRODUCTION

Bone is a vital, dynamic connective tissue which has evolved to reflect a balance between its two major functions, provision of mechanical integrity for locomotion and protection and involvement in the metabolic pathways associated with mineral homeostatis. In addition, bone is the primary site of hemopoiesis and recent findings support its important role as a component of the immune system [1]. Bones continuously mend and rebuild themselves by opposing actions of two types of cells, the 'osteoblasts' that form bone and the 'osteoclasts' that resorb (destroy) bone. When the activity of the bone destroying osteoclast cell outpaces that of bone forming osteoblasts, the bottom line is bone loss and the result is osteoporosis.

The term osteoporosis entered the medical parlance in France and Germany during the past century; it implied a histological diagnosis "porous bone". Thus osteoporosis can be defined as a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. Osteoporosis and related fractures represent major public health concerns that will only increase in importance as life expectancy increases and the population ages. It has been recognized as a global problem by the World Health Organization (WHO) [2].

Osteoporosis is of two forms- primary i.e. idiopathic and secondary. Primary osteoporosis is classified into type I and type II osteoporosis. Type I is referred to post menopausal osteoporosis which is the main type affecting women, characterized by rapid bone loss and affects women after the menopause, mainly in trabecular bone and is associated with vertebrae and distal radio fractures whereas type II also termed as 'senile osteoporosis' occurs due to chronic deficiency of calcium, increase in parathormone activity and decrease in bone formation and is associated with aging. On the other hand secondary type results from inflammatory processes, endocrine changes, multiple myeloma, sedentariness and the use of drugs such as heparin, corticoid and alcohol [3]. Prevention is the main treatment of osteoporosis, for which bone mass peak and the prevention of postmenopausal reabsorption are critical elements. The common treatment of osteoporosis includes calcium consumption as calcium salts, vitamin D supplements, and hormone reposition [4], the use of calcitonin to modulate serum levels of calcium and phosphorous [5], the use of bisphosphonate, mainly alendronates [6], use of ipriflavone and sodium fluoride [7], besides physical activity to strengthen muscles, stimulate osteoblasts formation and prevent reabsorption.

Pharmacological agents used to manage osteoporosis act by decreasing the rate of bone resorption, thereby slowing the rate of bone loss, or by promoting bone formation. Since there is broad spectrum effect of osteoporosis in the medical system hence an increasing demand is sought in the alternative system of medicine to design strategies to prevent and cure this global problem. From a medical and economical view it would, therefore, be desirable to explore antiosteoporotic leads obtained from natural sources to prevent osteoporosis. Pereira *et al* [8] have reported sixty nine crude plant extracts screened for antiosteoporotic activity.

PLANT FORMULATIONS

Various plants formulations have been screened and studied for the antiosteoporotic activity. *Glycine max* commonly known as soyabean is a plant which is exhaustively studied for its antiosteoporotic activity. Its ethanol extract as well as its constituents which are isoflavones and lignans are very active antiosteoporotic agents, which has already been proved by the number of studies. The effect of soybean ethanol extract on the activity of osteoblasts MC3T3-E1 cells has been studied. The extract increased survival ($P<0.05$) and DNA synthesis ($P<0.05$) of MC3T3-E1

cells at the concentration range of 0.01-.0.1 g/l in a dose dependent manner. Soy extract at the concentration of 0.05 g/l increased alkaline phosphatase (ALP) activity ($P<0.05$) and collagen synthesis ($P< 0.05$) of MC3T3-E1 cells. The anti-estrogen tamoxifen eliminated the stimulation of MC3T3-E1 cells on proliferation, ALP activity and collagen synthesis by soy extract 'indicating that the main action of soy extract on the osteoblastic MC3T3-E1 cells is similar to that of estrogen effects. Therefore, soy extract has a direct stimulatory effect on bone formation in cultured osteoblastic cells *in vitro* [9]. The total coumarins from the fruits of *Cnidium monnieri* increased bone mineral density of femur metaphysis of osteoporotic rats and uterus weight. The alkaline phosphatase activity and inorganic serum content were decreased. The levels of estradiol, osteocalcin, and calcitonin in serum were also increased, but there was no effect on calcium concentration in serum. Thus proving that total coumarin fraction has antiosteoporotic effects in ovariectomized rats by inhibiting bone turnover and increasing the production of estradiol and calcitonin [10].

A herbomineral formulation OST-6 (osteocare) was evaluated for its inhibitory effect on the progress of bone loss induced ovariectomy in rats. Each gram of OST-6 contains *Terminalia arjuna* (bark 250 mg), *Withania somnifera* (root 250 mg), *Commiphora mukul* (gum resin 280 mg) and praval bhasma (220 mg). Ovariectomized rats were administered with OST-6 at 250 and 500mg/kg b.wt. orally daily for 90 days. On 91st day, ovariectomized rats showed reduced bone mineral content and increased serum alkaline phosphatase levels, excretion of urinary calcium and pyridinium cross links levels. Histologically, sections revealed narrowed and disappearance of trabeculae and widened medullary spaces. The total numbers of tararate–resistant acid phosphatase (TRAP) positive cells were significantly increased both in vivo and in vitro methods. OST-6 at a dose of 500mg/kg, significantly improved bone mineral contents, serum alkaline phosphatase levels, reduced the elevated urinary calcium and pyridinium cross links excretion, number of TRAP positive cells and reversal of above mentioned histological features [11]. A Chinese herbal medicine 'Hoehu ekki to' (Bu-zong-yi-gi-tang) is composed of ten herbal medicines: a mixture consisting of 4.0g of *Astragalus* roots (Ougi), 4.0g of *Atractylodes lanceae* rhizome (soujyutsa), 4.0g of *Panax ginseng* roots (Ninjin), 3.0g of *Angelica* roots (Touki), 2.0g of *Bupleuri* roots (Saiko) 2.0g of *Zyzyphus* fruits (Taisou), 2.0g of *Aurantis nobilis* pericarp (Chinpi), 1.5g of *Glycyrrhiza* roots (Kanzou), 1.0g of *Cimifugae* rhizome

(Shouma) and 0.5g of *Zingiberus* rhizome (Shoukyou) was prepared, from which 5.0g 'Hoehu ekki to' was extracted with hot water, filtered, lyophilized, and stored at 4 ^{0}C. This medicine has been used for the treatment of oligospermia and as a post operative medication in Japan, on bone loss in rats treated with a gonadotropin releasing hormone (GnRH) agonist. The administration of GnRH agonist reduced the bone mineral density in the whole femur to 91.0% of that in the control group. However, administration of conjugated estrogens and 'Hoehu ekki to' increased the serum concentrations of estradiol 16.8 and 5.3 fold respectively compared with the concentration in the GnRH agonist treated group, resulting in the augmentation of the bone mineral density to 110.3% and 106.2% respectively. 'Hoehu ekki to' enhances the reduced BMD and causes a slight elevation of the serum estradiol levels in the chemically castrated rats [12]. The methanol extract of stems of *Sambucus sieboldiana* inhibited bone resorption in organ culture. The ethyl acetate fraction of the methanolic extract inhibited the PTH stimulated bone resorption of neonatal mouse bones, and inhibitory activity was more potent than those of other fractions. Oral administration of ethyl acetate fraction (50 and 100mg/kg/d) to ovariectomized rats prevented the decrease of bone mineral density (BMD) of the lumbar (L_{2-4}) vertebra, indicating that ethyl acetate fraction is effective in vivo. Furthermore, the ethyl acetate fraction (50, 100, and 150mg/kg/d) decreased the calcium level in low calcium dietary rats [13]. The effect of herbal medicines on trabecular bone area were studied using OVX rats as animal model of Type I osteoporosis and SAM P6 as that of Type II osteoporosis. RBC, Hb and Hematocrit (Hct) were counted using coulter method. Each traditional boiling water extract of roots of *Achyranthus* sp., *Psoralea* sp., *Rehmannia* sp., fruits of *Cornus* sp. and a systemic extract of *Astragalus* roots was given 5g/kg/day, p.o. for 30 days in a group of 4-5 OVX rats. One ml of blood was taken by tail vein at day, 0, 7, 14, 21 and 30 days after administration of extract. The traditional hot water extract of *Cervi parvum* cornu (Cervi) was given the same dose as described above for 14 days in a group of 10 SAM P6 mice. In Type I OVX rats administration of *Astragalus*, *Rehmannia*, and *Cornus* preparation showed decreased in RBC, Hb, and Hct. In Type II administration of *Cervi* increased RBC and Hct and that of *Astragalus* preparation elevated RBC. In type I, any administration of herbal medicine used in the study did not elevate trabecular bone area significantly except *Cornus* preparation showed a trend of increase in trabecular bone area [14]. The ethanol extract of the plant *Cissus quadrangularis* commonly

known as hadjod for its bone fracture healing property was evaluated for its antiosteoporotic activity in ovariectomized rat model of osteoporosis at two different dose levels of 500mg and 750mg/kg/day. Healthy female albino rats were divided into five groups of six animals each. First group was sham operated and served as control. All the remaining groups were ovariectomized. Group 2 was fed with equivolume of saline and served as ovariectomized control. Groups 3-5 were orally treated with raloxifen (5.4mg/kg) and with the ethanol extract of *Cissus quadrangularis* (500 &750 mg/kg) respectively. The findings assessed on the basis of biomechanical, biochemical and histopathological parameters showed that the ethanol extract of the plant had a definite antiosteoporotic effect [15]. The preventive effect of a herbal formulation " Dae-Bo-Won-Chun" (DBWC), on the progress of bone loss induced by ovariectomy (OVX) was studied in rats. From light microscope analyses, porous or erosive appearances were observed on the surface of trabecular bone of tibia in ovariectomized rats, whereas those of the same bone in sham-operated rats were composed of fine particles. The trabecular bone area and trabecular thickness in ovariectomized rats, decreased by 50% from those in sham-operated rats, these decreases were completely inhibited by administration of DBWC at the concentration of 10mg/kg per day for 7 weeks. The mechanical strength of the neck of the femur was decreased by ovariectomy, and this was significantly suppressed by the administration of DBWC. Serum phosphorus, alkaline phosphatase and thyroxine levels in ovariectomized rats increased compared with those in sham operated rats, and increases were completely inhibited by the administration of DBWC. These results, strongly suggest that DBWC is effective in preventing the development of bone loss induced by ovariectomy in rats [16].

In this review we have discussed plant-derived compounds with antiosteoporotic activity. The review is organized according to chemical classes.

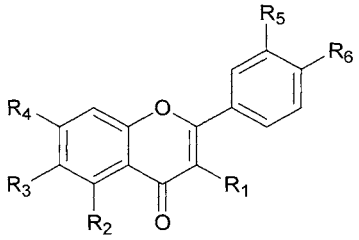
FLAVONOIDS

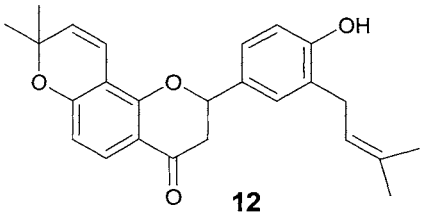
Flavonols, flavanones, flavanes and chalcones

Two flavonoid glycosides eupalitin 3-*O*-β-*D*-galactopyranosyl-(1→2)-β-*D*-glucopyranoside (**1**) and eupalitin 3-*O*-β-*D*-galactopyranoside (**2**) were isolated from the methanolic extract of *Boerhavvia repens*

(Nyctanginaceae). These compounds showed inhibitory activity on PTH-stimulated bone resorption at a concentration of 200μM. The inhibitory activity was found more than ipriflavone at this concentration [17]. Icariin (**3**) was extracted from the aerial parts of plant *Epimedium koreanum* (Berberidaceae) with hot water and purified by hydrophobic interaction chromatography, ion-exchange chromatography and finally by crystallization. This product increases bone P and Ca contents, bone mineral density and femur strength in ovariectomized subjects. It is used for the prevention and treatment of osteoporosis [18]. Kaempferol (**4**) and quercetin (**5**) are major naturally occurring flavonols found in many plants. Kaempferol exert a potent inhibitory effect on *in vitro* bone resorption of osteoclasts cells prepared from rabbit long bones at a concentration as low as 0.1μM. The IC_{50} measured for kaempferol was 1.6μM. Inhibitory effect of quercetin was tested at different concentrations ranging from 0.1 μM to 100μM, the inhibitory concentration IC_{50} measured for quercetin was 5.3 μM [19]. Rutin (**6**) (quercetin-3-*O*-glucose rhamnose), distributed in many plants, inhibits ovariectomy induced osteopenia in rats. Addition of rutin (0.25%) to the diet given to the OVX subjects inhibits estrogen deficiency induced femoral trabecular bone loss, both by slowing resorption and by increasing osteoblastic activity, resulting in increase in femoral strength. Rutin can be attractive candidate as a cheap therapeutic agent against osteoporosis [20]. Licoflavone A (**7**) and prenyllicoflavone A (**8**) are naturally occurring flavones, prepared synthetically and tested for antiosteoporotic activity. These act as inhibitors of bone resorption pits formation. The activity was evaluated in osteoclasts cells [21]. Bavachalcone (**9**) and isobavachalcone (**10**) are obtained from the acetone extract of the seeds of *Psoralea corylifolia* (Leguminosae). They are useful as bone strength enhancing and calcifying agents therapeutically. They antagonized the predinisolone induced osteoporosis in male rats [22]. Hesperidin (**11**) is a wide spread flavonoid occurring in many plant varieties, prevented bone loss in rats, and the effects were due to a decrease in bone resorption coupled with an increase in the osteoblastic activity [23]. Shinflavanone (**12**) a component of licorice (*Glycyrrhiza glabra*, Leguminosae), is a strong inhibitor (IC_{50} = 0.70μg/ml) of bone resorption pits formation by osteoclast like cell induced by 1α, 25-dihydroxy vitamin D_3 [24]. Methanolic extract of D*rynaria fortunei* rhizomes (Polypodiaceae) showed potent proliferative activity in the MCF-7 and ROS 17/2.8 osteoblasts like cells. A bioassay guided separation of this fraction yielded (-)-epiafzelechin (**13**), (-)-

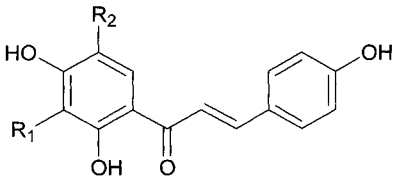
epiafzelechin-3-*O*-β-*D*-allopyranoside (**14**), (-)-epiafzelechin-3-*O*-(6"-*O*-acetyl)-β-*D*-allopyranoside (**15**), 4β-carboxymethyl-(-)epiafzelechin methyl ester (**16**), 4β-carboxymethyl-(-)epiafzelechin sodium salt (**17**), naringin (**18**), (-)-epiafzelechin-(4β→8)-4β-carboxymethyl epiafzelechin methyl ester (**19**) and (-)epiafzelechin-(4β→8, 2β→O→7)-epiafzelechin-(4β→8) epiafzelechin (**20**). Of these only compound **15** stimulated the proliferation of MCF-7 cells in a dose dependent manner, in the concentration range of 10^{-15} to 10^{-6}M, and compounds **18** and **19** showed significant proliferation in the concentration range of 10^{-15} to 10^{-12}M. However, the compounds **13**, **14**, **16**, **17** and **20** accelerated the cell proliferation at very low concentrations of 10^{-15}M, but MCF-7 proliferation effects were decreased above concentrations > 10^{-15}M. Compounds **17-20** stimulated the proliferation of the ROS 17/2.8 cells in a dose dependent manner, in the concentration range of 10^{-15} to 10^{-9}M, with compounds **13-15** exhibiting somewhat moderate proliferative activities. However, compound **16** had a lesser proliferative effect in the range of 10^{-15} to 10^{-6}M. The proliferative effect was much stronger than that of E_2 and genistein [25]. Catechin (**21**) and catechin gallate (**22**) are present widely in many plants. Their main role is in prevention of osteoporosis rather than in treatment [26].



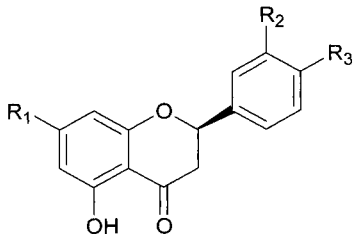


12

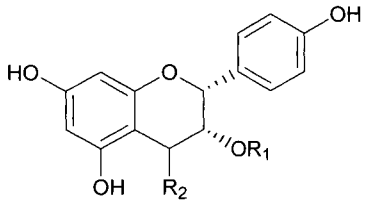
1: R_1=O–β-D-Gal (1-2)O–β-D-Glu, R_2=R_6=OH, R_3=R_4=OCH_3, R_5=H
2: R_1=O-β-D-Gal, R_2=R_6=OH, R_3=R_4=OCH_3, R_5=H
3: R_1=O-α-L-Rham, R_2=OH, R_3=R_5=H, R_4=O–β-D-Glu, R_6=OCH_3
4: R_1=R_2=R_4=R_6=OH, R_3=R_5=H
5: R_1=R_2=R_4=R_5=R_6=OH, R_3=H
6: R_1=O-rutinose, R_2=R_4=R_5=R_6=OH, R_3=H
7: R_1=R_4=R_6=OH, R_2=R_3=H, R_5=CH_2CH=CMe_2
8: R_1=R_4=R_6=OH, R_2=H, R_3=R_5=CH_2CH=CMe_2



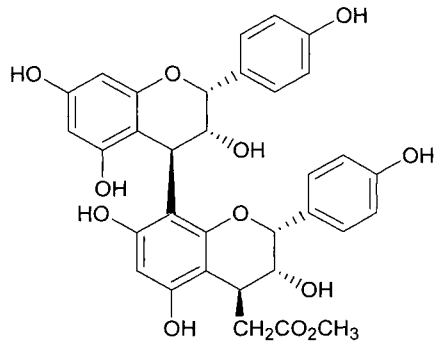
9: R_1=H, R_2=CH_2CH=CMe_2
10: R_1=CH_2CH=CMe_2, R_2=H



11: R_1=O-rutinose, R_2=OH, R_3=OCH_3
18: R_1=O-neohesperidose, R_2=H, R_3=OH



13: R_1=H, R_2=H
14: R_1=allose, R_2=H
15: R_1=acetylallose, R_2=H
16: R_1=H, R_2=CH_2CO_2H
17: R_1=H, R_2=CH_2CO_2Na



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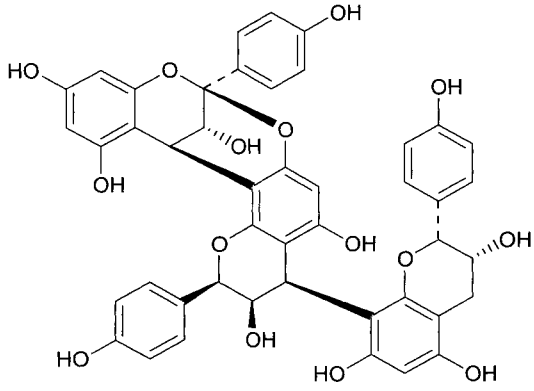
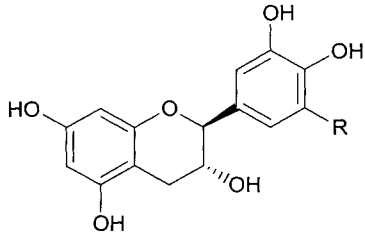


20



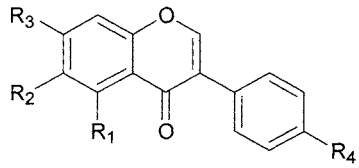
21: R=H
22: R=OH

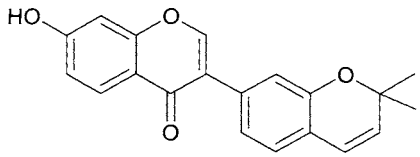
Isoflavones

Isoflavones are the most active antiosteoporotic compounds behaving like estrogens, therefore, termed phytoestrogens. Corylin (**23**) a constituent of *Psoralea coryfolia* (Leguminosae) was investigated for bone forming activity on osteoblasts like UMR cells. A cell differentiation bioassay measuring alkaline phosphatase (ALP) activity was developed using

[27]. Daidzein (**24**), genistein (**25**), glycitin (**26**), glycosides of daidzein and genistein namely daidzin (**27**) and genistin (**28**) are constituents of soybean (*Glycine max*, Leguminosae) and are effective antiosteoporotic agents. The effect of daidzein was compared with that of 17 β-estradiol on the development and activity of osteoclasts *in vitro*. Nonadherent porcine bone marrow cells were cultured in the presence of 10^{-8}M of 1, 25-dihydroxy vitamin D_3 with or without 10^{-8}M of daidzein, 10^{-8}M of 17 β-estradiol for 9-11 days. The number of osteoclasts formed in response to vitamin D_3 was reduced by 58 ± 8% by daidzein and 52 ± 5% by estrogen ($p<0.01$). The area resorbed by mature osteoclasts was reduced by 39 ± 5% by daidzein and 42 ± 6% by estradiol ($p<0.01$). Both compounds also inhibited the vitamin D_3 induced differentiation of osteoclasts progenitors 53 ± 8% by daidzein and 50 ± 7% by estradiol, inhibits osteoclasts differentiation and activity [28]. Genistein prevents bone loss caused by estrogen deficiency without substantial effects on the uterus in OVX mice by normalizing the accumulation of pre-β lymphocytes in bone marrow and suppressing increased bone resorption as 17β-estradiol (E_2). It was found that with a submaximal dose of 0.4 mg/day of genistein given subcutaneously to OVX rat, the BMD was significantly higher than to the OVX rat not given the dose. Further it was found that the combined intervention of moderate exercise and the submaximal dose of genistein administration show a cooperative effect in preventing bone loss in OVX mice [29]. Glycitin, daidzin and genistin prevented bone loss at a dose of 50 mg/kg/d like estrone. But daidzin and glycitin are effective in reversing the unfavorable changes of lipid due to suppression of bone turnover, as in the case of estrone, but genistin has a different mechanism of action [30]. The combination of these three isoflavones was formulated and termed 'fujiflavone P40'. Fujiflavone P40 is soyabean product which consists of 24% daidzin, 16.5% glycitin and 5.9% genistin. Its preventive effect against bone loss and periodontal alteration was evaluated in OVX rat model [31]. Tectorigenin (**29**) was isolated from the rhizome of the plant *Belamcanda chinensis* (Iridaceae). Binding studies with recombinant human ERα and ERβ were performed to show that tectorigenin binds to both receptor subtypes. In ERα-expressing MCF-7 and ERβ-expressing MDA-MB231 reporter gene transfected cells tectorigenin causes transactivation with EC_{50} of 3.5μM and 0.4μM respectively. When given intravenously 7mg/animal to ovariectomized rats, it inhibits pulsatile pituitary LH secretion. In postmenopausal women estrogen-unopposed LH pulses correlates with hot flushes. Therefore, suppression of pulsatile LH

may be beneficial in women suffering from hot flushes. It had estrogenic effects in the bone and on bone mineral density of the metaphysis of the tibia. Tectorigenin had a strong hypothalamotropic and osteotropic effect but no effect in the uterus [32].



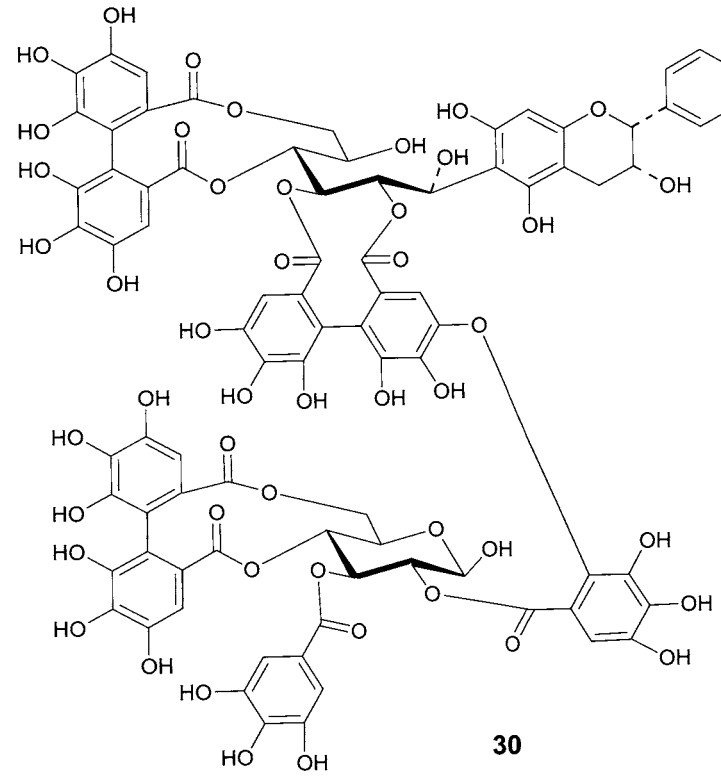


23

24: R_1=R_2=H, R_3=R_4=OH
25: R_1=R_3=R_4=OH, R_2=H
26: R_1=H, R_2=OCH_3, R_3=O–β-D-Glu, R_4=OH
27: R_1=R_2=H, R_3=O–β-D-Glu, R_4=OH
28: R_1=R_4=OH, R_2=H, R_3=O–β-D-Glu
29: R_1=R_3=R_4=OH, R_2=OCH_3

Tannins

Tannins are the complex flavonoid compounds. Some of these showed good results when evaluated for the antiosteoporosis. Camelliatanin D (**30**) was isolated from leaves and fruits of *Camellia Japonica* (Theaceae). It inhibited elevation of calcium concentration induced by PTH rp (1-34), with the IC_{50} of 2.4 x 10^{-7} M. Activity was done on cultured mouse calvaria [33]. Epigallocatechin (**31**) and epicatechin (**32**) are the constituents of *Rosa* sp. (Rosaceae); distributed widespread in other plants also. They behave as a bone sorption inhibitor [26].



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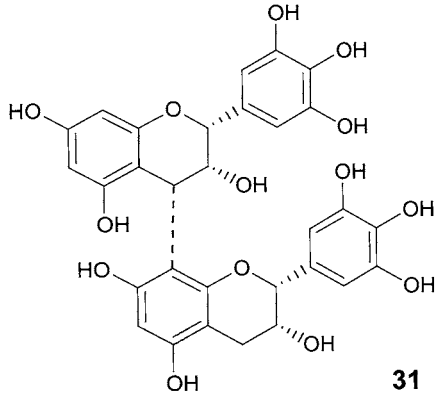

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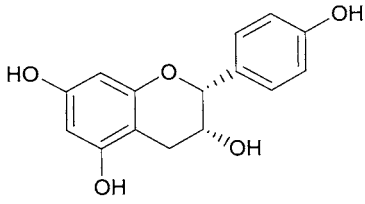
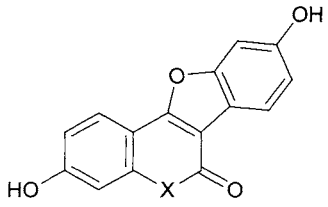


32

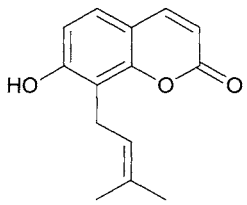
COUMARINS

Coumarins are active molecules against osteoporosis, they also behaves as estrogens, therefore, termed phytoestrogens as isoflavones and lignans.

Coumestrol (**33**) a constituent of *Glycine max* (Leguminosae), behaves as a weak phytoestrogen. In order to eliminate the estrogenic activity, a compound KCA-012 (**34**) was synthesized with structural similarity. It inhibited parathyroid hormone, 1α, 25-dihydroxy vitamin D_3 and prostaglandin E_2-induced bone resorption of cultured fetal rat bones. It also increased the calcium content of 9-day-chick embroyonic femur from 29 ±1μg to 36 ±2μg at the concentration of 2 x 10^{-6}M cultured in vivo. KCA-012 did not show any estrogenic activity as determined by an increase in the uterine weight of OVX rats, whereas coumestrol did, since subcutaneous administration of coumestrol 100mg/kg/twice a day for 3 days resulted in a marked increase in uterus of OVX rats. On the contrary, KCA-012 (10 or 100 mg/kg/twice a day) was ineffective, indicating that it has no estrogenic activity. These results indicate that coumestrol structurally related KCA-012 has no estrogen activity and has unique effects of inhibiting bone resorption and stimulating bone mineralization [34]. Osthole (**35**), bergapton (**36**) and imperatorin (**37**), the constituents of the fruits of *Cnidium monnieri* (Apiaceae) were tested for the proliferation of osteoblasts like UMR 106 cells *in vitro*, where osthole significantly promoted the cells' activity, bergapton and imperatorin were less effective as compared to osthole [35].



33: X=O
34: X=NH



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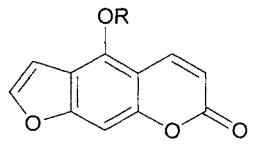
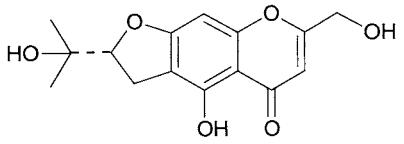


36: R=OCH_3
37: R=$CH_2CH{=}CMe_2$

CHROMONE

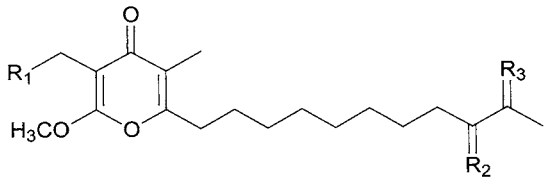
A chromone named angelican (**38**) obtained from *Cimifuga* sp. (Ranunculaceae) possess antiosteoporosis potential. This compound administered at 100mg/kg/day to rats for 6 weeks inhibited the bone density decrease compared to controls [36].



38

PYRONES

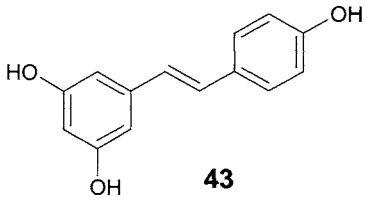
Four γ-pyrones oxopodopyrone 9' (**39**), oxopodopyrone-8-methyl-9' (**40**), oxopodopyrone 10' (**41**), oxopodopyrone-8-methyl-10' (**42**), were isolated from the methanol extract of leaves of *Gonsystylus keithii* (Thymeliaceae). These compounds markedly inhibited the bovine parathyroid hormone (PTH)-induced Ca release from neonatal mouse calvaria *in vitro*. The inhibitory activities of **39** to **42** were tested at the concentration of 0.01μg/ml, 0.1μg/ml, 1.0μg/ml and 10.0μg/ml each, the inhibitory activities of these compounds were assumed to be the same and were found to be more potent than those of calcitonins, etidronate and ipriflavone [37].



39: R_1= H, R_2 = O, R_3 = H & H
40: R_1 = CH_3, R_2 = O, R_3 = H & H
41: R_1 = H, R_2 = H & H, R_3 = O
42: R_1 = CH_3, R_2 = H & H, R_3 = O

STILBENES

Trans-resveratrol (**43**) extract prepared from *Polygonum cuspidatum* (Polygonaceae) showed reduction of reperfusion-induced ventricular fibrillation (*in vitro*) from its control value of 83% to 75% when rats were orally treated with 50mg/kg and 100mg/kg dose. Thus, it can be a potential alternative to conventional HRT for cardioprotection and osteoporosis prevention [38].

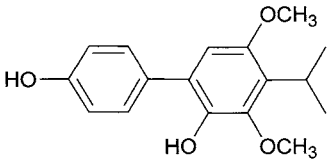


43

BENZENOIDS

Simple phenolic or benzenoid compounds displaying the activity are discussed below. BE-25327 (**44**) is manufactured from the cultivation of the microorganism *Pencillium purpurogenum* (Trichomaceae). This compound and its salts are found useful for the treatment of gynecological disorders, osteoporosis, prostrate cancer and prostrate hypertrophy. BE-25327 inhibited binding of (^{125}I) esteradiol to receptor, with an IC_{50} of 0.73 μM [39]. Coniferyl alcohol (**45**) was obtained from the ethyl acetate fraction of stems of *Sambucus sieboldiana* (Caprifoliaceae). It has inhibitory effect on PTH stimulated bone resorption at concentration of 20 μM and 200μM. Its effect is comparable to that of ipriflavone at the concentration of 200μM. The effect of this compound on BMD in the OVX mice for 4 weeks was investigated. The OVX caused a significant decrease in BMD which was increased by coniferyl alcohol at 10 and 30 mg/kg/d [13]. Gallic acid (**46**) was isolated from the ethanol extract of *Rosa* sp. (Rosaceae). Its tablets are formulated for the treatment of osteoporosis since it is found useful for sorption related diseases [26]. Isoferulic acid (**47**) was isolated from *Cimifuga sp.* (Ranunculaceae), inhibits bone density decrease at 100 mg/kg/day to rats for 6 weeks [36]. Vanillic acid (**48**) and vanillin (**49**) were isolated from the ethyl acetate fraction of the stems of *Sambucus sieboldiana* (Caprifoliaceae). They

exert inhibitory effects on bone resorption stimulated by PTH in neonatal mouse bone (in vitro) at the concentration of 20 and 200 μM. Vanillic acid is more potent than vanillin and has a significant inhibitory effect on the decrease of BMD in OVX mice (*in vivo*). The BMD of OVX mice treated with vanillic acid at the doses of 50 and 100 mg/kg/d was significantly greater than that of the OVX group, but not dose dependent [13]. Diospongin B (**50**) and diospongin C (**51**) are diarylheptanoids isolated from the aqueous extract of rhizomes of *Dioscorea spongiosa* (Dioscoreaceae). These compounds exerted potent inhibitory activities on bone resorption induced by parathyroid hormone in a bone organ culture system. Bone resorption was obtained as the percentage of total ^{45}Ca that was released into the medium during the culture. At 200μM the percentage release of Ca was 30.5 ± 0.4 and 19.1 ± 1.6 for the compound **50** and **51** respectively [40].



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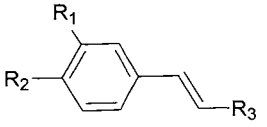
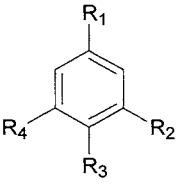
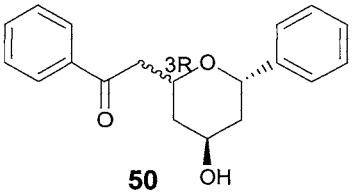


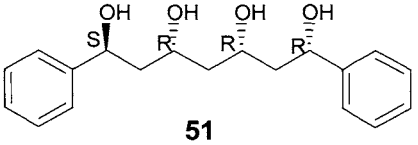
45: R_1=OCH_3,R_2=OH, R_3=CH_2OH
47: R_1=OH, R_2=OCH_3, R_3=CO_2H





50

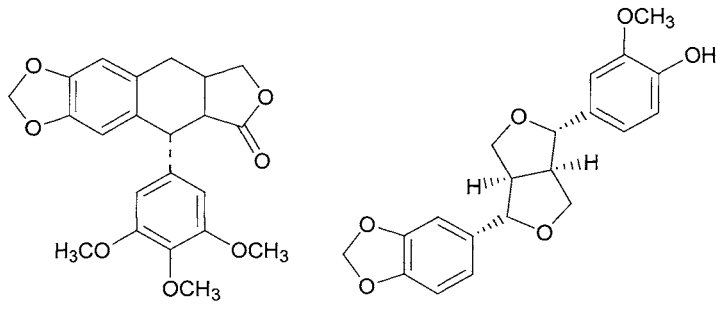
46: R_1=CO_2H, R_2=R_3=R_4=OH
48: R_1=CO_2H, R_2=OCH_3, R_3=OH, R_4=H
49: R_1=CHO, R_2=OCH_3, R_3=OH, R_4=H

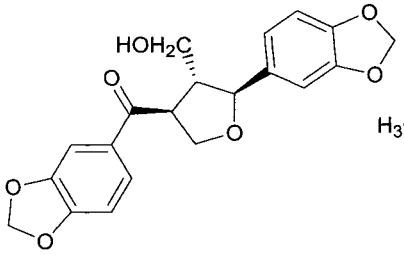


51

LIGNANS

Lignans are also termed as phytoestrogens having the property of estrogens. There are some lignans isolated from plants exhibiting the antiosteoporotic activity. Desoxypodophyllotoxin (**52**) isolated from *Podophylum peltatum* (Berberidaceae) exhibited the activity when tested *in vitro*. The crania of new born mice were cultured in a medium containing PTHrp (1-34) at concentration of 10^{-8}M and desoxypodophyllotoxin at the concentration of 1 x 10^{-5}M to show free Ca and P levels in the supernatant of the culture medium of 9.86 mg/dL and 3.37 mg/dL respectively Vs 11.95 mg/dL and 4.34 mg/dL respectively for controls cultured in the absence of this compound [41]. Piperitol (**53**), sesaminone (**54**) and syringaresinol (**55**) are the constituents of the aqueous extract of rhizomes of *Dioscorea spongiosa* (Dioscoreaceae). These compounds exerted potent inhibitory activities on bone resorption induced by parathyroid hormone in a bone organ culture system. Bone resorption was obtained as the percentage of total ^{45}Ca that was released into the medium during the culture and the drug elcitonin was used as standard. At 200μM the percentage release of Ca was 15.1 ± 1.5, 13.6 ± 1.0 and 23.3 ± 1.9 for the compound **53**, **54** and **55** respectively. The compounds **53** and **54** completely inhibited the calcium release [40]. Secoisolarice resionol diglycoside (**56**) is a useful intermediate for manufacturing female hormone like substances enterolactone and enterodiol, extracted from flax seeds (*Linum usitatissimum*, Linaceae) from alkaline alcoholic solution. The purified **56** is used for manufacturing foods and drinks efficacious against various symptoms caused by female hormone disorders such as menopause, osteoporosis, hyperlipemia, hypertension, obesity, depression and hot flash [42]. A lignan (**57**) isolated from the plant *Taiwania flousia* act as bone resorption inhibitor. The compound *in vitro* showed IC_{50} of 0.001 μg/ml against bone resorption [43].





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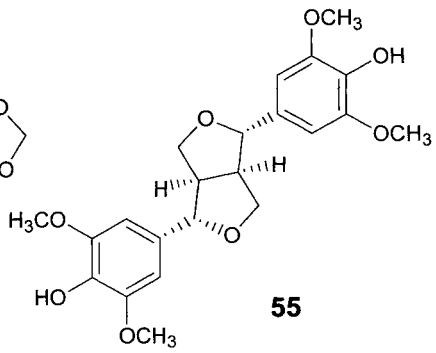
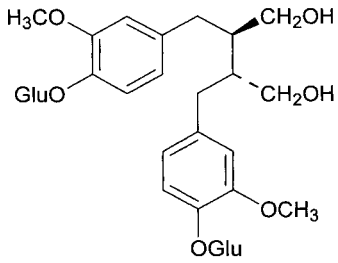
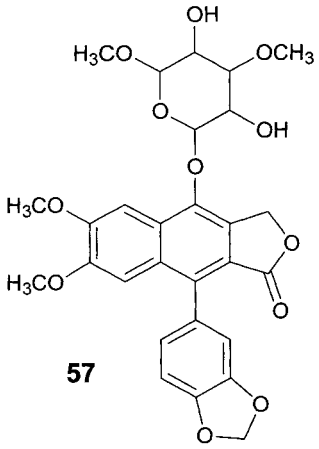


55



56



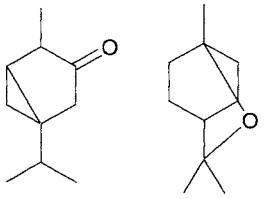
57

TERPENOIDS

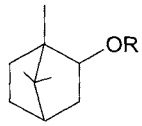
Terpenoids like flavonoids are also an important class, as there are number of compounds of this class that possess antiosteoporotic potential. Essential oils from *Salvia* species (Labiaceae) (sage oil) contain the monoterpenes thujone (**58**), eucalyptol (**59**), camphor (**60**) and borneol (**61**). A mixture of these 4 major monoterpenes occurring in 200mg of sage oil inhibits bone resorption by 26 ± 5% as compared to 27 ± 3% for 200mg sage oil. Thujone, eucalyptol and camphor tested singly at the doses of 80, 24, and 14 mg respectively inhibit significantly but not borneol at the dose of 10 mg. However, at the higher standard dose of 100mg/day, borneol strongly inhibits bone resorption. α-pinene (**62**), β-pinene (**63**), and bornyl acetate (**64**) of the pine oil (*Pinus* sp. Pinaceae), are also potent inhibitors of resorption. The *in vivo* models of these experiments are OVX rats and *in vitro* model are osteoclasts isolated from femur and tibia of 2 days old rats [44]. 2E, 6R-8-Hydroxy-2, 6 dimethyl -

2- octenoic acids (**65**) is isolated from a Chinese herb *Cistanche salsa* (Scophulariaceae). It suppressed the decrease of bone weight and the mechanical strength in the OVX mice. Its isomer was also synthesized and tested for the activity but it was found inactive [45]. Methyl (2E, 6R)-8-hydroxy-2, 6 dimethyl 2-octenoate (**66**) is methyl ether of the compound **65** isolated from the fallen leaves of *Catalpa ovata* (Bignoniaceae). This compound is also a useful agent against osteoporosis [46]. Geranyl geranyl stearate (**67**) a naturally occurring chain isoprenoid fatty acid ester possesses bone metabolism improving property. Bone metabolism improving effects are preferably based on inhibition of bone resorption and/or promotion of osteogenesis. The effects of this agent were examined in the osteoblastic MC3T3-E_1 cells [47]. Scopadulcic acid B (**68**) and scopadulciol (**69**) are tetracyclic diterpenoid isolated from *Scoparia dulcis* (Scrophulariaceae). These compounds were tested for their inhibitory effects on bone resorption in organ culture using neonatal mouse parietal bones. **68** inhibited the bone resorption dose dependently at the concentration range of 2 to 10 μM, while **69** showed a complete inhibition at higher dose than 0.5 μM. Thus **69** was the more potent inhibitor than **68**. The inhibitory effects of these diterpenoids on bone resorption may be due to the inhibition of osteoclast formation [48]. Triptolide (**70**) isolated from *Tripterygium wilfordii* (Celastraceae) possess antiosteoporotic property when tested *in vitro*. Its effects on proliferation capacity of osteoblasts were studied. Osteoblasts obtained from the calvaria of new born SD rats were cultured with various concentrations of **70**. The effects were detected by MTT methods. The proliferation rates of osteoblasts cultured for 2 days and 4 days were significantly inhibited by 10ng/ml and 0.01 ng/ml of **70** respectively [49]. Escin (**71**) was obtained from *Aesculus hippocastanum* (Hippocastanaceae) showed antiosteoporotic activity in the osteoporotic rat model created by ovariectomy. Escin at the dose of 35 mg/kg p.o., daily was administered for 4 weeks to 3-mo-old OVX and sham operated rats. It decreased the development of osteopenic skeletal changes in the OVX subjects. At the same dose it caused slight changes in the skeletal system of the sham operated rats, which was characterized by the increase in the bone formation processes [50]. Glycyrrhizin (glycyrrhetinic acid 3-*O*-[β-*D*-glucopyranosyl (1→2)-α-*D*-glucopyranoside]) (**72**) and glycyrrhetinic acid (**73**) are the constituent of *Glycyrrhiza glabra* (Leguminosae), they or their physiologically acceptable salts are useful for the treatment of malignant hypercalcemia, bone Paget's disease and osteoporosis. Glycyrrhizin inhibited PTH rp (1-

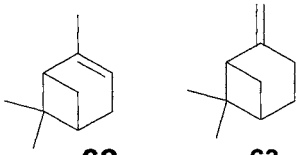
34)-induced release of Ca and inorganic P from cultured new bone mouse cranium [51]. Oleanolic acid (**74**) isolated from *Achyranthes bidentata* (Amarantaceae) has inhibitory effect of bone resorption stimulated by PTH. Different amino analogs of oleanolic acid were synthesized and their inhibitory activity on the formation of osteoclast like multinucleated cells (OCLs) induced by 1α, 25-dihydroxy vitamin D_3 was evaluated in a co-culture assay system with mouse bone marrow cells and osteoblast like cells. Elcatonin, a clinically available antiosteoporosis drug was used as a positive control. At 20 μM concentration of this compound, there was 27% inhibition of OCL formation relative to the control (100%) whereas samples cultured with elcatonin (2 μL) has 29.0 % of inhibition of OCL formation. The amino analogs were even more potent than the control [52].



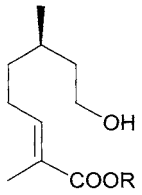
58 **59** **60**



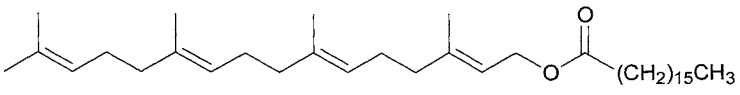
61: R=H
64: R=$COCH_3$



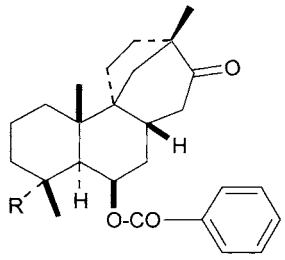
62 **63**



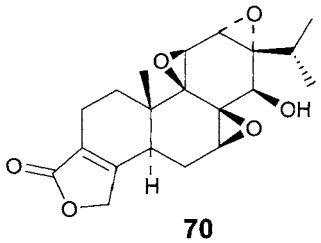
65: R = H
66: R = Me



67



68: R=CO_2H
69: R=CH_2OH



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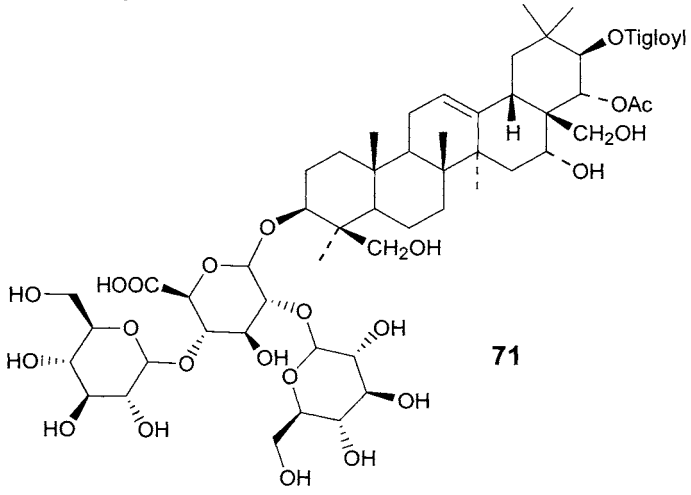

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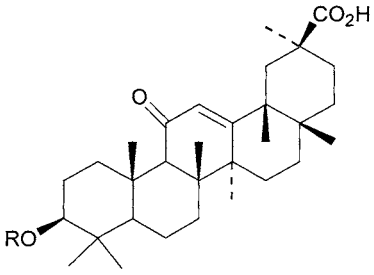
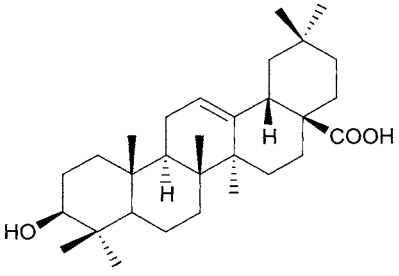




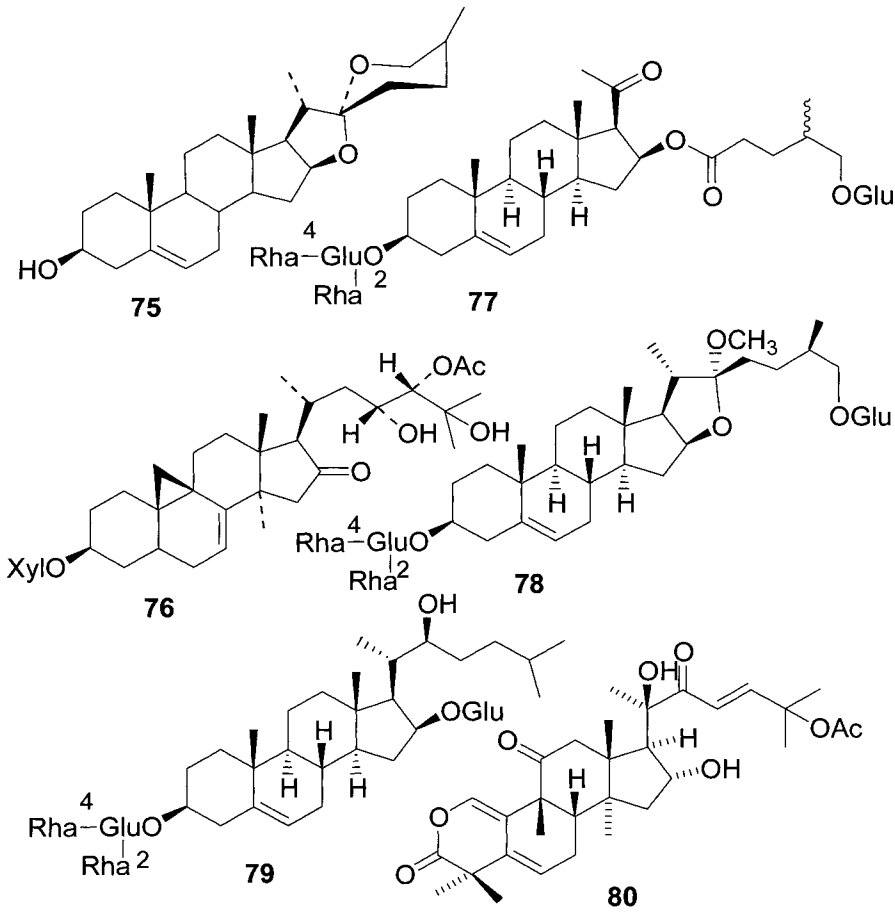
74

72: R=β-D-glucopyranosyl (1-2)Glu
73:R=H

STEROIDS

Many steroidal compounds are also promising antiosteoporotic drugs. They due to their structural resemblance to estrogen have been proved successful agents in postmenopausal osteoporosis. Diosgenin (**75**) a component of *Dioscorea* sp. (Dioscoreaceae), along with vitamin K, vitamin D and vitamin B_6 is formulated and given to a subject in need for

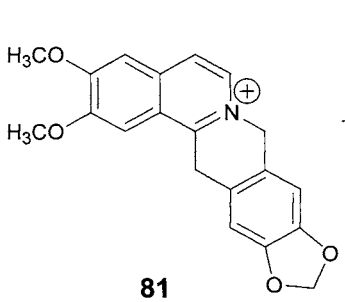
the treatment or prevention of osteoporosis [53]. The effect of mixture of ginsenosides obtained from stems and leaves of *Panax ginseng* (Araliaceae) was studied on ovariectomized rats by analysis of cancellous bone histomorphometry. High dose of ginsenosides (100mg/kg) greatly increased bone mass and had a tendency to decrease bone turnover when compared with OVX group. Ginsenosides partially prevented OVX induced cancellous bone loss by inhibiting osteoclast bone resorption and by a mild depression of bone turnover. Ginsenosides also showed protection of liver tissue and a preventive effect on bone loss in liver cirrhosis induced osteoporosis in mice. Ginsenosides did not inhibit active mineral bone formation hence differing from the estrogen treatment [54, 55]. 7, 8-Didehydro 24-O-acetyl hydro shengmanol 3-O-β-xyloside (**76**) was isolated from the rhizome *Cimifugae heracleifolia* and *Cimifugae foetida* (Ranunculaceae). It decreases blood serum Ca level in low calcium dietary rats. In ovariectomized rats it increases bone mineral density of the lumbar spine [56]. Hypoglaucin G (**77**), methylprotodioscin (**78**) and spongioside A (**79**) are the constituent of aqueous extract of rhizomes of *Dioscorea spongiosa* (Dioscoreaceae). These compounds showed potent inhibition against bone resorption induced by parathyroid hormone in a bone organ culture system. They were examined for their inhibitory activity at the concentrations of 200 and 20μM. At the concentration of 200μM, the compounds **77, 78** and **79** showed inhibitions of 66.6 %, 89.9%, and 63.6% respectively [57]. The compound **78** was also examined in the model of postmenopausal bone loss using ovariectomized rats. It was found that this compound at the dose of 50mg/kg/day significantly inhibited bone loss in bone mineral content and bone mineral density in total cancellous and cortical bones, and decrease in bone strength indexes induced by OVX, without side effects on the uterus [58]. Neocucurbitacin A (**80**) was isolated from the fruits of *Luffa operculata* (Cucurbitaceae). It possesses inhibitory activity of polyoma enhancer binding protein 2αA (PEBPLαA) and osteoclastogenesis inhibitory factor OCIF gene expression in human SAOS-2 osteoblast like cells, and B (2). OCIF- deficient mice exhibit severe osteoporosis. Exposure of SAOS-2 cells to 25 μM of this compound for 6 hours resulted in a significant decrease in PEBP2αA and OCIFm RNA levels. The compound can modulate the gene expression of these two factors which play important role in bone metabolism [59].



ALKALOIDS

Berberine (**81**) was isolated from the aqueous extracts of *Tsu-Kam-Gam*, a kampo formula used for the treatment of osteoporosis. Berberine inhibited the formation of OCLs and the bone resorbing activity of OCLs *in vitro*. At the concentration of 2 μM it showed an inhibitory effect on PGE_2 (prostaglandin EI_2) production in osteoblastic cells which results in inhibition of OCLs formation. It was found that oral administration of berberine at the daily dose of 30 or 50 mg/kg to OVX rats prevented a decrease in BMD of the lumbar vertebra without affecting the weight of uterus and plasma concentration of estradiol. These results suggest that berberine prevents a decrease in BMD *in vivo* by inhibiting osteoclastic bone resorption [60]. Biskoenigine 8, 8" (**82**) is a carbazole alkaloid isolated from the ethanolic extract of aerial parts of *Murraya koenigii* (Rutaceae). It was tested for antiosteoporotic activity in bioassays like

cathepsin B, CAT-B, carbonic anhydrase II and CA-II. It showed the activity in the CAT-B model with IC_{50} of 1.3 μg/ml [61].



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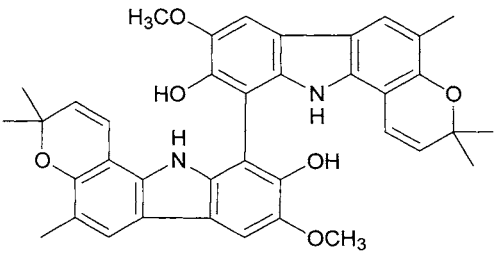


82

FATTY COMPOUNDS

Eicosapentanoeic acid (**83**) is a naturally occurring compound present in most of the plants. The OVX rats with osteoporosis were given a feed containing 0.62 % of this compound (42g/day) but containing no calcium for 6 weeks. Less bone loss in this case was observed than those given a feed containing no Ca and the compound [62]. *In vitro* effects of **83** were studied on MC3T3-E1osteoblasts like cells. The addition of MC3T3-E1cells to the acid caused increased calcium accumulation. When its effect on strength of femur in OVX rats and low calcium / vitamin D fed rats were studied, it was found that it abolished the effects of ovariectomy with consequent recovery of the bone strength.[63] The mixture of C_{22}-C_{38} linear saturated fatty alcohols which contain about 12% of octacosanol (**84**) are found useful for prophylactic and therapeutic treatment of osteoporosis. Rats were fed with this composition for 4 weeks to show breaking strength of bone 5.07 dyne, Vs 4.70 dyne for controls. These fatty alcohols improve mechanical strength of bone, and increase Ca and P contents of bone.[64]

$CH_3CH_2(CH{=}CHCH_2)_5CH_2CH_2COOH$

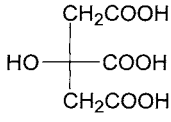
83

$CH_3(CH_2)_{26}CH_2OH$

84

CARBOHYDRATES

Citric acid (**85**) is present in many citrus plants. The effect of citric acid in lemon (*Citrus* sp. Rutaceae) juice was studied on osteoporosis. It was found that it increases Ca absorption through its chelating effect. Thus, osteoporosis can be prevented by frequent uses of lemon in daily meals [65]. Trehalose (**86**) is a disaccharide occurring in fungi, moulds, ergot, algae, yeast and insects. It is used as an effective ingredient in an orally or parentally administrable agent for antiosteoporosis. Trehalose acts by inhibiting the differentiation of osteoclasts, thus, inhibiting bone resorption [66]. Dietary xylitol (**87**) supplementation increased bone Ca and phosphorous in healthy rats, as well as protected against the decrease of bone minerals and bone density during experimental osteoporosis. Furthermore dietary xylitol reduces bone resorption in healthy rats and protects against the ovariectomy induced increase of bone resorption as measured by the urinary excretion of ^{3}H following [^{3}H]–tetracycline–prelabelling. It also increases trabecular bone volume in healthy rats, and protects against the loss of bone during experimental osteoporosis. Dietary xylitol is effective both in increasing bone mass in healthy rats, and in preventing bone loss in OVX rats, suggesting a favorable effect of xylitol on both main targets in the prevention of osteoporosis [67]. The xylooligosaccharides contain ≤ 50 wt % xylobiose (**88**) and ≥ 40 of xylooligosaccharides having a d.p. of ≥ 4. 0.1 to 10 weight parts of xylooligosaccharides are added to the 100 weight parts of health pet food. Xylooligosaccharides are prepared with xylanase from *Eucalyptus sp.* (Myrtaceae) containing broad leaves. This health pet food was found to improve calcium usage and prevent osteoporosis [68].



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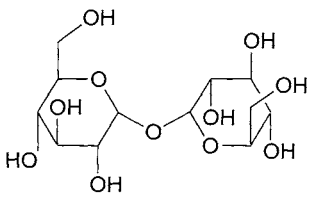

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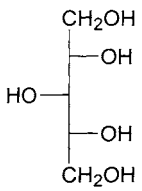

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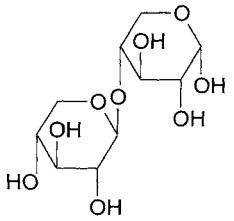
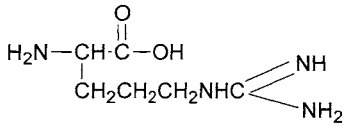


88

AMINO ACIDS AND PEPTIDES

L-arginine (**89**) is widely distributed in animal and plant kingdom. It exerts osteoprotective effects in rats. Rats on soy diet were treated with cyclosporin A, L-arginine, cyclosporin A + L-arginine. Control groups received a normal diet and the same pharmacological treatment. It was found that a soy diet prevents osteopenia only in the spinal cord (+30%) and confirm the protective effect of L-arginine in cyclosporine A induced osteopenia in whole body, pelvis and spine of rats on a normal diet (+31%, +55%, +55% respectively). Moreover these data showed that the osteoprotective effect of L-arginine in the whole body, pelvis and spine improves in the case of soy diet (+60%, +72%, +89% respectively) [69]. Arginine along with lysine (**90**) has a potential therapeutic effect in treatment of osteoporosis. Primary cultures of osteoblasts were used to investigate the effect of amino acids on gene expression, NO production and proliferation (MTT) of cells. Cells were isolated from the distal femurs of normal and osteopenic rats. Normal and osteopenic bone-derived cells were divided into 4 groups: control, Lys (0.587 mg/ml/d), Arg (0.625 mg/ml/d), and Lys + Arg (0.587 + 0.625 mg/ml/d). A significant increase of 10.4 % in NO production was observed in normal bone-derived osteoblasts treated with Lys + Arg when compared to the control group at 7 days. Also there was increase in collagen synthesis, MTT and cell count of osteopenic bone derived cells by 28.4 %, 27 % and 28.7 % respectively with the groups treated with Lysine + Arginine [70]. A peptide γ-L-glutamyl-*Trans*-S-1-propenyl-L-cysteine sulfoxide (**91**) was isolated from the aqueous ethanol extract of *Allium cepa* (Liliaceae). **91** inhibited the resorption activity of osteoclasts dose-dependently. Effect of **91** and calcitonin was studied on *in vitro* resorption activity. This compound was added to the medium at concentrations of 2, 4 and 8 mM. Calcitonin was used at the dose of 10^{-11}M. Two separate experiments were performed: one without the addition of parathyroid hormone and one in which to all cultures PTH (10^{-8}M) was added to stimulate bone resorption. The minimal effective dose of **91** in this model appears to be ~2mM in cultures not stimulated with PTH. In cultures stimulated with PTH this value falls between 2 and 4 mM [71]. WF14861 (**92**) is an amino acid 3-(N-(1-(N-(4-aminobutyl)-N-(3-amino propyl)-carbamoyl)-2-(4-hydroxy phenyl) ethyl) carbamoyl) oxirane-2-carboxylic acid. It was obtained from the culture mycelium of *Colletotrichum* sp. (Incertaesedis). It behaves as a cathepsine B and L inhibitor and also showed inhibitory activities against

bone derived crude protease and other cystine protease *in vitro*. The inhibition of Cathepsin B and L is dose dependent with IC_{50} value of 1.6 x 10^{-8} M and 1.1 x 10^{-9} M respectively. In addition WF 14861 inhibited mouse crude bone cathepsin with IC_{50} value of 4.0 x 10^{-8} M. The compound ameliorated the tissue damage and the bone destruction modes of low calcium diet fed mouse and adjuvant arthritis rat model. It lowered the plasma calcium concentration from 11.70 ± 0.33 mg/dl to 9.88 ± 0.57 mg/dl (84.0 ± 4.87 %) when injected at the concentration of 100mg/kg to low calcium diet fed mice [72].



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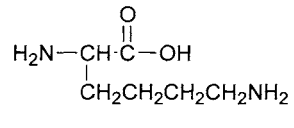

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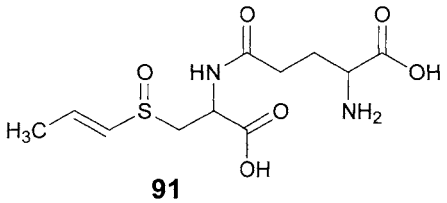

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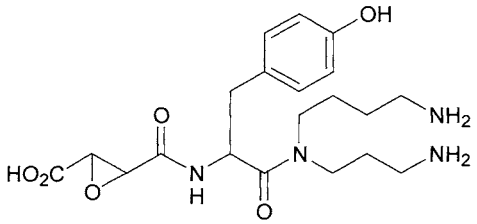


92

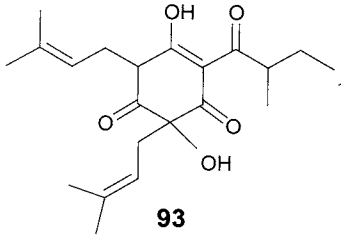
VITAMINS

Vitamins are distributed in whole plant kingdom. The important vitamins that play role in bone metabolism are vitamins B_6, C, D, E and K. Vitamin B_6 could function as a cofactor to build up cross links. Vitamin C is considered an essential cofactor of collagen formation. Vitamin D, the classical vitamin related to bone health, improves bone strength mainly by increasing intestinal Ca absorption, and reabsorption of Ca by the kidney. Similarly vitamin E is also found useful for prevention of osteoporosis. Vitamin K is required for the biological activity of several coagulation factors. This vitamin mediates the carboxylation of glutamyl residues on several bone proteins, notably osteocalcin [73].

MISCELLANEOUS

Besides the compounds discussed under various classes, there are few more compounds that belong to the classes other than those mentioned above hence these are dealt separately. Adhumulone (**93**), cohumulone (**94**), humulone (**95**), isoadhumulone (**96**) and isohumulone (**97**) are ingredients that are isolated from hop (*Humulus lupulus*, Cannabaceae). They have been found to exhibit antiosteoporotic activity by inhibiting bone resorption. They are made into a pharmaceutical composition in combination with a pharmaceutically acceptable carrier or excipient for treating osteoporosis [74]. The compound BE-26263 (**98**) is manufactured by the cultivation of microorganism *Scedosporium apiospermum* (Microascaceae). It is also used for the treatment of gynecological disorders, osteoporosis, prostrate cancer and prostrate hypertrophy. It inhibited binding of (^{125}I) estradiol to receptor with IC_{50} of 0.96 μM [75]. The product BR-606 (**99**) was extracted and purified from *Nardostachys jatamansi* (Valerianaceae) roots as bone sorption inhibitor useful for treatment of osteoporosis and hypercalcemia. This compound showed bone sorption inhibitor activity in isolated rabbit bone tissue cultures with IC_{50} value of 24 μg/ml [76]. Deoxymirosterol (**100**) was isolated from the root tuber of the plant *Pueraria mirifica* (Leguminosae). This compound or its pharmacologically active salts are useful for the treatment of postmenopausal osteoporosis, lipid metabolism disorder, menopausal syndrome and hypogonadism. Deoxymirosterol enhanced growth of MCF-7 cell at $\geq 10^{-8}$ M more strongly than estradiol [77]. Geniposidic acid **(101),** geniposide **(102)** and aucubin **(103** are the iridoid components of leaves and cortex of *Eucommia ulmoides* (Eucommiaceae). They were studied for the induction of growth hormone release by using rat pituatory cells. The proliferation of osteoblasts was assayed using a tetrazolium (MTT), alkaline phophatase (ALP) activity, and [^{3}H]-proline incorporation assays. The inhibition of osteoclasts was studied by using the co-culture of mouse bone marrow cells. The **101**, **102** and **103** increased proliferation of osteoblasts. In addition these compounds significantly inhibited proliferation of osteoclasts at the IC_{50} of 4.43 x 10^{-7}M. These components participate in each step of mechanism for activating osteoblast to facilitate osteogenesis, and suppress osteoclast activity to inhibit osteolysis [78]. Lycopene (**104**) is a carotenoid abundant in tomato (*Lycopersicon esculentum*, Solanaceae) showed effects on proliferation and differentiation of osteoblasts, the cells responsible for bone formation.

Human osteoblasts like cells osteosarcoma SaOS-2 cells were cultured for 24 hours, after which varying doses of a water dispersible microemulsion preparation of lycopene or vehicle of the same dilution were added. The cells were further cultured for 24 to 144 h and the cell numbers were counted. Lycopene at 10^{-6} and 10^{-5} M had significant stimulatory effects on cell numbers, compared with the corresponding vehicle treatment, at all time points from 24 h to 144 h. The effects of lycopene on activity of the differentiation marker alkaline phosphatase activity in the absence or presence of dexamethasone were shown to be dependent on osteoblasts of human origin [79].



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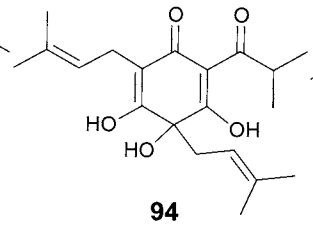

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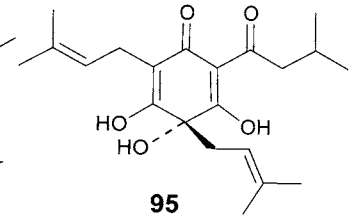

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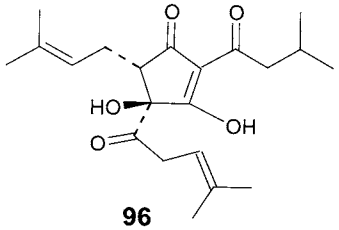

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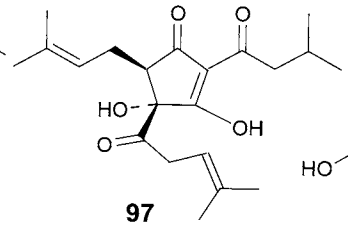

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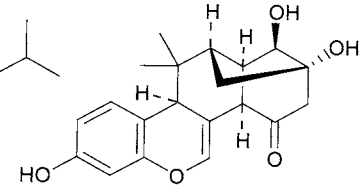

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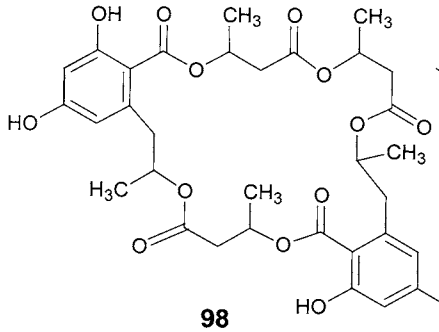

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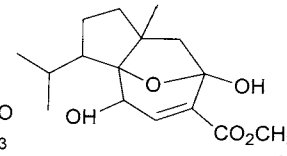

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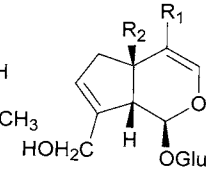

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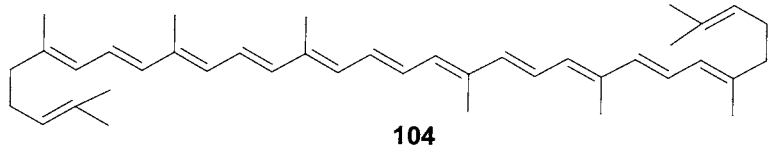
102: R_1=CO_2CH_3, R_2=H
103: R_1=H, R_2=OH



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CONCLUSIONS

Osteoporosis is emerging as a major health problem for aging society all over the world therefore, development of medicinal agents for the successful treatment of osteoporosis is required to assure a person a happy long life. The market of antiosteoporotic drugs is still occupied by synthetic drugs which are successful in combating the disease but each one of these is followed by side effects. Natural products covering a wide diversity of chemical structures have been reported to exhibit antiosteoporotic activity. These structural skeletons could promote useful scaffold or templates for the development of new antiosteoporotic drugs. Given the range of chemical structures and potency of biological activity presented in this review, it is clear that plant products will play an important role in the development of new generation of antiosteoporotic drugs.

ACKNOWLEDGEMENTS

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PLANTS WITH POSSIBLE ANXIOLYTIC AND/OR HYPNOTIC EFFECTS INDICATED BY THREE BRAZILIAN CULTURES - INDIANS, AFRO-BRAZILIANS, AND RIVER-DWELLERS

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ABSTRACT: This study shows that, in spite of the great biological and cultural potential in Brazil, there is, even today, no phytomedicines originating from this flora, as an alternative to allopathic anxiolytics and hypnotics prescribed by psychiatry. Thirty-nine plants with potential anxiolytic effects and 28 hypnotics were indicated in the course of ethnopharmacological surveys carried out with Afro-Brazilians and/or Quilombolas, the Caboclo population (river-dwellers), and Indians in Brazil. Practically no pharmacological studies have been found in the scientific literature as evidence of their popular use. From the phytochemical point of view, it is of interest to observe that flavonoids, essential oils, phenolic acids, and alkaloids are the chemical constituents predominantly present in these species, both in those indicated as anxiolytic, and the hypnotic.

INTRODUCTION

Biological and Cultural Aspects in Brazil

The interest in flora in Brazil can be traced back to the 16th century: countless European botanists and naturalists visited the country to study the landscape and flora as from the 17th century and on, until the end of the 19th century [1,2].

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In this contact, some of the medicinal uses of plants were brought to Brazil, above all, by the Portuguese: rosemary (*Rosmarinus officinalis* L.), while some of the Indian habits were taken from here to the European and African continents: consumption of peanut (*Arachis hypogaea* L.) is an example of this [3].

Brazil, with 8,547,403.5 km^2, possesses the richest flora in the world, with over 56,000 species of plants - almost 19% of the world flora. Estimates today point to 5-10 species of gymnosperms, 55,000-60,000 species of angiosperms, 3,100 species of bryophytes, 1,200-1,300 species of pteridophytes, and some 525 species of marine algae [4,2].

These species are distributed over five main types of biomas: the cerrado savannahs, the Mata Atlântica rain forest, the Amazon forest, pantanal wetlands, and caatinga semiarid scrublands, as can be seen in Fig. (**1**). In spite of all this wealth, the first two are among the ecosystems regarded as hotspots in South America [5].

Brazil is rich not only from the point of view of biodiversity, but also of cultural diversity and is inhabited by, at least, three types of population that live in the rural areas of the biomas cited above, many of them isolated geographically from official medical assistance.

Indigenous Ethnic Groups

Estimates show there are 218 Indian groups inhabiting Brazil - a total of some 370 thousand persons, approximately 0.2% of the total population of Brazil, speaking 180 different languages [6]. This population in Brazil was once, however, much greater. It is difficult to say for sure how many Indians there were in Brazil before colonization by Portugal in 1500 - an estimated 5 million. Three centuries subsequent to this contact with Europeans, this number was reduced to 1 million [7].

Specialists in healing in these ethnic groups are the shamans, that generally use plants that will alter their perception, above all, in rituals for healing, known by the academic community as shamanism. In addition to consuming these plants, that we refer to as hallucinogens, they prescribe as many more, with varied therapeutic purposes, to the sick that seek them out: this process is generally carried through with spiritual aid. In shamanism, a shaman may be in a state of trance, brought on by one of the hallucinogenic plants, and may extracts the disease from the sick in a materialized form.

Fig. (1). The five main biomas in Brazil, prior to human interference. Today, different parts of each of these have ceased to exist and made way for soybean monoculture, human occupancy, and grazing lands, amongst others.

A review by Rodrigues et al [8] shows there are ethnopharmacological surveys concerning 26 indigenous groups in Brazil, some of them occupy the borders of other South American countries [9-38]. The use of 307 plants with possible action on the CNS, with 25 classified as "hallucinogens" were recorded [8]. This review also includes 10 plants with potential anxiolytic and 7 with hypnotic effects, to be presented in the present study, below.

Quilombolas and Afro-Brazilian Groups

There are in Brazil an estimated 178 groups of descendants of Afro-Brazilian runaway slaves, called Quilombolas, who sought hideouts in

areas named Quilombos [39]. In addition, rural and urban areas in Brazil are occupied by Afro-Brazilians, resulting from slavery in Brazil from the 16th until the 19th century. An estimated 3,216,800 African slaves are believed to have been brought to Brazil at this time [7].

The slaves found great biodiversity in Brazil and set about deciphering same, to survive both physically and culturally. Adaptation to the new habitat and the new social conditions gave way to substitution of indispensable plants that were not found here [40]. When it was not possible to find these plants, the Black population developed strategies so that these species could be brought from Africa [40]. Species such as *Cola acuminata* (P. Beauv.) Schott & Endl. (obí), *Garcinia kola* Heckel (orobô kola nut) and *Elaeis guineensis* Jacq. (dendê oil palm) were introduced in Brazil [41].

This ethnic group includes a wealth of experts in healing: faith healers, mediums, "pai-de-santo" priests, "mãe-de-santo" priestesses, "babalorixás", among others. Rituals for healing may also vary, most of them occurring during the "candomblé" and "umbanda" ceremonies. These rituals involve spiritual therapy, divination, healing baths, and incenses [41]. In this therapy, a great number of plants are mixed together to make up each formula in a manner similar to that observed among the Caboclo population (river-dwellers), as opposed to that observed among the Indians [42].

Some studies on the therapeutic use of plants by the Black population have been carried out in Brazil: [3,40,41,43,44,45,46,47,48] and, recently, by Rodrigues & Carlini [49,50].

Mestizo Populations: River Dwellers

Derived from the miscegenation of European, Indian, and Black (including such as: Caboclo river dwellers, coastal Caiçara fishermen, Seringueiro rubber tappers and Jangadeiro raftsmen).

The Caboclo river dwellers living in the Amazon region inhabit the banks of the rivers and live from hunting, fishing, fruit-gathering, and subsistence agriculture. They usually possess a great deal of knowledge on the therapeutic use of plants and animals available to them within their environment [51].

Until ten years ago, over one half of the original population of Amazon Caboclos had been dislodged from their settlements and forced into nearby towns. In these cases all of the thousand-year adaptive wisdom

this population had acquired from the Indians to survive in a site as inhospitable as the Amazon forest, has been lost [7].

Specialists in healing among the Caboclo population include: faith healers, midwives, and healers, among others. In the course of healing practices, in addition to prescribing medicinal plants and animals, rituals are used: prayers, during which branches of specific plants such as vassourinha broom (*Scoparia dulcis* L.) are shaken around the body of the sick.

A record of plants utilized by Caboclos in the Amazon region for medicinal purposes has been compiled by some Brazilian researchers [52-59].

The ethnopharmacology was defined by Schultes [33] as a sub-area of the ethnobotany and refers to the medical or pseudomedical use of plants and animals by pre-literate societies.

Ethnopharmacological studies carried out in Brazilian forests are promising instruments for the discovery of new drugs: the high indices of biodiversity and endemism associated to a process of intense miscegenation resulted in considerable wealth of knowledge on the flora.

In addition, ethnographical work with these groups has shown us that the use of certain categories of plants by any one specific culture is related to their day-to-day needs. Use of hallucinogenic plants is more common, for instance, among Afro-Brazilians and Indians [42]; in healing ceremonies, Shamanism or "Umbanda"rituals, that require the use of plants to facilitate communication with spiritual guides [3,60].

Anxiolytic and Hypnotic Drugs: Psychopharmacology and Psychiatry

Psychoactive substances (including plants) are those that alter some aspect of the mind including behavior, mood, anxiety, cognition, and well-being [61]. They may be classified in three types: a) depressors of Central Nervous System (CNS) activity, such as neuroleptics, anxiolytics, and hypnotics; b) CNS stimulants - antidepressives and amphetamines, mainly; and, finally, c) disturbers of the CNS - hallucinogens [62].

Anxiolytics are substances that, as the name suggests, precipitate a break (lise) in anxiety, and hypnotics are substances that induce sleep. One same substance will generally serve both to reduce anxiety and induce sleep, depending on the dose employed. These drugs are classified

as CNS depressors, producing slower mental processes, reduced reflexes, deficient attention, and memory impairment. On prescribing this medication, the patient must be informed of the risks in driving and operating machinery. Because they are psychotropic drugs, they may potentially induce tolerance and dependence [63,64]. On prescribing a benzodiazepine (BDZ), a doctor should restrict use of same to short periods of time - from 2 to 4 weeks at the most, and only within the framework of severe anxiety or insomnia [65]. In addition, the risk of interaction with other substances is high, particularly with alcohol [66]. The search for new substances to reduce anxiety, that will entail fewer damages than the BDZs are promising prospects in the field of medical therapeutics.

Anxiolytics act together with the gabaergic receptors facilitating coupling to Gama Amino Butiric Acid (GABA) - the main neurotransmissor inhibitor to the CNS, to GABA A receptors. This is an ionotropic receptor that, coupled to GABA, opens channels of chloride, increasing the influx of these anions and rendering the neuron hyperpolarized, reducing nervous transmission [67].

BDZs are among the most prescribed drugs worldwide. Consumption of these drugs is believed to double every five years [68]. In 1999, Brazilian consumption of BDZs was approximately of 20 DDDS (defined daily doses), similar to that of the U.S. [69].

A recent review reported by Ernst [70] assesses the conditions of the various plants, amongst these Blue skullcap (*Scutellaria lateriflora* L.); Gotu kola (*Centella asiatica* (L.) Urb.); Guaraná (*Paullinia cupana* Kunth); Kava-kava (*Piper methysticum* G. Forst.); Lemon grass (*Cymbopogon citratus* (DC.) Stapf); Passion flower (*Passiflora incarnata* L.) and Valerian (*Valeriana officinalis* L.). Only the use of Kava-kava seems consistent as to an anxiolytic effect, comparable to that of BDZs. The author concludes stating that today there are no well documented studies on alternatives to conventional anxiolytics [70]. Several studies have reported the safety and advantages of the use of Kava-kava in relation to the BDZs in that they do not induce dependence and are well tolerated by the patients [71]. There are, however, in scientific literature, reports of cases of hepatitis and hepatic failure produced by chronic use of Kava-kava or even ataxia with acute use of same [72]. Finally, kavalactones from extracts of Kava-kava have been identified as powerful inhibitors of various enzymes of the CYP450 system, which may result in

severe complications in the use of this plant with certain types of medication [73].

Synergism produced by concomitant use of two or more plants popularly recommended as "tranquilizers" is of interest as, for instance, simultaneous use of Kava-kava extract and Passiflora extract [74]. The tendency is to mix extracts from various plants into one single product, also a practice in Brazil. One example is a medication advertised wholesale in the media in Brazil to spread the message that: to feel "ever so relaxed", just take *Passiflora alata* Ailton, *Erythrina mulungu* Mart. ex Benth, and *Crataegus oxyacantha* Linné. The product described as phytomedicine does not include one fundamental piece of information - namely, a 16% alcoholic content. So, what exactly does bring on the relaxed feeling: the plant extracts or the alcohol?

The Phytochemical Approach in the Study of Medicinal Plants

Plants are regarded as an important source of biologically active natural products, many of which are models for synthesis of a great number of medicines. Researchers of natural products are impressed by chemical constituents found in nature in an almost unbelievable range of structures and physico-chemical and biological properties [75]. With the prospect of obtaining new medicines, two aspects distinguish products of natural origin from the synthetic: molecular diversity and biological function. Molecular diversity of natural products is much superior to that derived from processes of synthesis, that in spite of considerable advances, are as yet limited. In addition, as the product of organisms that are very similar to the metabolism of mammals, natural products often show properties additional to the antimicrobian associated to them [76]. The quality of a phytomedicine is ensured through the characterization of their chemical constituents, or pharmacological activity. None of the alternatives are swift or of rapid execution. The most secure option would be to identify and determine the concentration of active substances, which is not always possible because of the great number of components present in the extract. Use of marker substances, listing, for instance, the concentration of the more abundant substances, or that of chemical groups with biological activity is an alternative to be validated.

Anxiolytic Drugs: Animal Models for Study

Several animal models have been developed in the study of anxiety in order to verify the effectiveness of new drugs, study their mechanisms of action, or investigate the pathophysiological phenomena involved in anxiety. These models are based on punishment-conflict procedures, such as the Geller and Steifer model [77], or, as the model for discrimination of the convulsive effects of penthylenotetrazol, or, as yet, in exploratory behaviors such as the Elevated Plus Maze (EPM) [78] model, and also fear of a new situation such as a neophobic reaction in rats. Amongst the most utilized, we may cite the EPM, the neophobia test, and the open field.

The EPM was utilized for the first time by Handley and Mithani, in 1984, as from the model developed by Montgomery, in 1955. They observed that rats placed in an elevated maze, consisting of two open arms and two closed arms showed a preference for the closed arms. In addition, these authors observed that anxiolytic drugs such as diazepam (a BDZ) increased the number of entrances of the animals into the open arms of the maze, where anxiogenic drugs reduced the number of entrances into these arms. Among the advantages of this model are simplicity of use, low cost of assembly and maintenance, allowing an assessment both of the anxiolytic and of the anxiogenic substances [79].

Another very simple test to evaluate substances with anxiolytic action is the Test of Neophobia in Rats [80]. When rats are exposed to new food in their natural habitat, or when their usual food is placed in a new environment, or when both occur, namely, food and environment are both new, the animals' intake of food is reduced (neophobic reaction). This seems to be the result of a reaction of "fear" or of "anxiety" on the part of the animal to external stimuli: ultimately, the anxiolytics should be able to block the reaction, as, in fact, they do.

A response to anxiety is a subjective answer inherent to the human species; for this reason, the term "emotionality" or "emotional response" has often been used to refer to the association of responses generated by animals exposed to new situations or that are elicited by several noxious stimuli.

The open field is one of the oldest models used for the study of emotionality in animals, and was used for the first time in 1934 by Hall. The procedure consists in observing locomotor activity, rearing, freezing, and defecation by animals subjected to a circular arena divided into 12

peripheral and 6 central quadrants. In addition, some of the variables present in the environment as, for instance, continuous noise or increased lighting, increase the emotional reactivity in animals and, for this reason, are part of the procedure most commonly utilized for open field [81,82]. Open field, as in the case of other tests formerly described, is also a model characterized by simplicity and ease of application - much utilized to assess possible anxiolytic action of one specific substance.

To evaluate the hypnotic effect, the same models can also be utilized, besides one more specific, the sleeping time.

Plants with possible anxiolytic and/or hypnotic effects indicated by three Brazilian cultures - Indians, Afro-Brazilians, and River-Dwellers.

As in a previous review [8], one strong limitation to analyzing the data found in this study resides in the interpretation of the researchers concerning the uses indicated by the cultures involved in each one of the ethnopharmacological surveys. To correlate one term in ethnomedicine with one in official use is not always an easy task: for some terms, researchers must resort to a type of "translation", a type of "ethnopharmacological puzzle" where one of the greatest challenges to researchers is the absence of medical professionals to follow up on field works and thus contribute to establish this correlation.

Twenty-three publications were consulted for this review: 10 scientific articles, 8 books, and 5 Master's degree and Ph.D. degree theses. Of studies carried out amongst Indians, Afro-Brazilians and/or Quilombolas, and Caboclos that inhabit 4 of the 5 principal biomas in Brazil (caatinga, the Amazon forest, cerrado and Mata Atlântica) almost one half (10) are studies among Afro-Brazilians and/or Quilombolas; another 7 among Indians, and 6 among Caboclos. These data must be considered in analyzing the contributions from each group to the present study.

Plants with Possible Anxiolytic Effects

Table 1 shows that 39 species were indicated for uses with apparent anxiolytic effect/action. The main uses described in literature seem synonymous - as a tranquilizer, as a tranquilizer for children, for anxiety, a tranquilizer for nerves, for nervous excitation, nervous disturbances, to

relax, for child nervousness, children crying for feeling unwell, for emotionally unstable people, for irritability, for nervous diseases, and nervousness.

These 39 species belong to 22 taxonomic families, the most important being: Asteraceae (5 species indicated), Fabaceae (4), and Melastomataceae (4); with 48.7% of these native to Brazil.

The species most often cited in the publications consulted were *Lippia alba* (Mill) N.E.Br. (cited in 7 of these) and *Passiflora edulis* Sims. (4).

Among the plants indicated for anxiety, 21 were indicated by Afro-Brazilians and/or Quilombolas; 15 by Caboclos and by 10 Indian groups with some species indicated by more than one culture. Most of the publications available and consulted in this study focus on the first ethnic group.

Table 1: Thirty-nine (39) plants with possible anxiolytic effects utilized by three cultures in Brazil - Indians, Afro-Brazilians and Caboclos - and respective pharmacological and phytochemical studies published in the scientific literature.

Family (n. species) *Species* - vernacular name	**Use described in the literature consulted**	**Part utilized and form of use as described in the literature consulted**	**Cultures cited in the literature consulted**	**Phytochemical studies found in the literature**	**Pharmacological studies found in the literature**
Amaranthaceaea (1)					
Celosia argentea L. - suspiro	Tranquilizer	No information	Quilombolas [48]	Betalains [84,85]	Anti-diabetic activity [248]; anti-metastatic and immunomodulating properties [249]; immunostimulating activity [250]; hepatoprotective effects [251]
Annonaceae (2)					
Annona muricata L. - graviola	Tranquilizer	No information	Caboclos [59]	Acetogenins [86,87]; neurotoxic benzylisoquinoline derivatives [88]; essential oil [89,90]; alkaloids [91,92]; flavonoids and terpenoids [93]	striatal neurodegeneration [252]; atypical parkinsonism [253]; antileishmanial activity [254]; antidepressive activity [91]
Guatteria scandens Ducke [N] - Cipó-luira, cipó-iuira	As a tranquilizer for children	Bark (bathing)	Caboclos [52]	Alkaloids [94,95]	
Apiaceae (1)					
Pimpinella anisum L. - erva-doce	For anxiety	Raw leaves to be chewed or leaf tea, for flatulence	Afro-Brazilians [41]	Flavonoids [96]; essential oil [97,98]; coumarins [99]	
Apocynaceae (1)					
Tabernaemontana sananho Ruiz & Pav. [N]	For a calming effect		Indians from the Brazilian Amazon [83]	No phytochemical data	
Asteraceae (5)					
Baccharis uncinella DC. [N] -vassoura-do-campo	Tranquilizer for the nerves	Leaves and flowers (decoction , ingested)	Xokleng Indians [36]	No phytochemical data	
Lactuca sativa L. -	Nervous	Leaf (Infusion)	Afro-Brazilians	Phenolic acids [100]; triterpenoids,	

alface	disturbances		(Northeast Brazil) [45,46]	saponins [101,102]; flavonoids [103]	
Matricaria chamomilla L. - camomila	Nervous disturbances	Flower (infusion)	Afro-Brazilians (Northeast Brazil) [45,46]	Flavonoids [104,105]; essential oil [106]; acylglycerols, linoleic and linolenic acids [107]; coumarins [108,109]; sesquiterpene lactone [110]	inhibits both development of morphine dependence and expression of abstinence syndrome [255,256]; anxiolytic effect [257]; sedative as well as spasmolytic effects [258]
Mikania amara (Vahl) Willd.[N] - Cipó-catinga)	As a tranquilizer	Leaf (bathing)	Caboclos [52]	No phytochemical data	
Tagetes erecta L. - cravo-de-defunto	Diseases of the nerves	Flowers (infusion, ingested)	Caboclos [56]	Fatty acids [111,112,113]; essential oils [114,115]	
Caryophyllaceae (1)					
Dianthus caryophyllus L. - cravo-branco	Nervous disturbances	Flower (Infusion)	Afro-Brazilians (Northeast Brazil) [45,46]	Phenolic acids [116]; essential oils [117]; anthocyanins [118,119]	
Crassulaceae (1)					
Kalanchoe brasiliensis Cambess.[N] - folha-da-costa	As a tranquilizer	Leaf	Afro-Brazilians [43]	Flavonoids [120]	Immunomodulatory and anti-inflammatory effects [259,260]
Fabaceae (*sensu lato*) (4)					
Erythrina corallodendron L. - mulungu	As tranquilizer for nervous excitement	Bark	Afro-Brazilians [44]	No phytochemical data	
Erythrina poeppigiana (Walp.) O.F. Cook - mulungu	To relax	Leaf (tea)	Afro-Brazilians [41]	Isoflavonoid [121,122,123]; arylbenzofuran [124], erythrinan alkaloids [125,126]	Antibacterial properties [121]; antimicrobial activity [123]
Erythrina velutina Willd.[N] - mulungu	As a tranquilizer	Relief bathing and bottled brews (ingestion)	Afro-Brazilians [44]	Flavonoids [127,128]	Anti-nociceptive activity [261,262] ; central nervous system effects [263,264]
Mimosa camporum Benth. - juqueri-manso	As a tranquilizer for children	Leaves and roots (bathing)	Caboclos [52]	No phytochemical data	
Lamiaceae (3)					

Melissa officinalis L. - erva-cidreira	Irritability	Tea	Terena Indians [13]	Flavonoids [129,130]; essential oils [131,132,133,134]; phenolic acids [135,136,137]	
Mentha piperita subsp. *citrata* Briq. - hortelã	Tranquilizer	Branch (infusion, ingested)	Caboclos [57]	Essential oils [138,139]; flavonoid glycosides [140,141]; menthol [142]; menthofurolactone [143,144]; pyridine-derivatives [145]; mintlactone [146]; flavones [147]; triterpenes [148]; terpenoids [149]; sesquiterpenic hydrocarbons [150]; menthofuran [151,152].	Antimicrobial and antioxidant activities [138,265]; antiallergic effect [140]; anti-nociceptive and anti-inflammatory effects [266]
Pogostemon cablin (Blanco) Benth. – oriza, patchouli, patcholi, patchuli	Tranquilizer, for a calming effect	Leaf (decoction or infusion, ingested)	Caboclos [51,56]	Essential oils [153,154,155,156]; patchoulic alcohol [157,158]; sesquiterpene hydroperoxides [159]; flavonoids [160,161]; tetracyclic sesquiterpene [162,163,164].	
Malpighiaceae (1)					
Camarea ericoides A. St.-Hil. [N] - erva-doce-do-campo	Tranquilizer	The whole plant (decoction, ingested)	Quilombolas*	No phytochemical data	
Melastomataceae (4)					
Henriettea granulata O. Berg & Triana [N] – Pöra	Irritability in children, children crying	Leaves (infusion, bathing)	Tiriyó Indians [14]	No phytochemical data	
Miconia holosericea (L.) DC. [N] - Pöra-imö	Irritability and for children who are unwell	Leaves (decoction, bathing)	Tiriyó Indians [14]	No phytochemical data	
Miconia rubiginosa (Bonpl.) DC. [N] - pöra-imö	Irritability and for children crying because they are unwell	Leaves (decoction, bathing)	Tiriyó Indians [14]	Triterpenoids [165]	
Tococa formicaria Mart. [N]	As a tranquilizer	Branches (bathing)	Pareci Indians [25]	No phytochemical data	
Meliaceae (1)					
Cedrela odorata L. [N] - Chega-te-a-mim	As a tranquilizer	Bark (bathing)	Caboclos [52]	Essential oils [166,167]; flavonoids [168,169]; tetranortriterpenoids [170]; triterpenoids [171,172]	Antimalarial activities [168,169]
Monimiaceae (1)					

Siparuna guianensis Aubl.[N] - capitiú	Tranquilizer for children	Leaf (bathing the body)	Caboclos [51]	Essential oil [173,174]; oxoaporphine alkaloids [175]; flavonoids [176]	
Moraceae (1)					
Ficus doliaria (Miq.) Miq.[N] - gameleira	For a calming effect	Latex	Afro-Brazilians	No phytochemical data	
Nymphaeaceae (1)					
Nymphaea alba L. - òsibàtà	As a tranquilizer	Leaf	Afro-Brazilians [40]	Anthocyanins [177]	
Passifloraceae (1)					
Passiflora edulis Sims[N] - maracujá, maracujá-de planta	Tranquilizer, irritability	Flower or leaf (decoction, ingested)	Caboclos [53,57,59]; Xokleng Indians [36]	Flavonoids, glycosides, alkaloids [178]; triterpenoids [179]; saponins [180]; norterpenoids derivatives [181]; Cyanogenic glucosides [182]; carotenoids [183]; 3-Methyl-2-butanone [184]; phenolic compounds [185]	
Poaceae (1)					
Cymbopogon citratus (DC.) Stapf - capim-santo, capim-cidró	Tranquilizer, for a calming effect on the nerves, irritability	Leaf or the whole plant (decoction, ingested)	Quilombolas [50,54]; Xukuru Indians [37]; Caboclos [57,59]	Flavonoids [186,187]; essential oils [188,189]; triterpenoids [190]	
Rutaceae (3)					
Citrus aurantifolia (Christm.) Swingle - lima	Tranquilizer	No information	Caboclos [59]	Essential oils [191,192]; flavonoids [193,194]	
Citrus aurantium L. - laranja	Nervous disturbances	Leaf (Infusion)	Afro-Brazilians (Northeast Brazil) [45,46]	Alkaloids [195,196]; adrenergic amines [197,198]; flavonoids [199,200]; Coumarins, fatty acids [201,202]; polyphenolic compounds [203]; essential oils [204,205]	Suppressive effect [267]; ischemic stroke [268]; cardiovascular changes [269]; weight loss [267]; induction of apoptosis [270]; anxiolytic and sedative effects [271]; adrenergic agonists [272], antiobesity [273,274]; anti-inflammatory activity [275]
Citrus sinensis (L.) Osbeck - laranjeira, laranja	Tranquilizer	Leaf (infusion or decoction, ingested)	Caboclos [57,59]; Quilombolas [54]	Flavonoids [206,207]; essential oils [208,209]; phenolic compounds; Coumarins, fatty acids [210,201]; quinoline alkaloids [211,212];	Radical scavenging activity [276]; antioxidant activity [277]; inotropic effect [278]

				anthocyanins [213,214]	
Schizaeaceae (2) - ferns					
Lygodium venustum Sw.[N] - abre-caminho	For emotionally unstable people who are irritable	The whole plant (relief bathing)	Afro-Brazilians (Northeast Brazil) [47]	No phytochemical data	
Lygodium volubile Sw.[N] - abre-caminho	For emotionally unstable people who are irritable	The whole plant (relief bathing)	Afro-Brazilians (Northeast Brazil) [47]	No phytochemical data	
Solanaceae (1)					
Physalis angulata L.[N] - camapu	Nerve diseases	Root (tea, ingested)	Caboclos [56]	Steroids [215,216]; flavonoids [217]; alkaloids [218]	
Verbenaceae (2)					
Lippia alba (Mill.) N.E. Br.[N] - cidreira, erva-cidreira, melissa, carmelitana	A calming effect, to relax, tranquilizer	Leaves (decoction)	Afro-Brazilians [3,41]; Xukuru Indians [37]; Caboclos [52,56,59]; Quilombolas [48]	Alkaloids [219]; saponins [220]; sterols, flavonoids [221]; essential oils [222,223]; prenylated naphtoquinones [224]; iridoids [225]; terpenoids [226,227]; phenolic acids [228]	
Vitex agnus-castus L. - alecrim-de-angola	Tranquilizer	Flowers and seeds (prepare a beverage)	Afro-Brazilians [3]	Phenolic compounds [229]; essential oil [230,231]; iridoidal glycosides [232,233]; flavonoids [234,235]; glucosides [236]; diterpenoids [237,238]; labdane diterpene lactam [239]; phenolic acids [240]; ketosteroids [241]	Induces apoptosis [279,280]; dopaminergic activity [281]; treatment of disorders of the female sexual cycle [282]
Zingiberaceae (1)					
Alpinia zerumbet (Pers.) B.L. Burtt & R.M. Sm. - leopoldina	Nervousness	Tea	Afro-Brazilians [41]	Essential oils [242,243]; kava-pyrones [244]; flavonoids [245]; labdane diterpenes [246,247]	

[N] native to Brazilian flora.

* Personal information obtained by author E.R., in a study not yet published.

A survey made on the main data base and an extensive analysis of the scientific literature available, showed that some plants with an anxiolytic and/or hypnotic effect utilized by the three Brazilian cultures exert an effect confirmed by scientific studies. Outstanding among these plants are those of the genus *Erythrina* - a genus well known in Brazil and in various parts of the world. Some authors [283,284] described the depressor activity on the CNS. Vasconcelos et al. [264] found that the hydroalcoholic extracts of *E. velutina* Wild and *E. mulungu* Mart. ex Benth did not interfere with the results of the EPM and did not, therefore, present an anxiolytic effect; however, the data obtained by these authors showed a depressor action on the CNS, in that rats subjected to the Open Field test subsequent to the administration of these plants; the animals showed a reduction in locomotor activity, in rearing and grooming. Onusic et al [285], on the other hand, observed an anxiolytic effect in rats subsequent to acute administering of a hydroalcoholic extract of *E. mulungu* Mart. ex Benth. The action of another plant of this genus (*E. poeppigiana* (Walp) O.F.Cook), utilized by Afro-Brazilians as an anxiolytic, is not confirmed in scientific literature: on the other hand, reports concerning this plant emphasize its antibacterial and muscular block action [286,287].

Melissa officinalis L. is a plant widely utilized for its mild sedative/hypnotic properties [288,289], and is also employed as an anxiolytic by Brazilian cultures related to this study. In high doses, this plant may present peripheral analgesic activity, obtained by reducing the acetic acid-induced pain (writhing test) [288]. Its essential oils possess an antioxidant and anti-tumoral effect [290,291]. Clinical studies with this plant [292-294] also showed that administering same on healthy volunteers reduced stress induced in a laboratory, in addition to modulating mood and augmenting calmness, although it may produce damage to cognitive functions. Some studies show that the extract of *Melissa officinalis* L. also acts as a stimulant of the immune system [295].

One other plant much cited (7 citations) by the cultures, in addition to being widely utilized in popular Brazilian medicine, is *Lippia alba* (Mill) N.E.Br. as to its sedative properties [296,297]. Some studies reported that this plant also has a myorelaxing effect [296] and its essential oils may present both a possible anxiolytic [298] and anticonvulsant effect [299].

The genus *Passiflora* includes approximately 500 species and, as per the last two plants cited (*Melissa officinalis* and *Lippia alba*), is a genus well known [178]. In the specific case of *Passiflora edulis* Sims., some studies have shown its anxiolytic effect when assessed through the EPM [300]. A comparative study involving two species of this genus by Dahwan et al [301] showed that, although the methanolic extract of *P. edulis* Sims. presented an anxiolytic effect, this effect is less potent if compared to that observed with *Passiflora incarnata* L.

Some of the plants listed as anxiolytic by the three cultures are also listed as hypnotic, amongst these, *Lippia alba* (Mill) N.E.Br., the species of the *Citrus* and of the *Erythrina* genus. This is owing to the fact that, often, the hypnotic and anxiolytic effects are similar, in that one and the same substance may present both effects, depending on the dose utilized. On the other hand, some plants listed in Table 1 did not have an anxiolytic effect confirmed by the survey in literature, and others have apparently not been studied up to the present time.

A Phytochemical Approach

The phytochemical survey carried out with the species indicated, to which were attributed an anxiolytic effect showed that the main constituents found were the flavonoids, essential oils, as shown in Fig. (**2**), followed by the phenolic acids, sesquiterpenoids, triterpenoids, and alkaloids.

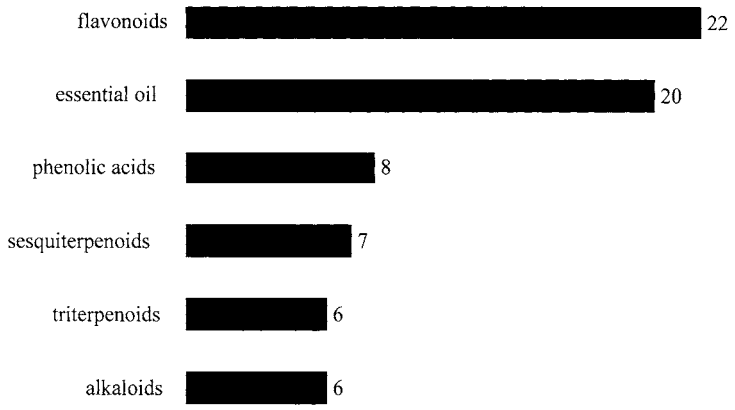


Fig. (2). Number of species with possible anxiolytic effects indicated by the cultures under study that present respective chemical constituents (in this figure represented only by those constituents with at least six species cited).

The flavonoids represent one of the most important and diversified phenolic groups among products of natural origin. This class of compounds is broadly distributed in the plant kingdom. Flavonoids such as 6-Methylapigenin and hesperidin exert activity on the CNS [302]. *Passiflora incarnata* L. has been used to cure anxiety and insomnia since time immemorial. The flavonoids of *P. incarnata* L. [301] and of *Turnera aphrodisiaca* Ward showed anxiolytic activity [303]. The behavioral effects of acute administration of two flavonoids, apigenin and chrysin, contained in *Matricaria chamomilla* L. and in *P. incarnata* L., respectively, were studied in rats. Chrysin exhibited a clear anxiolytic effect [255].

According to the chemical structure, the anxiolytic agents used in allopathic medicine may be divided into three classes: carbamate of propanodiol and related compounds, BZDs and several others compounds. The most effective are the BDZs Chlordiazepoxide, Fig. (**3**), which was commercialized in 1960 as a therapeutic innovation for the treatment of anxiety. As from the identification of its property, dozens of new BDZs derivates were commercialized, including diazepam, Fig. (**3**), one of the medicines most prescribed worldwide.

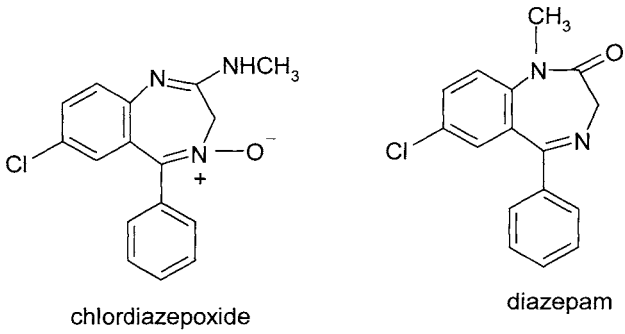


Fig. (3). Chemical structures of chlordiazepoxide and diazepam.

Flavonoids: A New Family of BDZ Receptor Ligands

BDZs are the most widely prescribed class of psychoactive drugs in current therapeutic use, despite the important unwanted side-effects that they produce such as sedation, myorelaxation, ataxia, amnesia, ethanol and barbiturate potentiation, and tolerance. The existence of a new family of ligands with a flavonoid structure was recently demonstrated in the

search for safer BDZ-receptor (BDZ-R) ligands. First isolated from plants used as tranquilizers in folk medicine, some natural flavonoids have proved to possess a selective and relatively mild affinity for BDZ-Rs and a pharmacological profile compatible with a partial agonistic action. As a logical extension to this discovery, various synthetic derivatives of those compounds, such as 6,3'-dinitroflavone, were found to have a very potent anxiolytic effect, not associated with myorelaxant, amnestic or hypnotic actions [304].

A large number of structurally different classes of ligands, many of them sharing the main characteristics of the BDZ nucleus, are active in the modulation of anxiety, sedation, convulsion, myorelaxation, hypnotic and amnesic states in mammals. These compounds have a high affinity for the BDZ binding site (BDZ-bs) of the GABA(A) receptor complex. Flavonoids found to be ligands for the BDZ-bs have been compared with the classical BDZ diazepam [305]. Structure-activity relationships utilizing synthetic flavonoids with different 2' substituents on the flavone backbone supported that 2'-hydroxyl-substitution is a critical moiety on flavonoids with regard to BDZ receptor affinities [306].

Volatile oils are compound mixtures of volatile, lipophylic substances that are generally odoriferous and liquid. The name volatile oils is derived from some of their physico-chemical characteristics such as, for example, to being generally liquid and oily in appearance at room temperature. Their main characteristic is volatility. Another important characteristic is a pleasing and intense aroma for the majority of volatile oils - also known as essences. In water, volatile oils present limited solubility, but that is sufficient to aromatize aqueous solutions [307]. Angelica essential oil (*Angelica officinalis* Moench.), as does diazepam, exhibits an anxiolytic-like effect [308]. Essential oil from *Tagetes minuta* L. may exert a negative modulation on the GABAergic function without affecting the learning ability [309].

No article was found reporting anxiolytic activity of phenolic acids. However, the anxiolytic activity of the alkaloids is known. Anxiolytic properties may be a crucial feature of newer antipsychotics associated with the improvement of negative symptoms in schizophrenic patients. The indole alkaloid alstonine acts as an atypical antipsychotic in behavioral models, but differs in its dopamine and serotonin binding profile [310]. Behavioral effects of psychollatine, a glycoside indole monoterpene alkaloid isolated from *Psychotria umbellate* Thonn., was investigated in models of anxiety, depression, memory, tremor, and

sedation related to 5-Hydroxytryptamine (5-HT) and/or GABA neurotransmission. The effects of psychollatine on the CNS involve serotonergic 5HT2 (A/C) receptors [311].

Plants with Possible Hypnotic Effects

Twenty-eight species were indicated for the following uses: insomnia, severe insomnia, sedative, hypnotic, sedative for children, for the elderly who find difficulty in sleeping, to induce sleep (Table 2).

These species belong to 17 taxonomic families, the most important being: Fabaceae (6 species cited) and Rutaceae (3); 57% of these belong to the Brazilian flora. The species with greater frequency of citation in the publications consulted were: *Lactuca sativa* L., *Lippia alba* (Mill.) N.E.Br. and *Citrus aurantium* L. (3 citations each).

In the same way as in the category of anxiolytic plants, the majority of plants were indicated by the Afro-Brazilians and/or Quilombolas (15 in all), with 8 indicated by Indians, and 6 by Caboclos.

Table 2: Twenty-eight (28) plants with possible hypnotic effects utilized by three cultures in Brazil - Indians, Afro-Brazilians and Caboclos - and respective pharmacological and phytochemical studies published in the scientific literature.

Family (n. species) *Species* - vernacular name	**Use described in the literature consulted**	**Part utilized and form of use as described in the literature consulted**	**Cultures cited in the literature consulted**	**Phytochemical studies found in the literature**	**Pharmacological studies found in the literature**
Annonaceae (1)					
Annona muricata L. - graviola	Severe insomnia	Leaves (infusion or decoction, ingested)	Caboclos [56,57]	Acetogenins [86,87]; neurotoxic benzylisoquinoline derivatives [88]; essential oil [89,90]; alkaloids [91,92]; flavonoids and terpenoids [93]	Striatal neurodegeneration [252]; atypical parkinsonism [253]; antileishmanial activity [254]; antidepressive activity [91]
Asteraceae (2)					
Lactuca sativa L. - alface	Insomnia, sedative	Leaf (tea)	Terena Indians [13]; Afro-Brazilians (Northeast Brazil) [45,46]	phenolic acids [100]; triterpenoids, saponins [101,102]; flavonoids [103]	
Matricaria chamomilla L. - camomila	Sedative	Flower (Infusion)	Afro-Brazilians (Northeast Brazil) [45,46]	Flavonoids [104,105]; essential oil [106]; acylglycerols, linoleic and linolenic acids [107] ; coumarins [108,109]; sesquiterpene lactone [110]	Inhibits both development of morphine dependence and expression of abstinence syndrome [255,256]; anxiolytic effect [257]; sedative as well as spasmolytic effects [258]
Burseraceae (1)					
Commiphora leptophloeos (Mart.) J.B. Gillett [N] - emburana	Insomnia	Seeds (decoction, ingested)	Quilombolas*	No phytochemical data	
Caryophyllaceae (1)					
Dianthus caryophyllus L. - cravo-branco	Sedative	Flower (Infusion)	Afro-Brazilians (Northeast Brazil) [45,46]	Flavonoids [313,314]; phenolic acids [116]; essential oils [117]; anthocyanins [118,119]	

Fabaceae (*sensu lato*) (6)					
Cassia multijuga Rich.[N] - topeiuia	As a sedative for children	Leaves	Tenharins Indians [56]	Anthraquinones [315,316]; anthraquinones glycosides [317]; chromone glycosides [318,319]; flavonol glycoside [320]	
Erythrina mulungu Mart. ex Benth[N] - mulungu	Hypnotic and sedative	Bark (decoction)	Afro-Brazilians [44]	No phytochemical data	
Erythrina poeppigiana (Walp.) O.F. Cook - mulungú	Soporific	Leaf (tea)	Afro-Brazilians [41]	Isoflavonoid [121,122,123]; arylbenzofuran [124]; erythrinan alkaloids [125,126]	
Erythrina speciosa Andrews[N] - mulungu	Hypnotic and sedative	Bark (decoction)	Afro-Brazilians [44]	Flavonoids [321]	Antibacterial activity [321]
Mimosa hirsutissima Mart. var *barbigera* (Benth) Barneby[N] - durme-durme	Insomnia	Leaves (decoction, ingested)	Quilombolas*	No phytochemical data	
Mimosa pudica L.[N] - cipó-dorme-dorme	Insomnia	Leaves and vines (decoction, ingested)	Xokleng Indians [36]	Tannins, steroids, alkaloids, triterpenes [322,323]; flavonoids [324,325,326,327,328]; saponins [329]; bufanolide [330]; mimopudine [331,332]	
Flacourtiaceae (1)					
Casearia sylvestris Sw.[N] - chá-de-frade	Insomnia	Leaf (decoction, ingested)	Quilombolas*	Terpenes [333]; essential oil [334,335]; diterpene [336]; clerodane diterpenoids [337,338]	Antiulcer activity [354]; abortive activity [338]
Lamiaceae (1)					
Pogostemon cablin (Blanco) Benth. - oriza, patchouli, patcholi, patchuli	Sedative	Leaves (infusion, ingested)	Caboclos [56]	No phytochemical data	
Malpighiaceae (1)					
Camarea ericoides A. St.-Hil.[N]	Against insomnia	The whole plant	Quilombolas*	No phytochemical data	

- erva-doce-do-campo		(decoction, ingested)			
Malvaceae (2)					
Hibiscus rosa-sinensis L. - pampola	Against insomnia	Flowers (infusion)	Caboclos [56]	Phenolic acids [339]; fatty acid methyl esters [340,341]; anthocyanins [342]	Against oxidative damage [355,356]; hypoglycemic activity [357,358]
Urena lobata L.	Sedative	No information	Indians of the Brazilian Amazon [312]	No phytochemical data	Antibacterial activity [359]
Marcgraviaceae (1)					
Marcgraviastrum elegans de Roon [N] - no-tê-wê-tá	For the elderly who find it difficult to sleep	Leaves and flowers (tea)	Kubeo Indians [83]	No phytochemical data	
Monimiaceae (1)					
Siparuna guianensis Aubl. [N] - negramina-branca	Insomnia	Leaf (decoction, ingested)	Quilombolas*	Essential oil [173,174]; oxoaporphine alkaloids [175]; flavonoids [176]	
Passifloraceae (2)					
Passiflora coccinea Aubl. [N] - maracujá-do-mato	Sedativo	Leaves (tea, ingested)	Caboclos [56]	Cyanogenic glycoside [343]	
Passiflora laurifolia L. [N]	To induce sleep	Leaves (decoction)	Kubeo Indians [83]	No phytochemical data	
Poaceae (1)					
Saccharum officinarum L. - cana-de-açucar	Insomnia	Culm (suco)	Caboclos [56]	Flavonoids [344,345]; long-chain aliphatic acids [347]; fatty acids [346,348]; phenolic compounds [349]; glycans [350]; triterpenoids and steroids [351]	Anti-inflammatory and analgesic effects [348]; antidiabetes [350]
Rubiaceae (2)					
Palicourea rigida Kunth [N] - erva-molar-fêmea	Insomnia	Leaf (decoction, ingested)	Quilombolas*	Alkaloids and iridoid glucosides [352]	
Rudgea viburnoides (Cham.) Benth. [N] - erva-molar-macho	Insomnia	Leaf (decoction, ingested)	Quilombolas*	No phytochemical data	
Rutaceae (3)					
Citrus aurantium L. - laranja-da-terra, laranja	Sedative	Leaf (Infusion)	Afro-Brazilians (Northeast Brazil) [41,45,46]	Alkaloids [195,196]; adrenergic amines [197,198]; flavonoids [199,200]; coumarins, fatty acids [201,202];	

				polyphenolic compounds [203]; essential oils [204,205]	
Citrus sinensis (L.) Osbeck (laranjeira)	Insomnia	Leaf (decoction, ingested)	Quilombolas*	Flavonoids [206,207]; essential oils [208,209]; phenolic compounds; coumarins, fatty acids [210,201]; quinoline alkaloids [211,212]; anthocyanins [213,214]	Radical scavenging activity [276]; antioxidant activity [277]; inotropic effect [278]
Pilocarpus pennatifolius Lem.[N] - ibirarta-iba	Sedative	No information	Indians of the Brazilian Amazon [312]	Essential oils[353]	
Solanaceae (1)					
Brugmansia insignis (Barb. Rodr.) R.E. Schult.	Sedative	Leaves	Indians of the Brazilian Amazon [83]	No phytochemical data	
Verbenaceae (1)					
Lippia alba (Mill.) N.E. Br.[N] - cidreira, erva-cidreira	To sleep, to induce sleep	Leaves (infusion or decoction, ingested)	Caboclos [51,52,57]	Alkaloids [219]; saponins [220]; sterols, flavonoids [221]; essential oils [222,223]; prenylated naphtoquinones [224]; iridoids [225]; terpenoids [226,227]; phenolic acids [228]	

[N] native to Brazilian flora.

* Personal information obtained by author E.R., in a study not yet published.

Some plants utilized as hypnotics and/or anxiolytics by the Brazilian cultures such as, for instance, *Citrus aurantium* L., were the object of contradictory studies. According to Carvalho-Freitas & Costa [271], the essential oils of this plant exert sedative and anxiolytic effects, whereas the hexane and dichlomethanic fractions present only the former effect. The anxiolytic effect of the essential oils of this plant was later confirmed by Pultirini et al. [360].

Although *Lactuca sativa* L. is one of the species most cited in this survey, there is no clear pharmacological evidence that this plant exerts a significant hypnotic effect. According to Bang et al [361], phytol, a diterpenoid isolated from the ethanolic fraction of this plant would raise the levels of GABA in the CNS by inhibiting the action of one of the enzymes responsible for degradation of this neurotransmitter, succinic semialdehyde dehydrogenase (SSADH). The result is a sedative effect on the body; in any case, other studies would be necessary to confirm the sedative action attributed to this plant.

Few studies have been found concerning the sedative effect of *Annona muricata* L. [362]: this plant appears to be potentially toxic, which would render use of same difficult [363].

Other plants have either been commented because they are in Table 1 or possibly, have been described in the item referring to the phytochemistry in this chapter, as a result of knowledge or isolation of some chemical component with a known pharmacological action already described. Those species that were not commented have not, as far as we have been able to ascertain, been the object of pharmacological studies.

A Phytochemical Approach

Mankind has, down the ages, made use of psychoactive plants and plant-derived products for spiritual, therapeutic, and recreational purposes. An investigation of psychoactive plants such as *Cannabis sativa* L. (marijuana), *Nicotiana tabacum* L. (tobacco) and analogues of psychoactive plant derivatives such as lysergic acid diethylamide (LSD) have provided insight into our understanding of neurochemical processes and diseases of the CNS. Many of these compounds are currently being used to treat a variety of diseases such as anxiety as, for instance, Kava-kava [364].

Kava-kava is a well-established hypnotic drug with a rapid onset of effect, adequate duration of action and minimal morning after-effects. As mentioned previously, however, reports of serious hepatotoxicity with this preparation have led to its being banned in most countries worldwide [365]. Kava-kava extract produced a significant increase in delta activity during non-REM sleep in sleep-disturbed rats, whereas a significant decrease in delta power during non-REM sleep was observed with flunitrazepam, a BDZ utilized as hypnotic [366].

In this review, the phytochemical studies found in the scientific literature for the plants included in Table 2, with possible hypnotic effects, also showed a prevalence of the flavonoid and essential oil, as shown in plants in Table 1, with possible anxiolytic effects, as can be seen in Fig. (**4**). These results emphasize the importance of these constituents in the treatment of diseases related to some deficiencies of the CNS, once more followed by the phenolic acids and alkaloids as shown in Fig. (**4**). These substances may act producing two effects, both as anxiolytics and as hypnotics, the difference being only in the dosage of the drug, as formerly cited.

Several studies describe the hypnotic activity of flavonoids. Apigenin is a flavonoid that showed sedative and antidepressant activity [367]. The flavonoids and indole alkaloids of *P. incarnata* L., also showed sedative effects [368]. Linarin, a flavonoid-isolated from *Valeriana officinalis* L. showed sedative and sleep-enhancing properties (Fernandez et al., 2004). The nonvolatile fraction of *L. alba*, extracted in ethanol, presented sedative and myorelaxing effects: among the extracts tested, these possess the highest flavonoid content [296].

Essential oils also present sedative activity, as do those from *Lippia alba* (Mill.) N.E.Br. and *Matricaria chamomilla* L. [255,257]. Citral, myrcene and limonene, constituents of essential oils from *Lippia alba* produced a potentiation of pentobarbital-induced sleeping time in mice which was more intense in the presence of citral [370].

No studies were found on the hypnotic activity of the phenolic acids; however, the hypnotic activity of alkaloids is known. D1-Tetrahydropalmatine (d1-THP), a naturally occurring alkaloid, has been intensively studied for its sedative and hypnotic effects. A putative explanation for its mechanism and target of action involves the dopaminergic neurotransmission system [371]. Reserpine, an alkaloid from *Rauwolfia serpentina* Benth. ex Kurz, was widely used for its antihypertensive action. However, its use has been reduced because of its

sedative symptoms [372]. Alkaloid rotundin [373] and the peptide alkaloids from *Zizyphus* spp. species exhibited sedative effects [374].

Fig. (4). Number of species with possible hypnotic effects, indicated by the cultures under study, that present the respective chemical constituents (only those constituents with at least four species cited have been shown in this illustration).

Alkaloids of the *Erythrina* Species

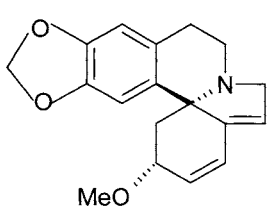
The *Erythrina* genus belong to the Fabaceae family and are in popular use in Brazil for their effects on the CNS.

Erythrina velutina Willd. at lower doses interferes with the mnemonic process for different tasks, while the sedative and neuromuscular blocking actions are the main effects at higher doses [263]. The hydroalcoholic extracts of *E. velutina* and *E. mulungu* Mart. ex Benth have depressant effects on the CNS which, at least partially, corroborates the popular use of these species as tranquilizers in Brazilian popular medicine [264].

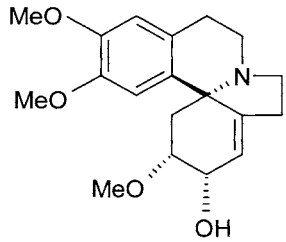
Through an analysis of four species of *Erythrina* (*E. mulungu*; *E. corallodendron* L.; *E. speciosa* Andrews and *E. velutina*), Camargo [44] explains that the use of these different species does not interfere with the results desired by users, since all of them presented alkaloids responsible for hypnotic and sedative effects in varying doses.

The study of alkaloids of the *Erythrina* genus is of assistance in grouping species, but only some of the species have been carefully examined [375]. Alkaloids are compounds, generally with heterocyclic nitrogen in the molecule, biosynthesized as from aminoacids.

Erythrina alkaloids, Fig. (**5**), have a structure which is very different, strictly speaking from the curares, but are substances with curare-like activity. Naturally-occurring curares are non-depolarizing (or competitive) neuromuscular blocking agents. Active only by the parenteral route, they compete with acetylcholine for the cholinergic receptors at the motor end-plate and prevent the formation of action potential, without modifying nerve conduction elsewhere, and without preventing muscular contraction in response to direct stimulation [376]. No phytochemical data were found for species *E. corallodendron* L. and *E. mulungu* Mart. ex Benth.



erythraine



erythratidine

Fig. (5). Erythrina alkaloids

Essential Oils

Essential oils were the second class of chemical constituents most recurrent among the species analysed in this review, both for anxiolytic and for hypnotic effects. The essential oil from *Citrus aurantium* L., commonly used as an alternative treatment for insomnia, anxiety, and epilepsy, showed anxiolytic and sedative effects [271].

There is some evidence that aromatic substances present in essence/volatile oils from certain plants might exert an anxiolytic or a relaxing effect, with improved mood.

In an interesting study developed by Almeida et al [377], the anxiolytic effects of inhaling rose oil on rodents was assessed, with diazepam as gold standard. Results showed that, subsequent to inhaling this essential oil, rats explored the open arms of the EPM more, as compared to the placebo group.

Studies with essential oils in human beings have also shown beneficial effects. For instance, anxiolytic effects were observed in patients awaiting dental care treatment in a waiting room previously aromatized with essential oils from *Citrus sinensis* (L.) Osbeck [378]. In another controlled study, 14 patients undergoing hemodyalisis benefited from hiba and lavender aromas and presented a feeling of calm [379]. Several studies on aromatherapy have attracted the attention of researchers in a study of chemical, pharmacological, and therapeutic properties of these substances [380].

According to this line of reasoning, Ballard et al [381] verified that aromatherapy with essential oils from *Melissa officinalis* L. was very effective in reducing the agitation normally observed in patients with several dementia, with consequent improved quality of life for these people.

One fact that is worthy of note relates to *Cymbopogon citratus* Staf. This is one of the plants most utilized in popular medicine in Brazil: many studies have been carried out with this plant. Some studies show that this plant produces evidence of an absence of hypnotic and anxiolytic effects [382], with no effect on nervous and gastrointestinal disorders [383]: it is a plant with no toxicity [384]. Anti-inflammatory action was observed [385], in addition to an analgesic effect confirmed by different authors [386-388]. Palmieri [389], however, observed an anxiolytic effect utilizing the essential oils of the plant, while studies by Leite et al. [382] and Carlini et al. [383] which did not verified this effect, utilized hydroalcoholic extract.

Since it is known that the potentiation of GABA(A) receptors by BDZ, barbiturate, steroids, and anesthetics induces the anxiolytic, anticonvulsant, and sedative activity or anesthetic effect, these results suggest the possibility that the intake of perfume or phytoncid through the lungs, the skin, or the intestines modulates the neural transmission in the brain through ionotropic GABA(A) receptors and changes the human frame of mind as do alcohol or tobacco [389]. Aromatherapy is an anecdotal method for modifying sleep and mood. The inhalation of essential oils may induce stimulative or sedative effects in mice [390], as a nonphotic method for promoting deep sleep and for producing gender-dependent sleep effects [391].

Within this framework, the attribution of essential oils to anxiolytic activity might relate not only to the oral route for administering, but also as an inhalant route: as the tea is being imbibed, mostly taken hot, part of

its active principles, essential oils, for instance, volatilize and may thus come into contact with the neurons of the vomeronasal organ which is considered an accessory olfactory system which can detect odorants and pheromones [392].

Cultural Peculiarities in the Therapeutic Use of Plants

Forms of Use

Some of the publications consulted for this review offered insufficient description of use, lacking information about route of administration and the recipes concerning the "medicines", as can be observed in Tables 1 and 2. In the cases in which they are described, recipes may include: teas (infusion or decoction), bottled brews, or extract - in natura. While, route of administration may be: ingestion and topical (in the form of bathing).

One of the uses frequently described during ethnopharmacological surveys is bathing, associated or not to the consumption of plants in the form of teas, bottled brews, etc. The liquid utilized in these cases is produced as from decoction, infusion, or even maceration prepared with the leaves, bark, roots, or other parts - in most cases with appropriate times for bathing: in the morning, at midday, or in the late afternoon. In some cases, the site on the body to be bathed is specified: only the head (in the cases of flu or headache) or the entire body (usually fever and flu). The brew is allowed to dry on the body post bathing.

Albuquerque and Chiappeta [47] explain that the Afro-Brazilians (of northeast Brazil) prescribe "relief bathing" for people who are emotionally unstable, and nervous: two ferns are used for this purpose *Lygodium volubile* Sw. and *Lygodium venustum* Sw.

Amarozo and Gely [52] call attention to the fact that the practice of bathing is most important among Caboclos. Furthermore, belief in the effectiveness of applying medication topically (bathing, poultice) is so great that, instead of taking some allopathic medicines by mouth, Caboclos usually dissolve tablets together with plants in the bath water to counteract flu and headache.

The effects against nervous excitation caused by the action of alkaloids and of some species of *Erythrina* spp. in baths have not yet been studied from the scientific point of view, but both the Afro-Brazilians and the Indians of the Amazon region resort to them [44].

Table 1 and another review [8] show that the therapeutics for the Pareci and Tiriyó Indians involve a considerable number of baths.

How can bathing be interpreted from the pharmacological point of view? These processes would lead to dermic absorption similar to the action of slow-release patches, since in bathing, the formula is allowed to dry on the body ?

Lipophylic substances such as diterpenoids, fatty acids, essential oils themselves, triterpenoids, of greater affinity with the skin which is lipophylic [393], are known to be absorbed by the skin. However, it is difficult to explain just how these constituents may be transported to the CNS, although lipophylic substances do cross the hematoencephalic membrane easier than the hydrophylic substances which are polar.

Number of Plants per Formula

A number of plants make up the composition of each formula in Afro-Brazilian and/or Quilombola therapeutics: the species *Erythrina velutina* Willd. in bottled brews is mixed with another three species: *Anadenanthera colubrina* (Vell.) Brenan (angico), *Stryphnodendron* sp (barbatimão), and *Schinus* sp (aroeira pepper tree) [44]. In "relief bathing", Afro-Brazilians also add another three plants to *Lygodium volubile* Sw. and *L. venustum* Sw.: *Petiveria alliacea* L (guiné), *Ruta graveolens* L. (arruda rue), and *Rosmarinus officinalis* L. (alecrim rosemary) [47].

In a similar way, an ethnopharmacological survey among the Caboclos of the Jaú National Park, in the Amazon forest, showed that every formula may present from one up to six ingredients (parts of plants and/or animals) [51].

This was also observed in Africa, among the Yorubas [394]; in India, in Ayurveda therapeutics [395], and among the Chinese [396]. Verger, (1996) tries to explain this logic: "The single plant may perhaps be compared to one letter in a word: on its own, it has no significance, but associated to other words, it contributes to the significance of the word." From the pharmacological point of view, this may signify that the association of plants could well have a synergic effect, as explained by some authors [397,398].

On the other hand, among the Indian ethnic groups, the opposite occurs, namely, the use of one single plant for each prescription and, in general, specifically, for one use [42].

A predominance was observed in the use of plants introduced into Brazil by Afro-Brazilians and/or Quilombolas (48.3%) and Caboclos (50%) in relation to Indians (28%), as can be observed in Table 3.

This peculiarity in the use of plant resources can be explained, in part, by the history of the occupation of Brazil itself, in that the ascendants came from other continents (Europe and Africa) and brought with them multiple influences, as also plant resources that, even today, are incorporated to the therapeutics of the Caboclos and Afro-Brazilians and/or Quilombolas

Among the Indians, 72% of the plants indicated by them in this study are native to the Brazilian flora. These data are congruent with a study carried out among the Krahô Indians where 100% of the plants used by them are originally from Brazil [49]. One of the reasons for this difference is the fact that the Indians invariably had recourse to "a stock of plants on Brazilian territory", and thus, investigated and still do investigate the plants; furthermore, they did not have to move across other continents in the course of history, different from the Afro-Brazilians and/or Quilombolas.

Table 3. Number of native plants and of plants introduced in Brazil utilized by each culture under analysis, with possible anxiolytic and/or hypnotic effects, as also the frequency with which species introduced were utilized.

Culture	Native	Introduced	Total	% introduced
Afro-Brazilian and/or Quilombola	15	14	29	48.3
Caboclo	10	10	20	50
Indian	13	5	18	28

Other Pharmacological Effects

A review of the literature showed that many of the plants cited by the three cultures cited in this survey present other pharmacological effects that are not necessarily anxiolytic or hypnotic, although, in the majority of cases, these plants exert action on the CNS, as can be observed in Tables 1 and 2. Thus *Lactuca sativa* L. exerts an antioxidating, [399] analgesic, and anti-inflammatory effect [101]; *Miconia rubiginosa* (Bonpl.) DC has an analgesic effect [165]. Molina et al., [400], working

with *Mimosa pudica* L., verified its antidepressive effect on the CNS, but could not confirm the anxiolytic effect of this plant. In a later study, Bum et al [401] reported anticonvulsant action of this plant, an effect that was also observed in *Pimpinella anisum* L. [402].

Analgesic and anti-inflammatory effects were reported for species *Sacharum officinarum* L. [403] and *Casearia sylvestris* Sw. [404]. Studies were also found showing the following pharmacological effects for some of the plants cited in this study: *Kalanchoe brasiliensis* Cambess., anti-inflammatory [405] and immunomodulator [406]; *Vitex agnus-castus* L., a dopaminergic effect [407]; *Mentha piperita* L., gastric antispasmodic action [408]; *Hibiscus rosa-sinensis* L., anticonceptional effect [409,410]; *Nymphaea alba* L., renal protector [411] and antinociceptive [412]; finally an anti-hypertensive effect [413] for *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm.

FINAL CONSIDERATIONS

Among the plants indicated in this survey, 67% were investigated from the phytochemical point of view: the flavonoids, essential oils, and alkaloids are important constituents for the anxiolytic and hypnotic effects attributed to these plants by popular lore.

Fifty per cent were investigated from the pharmacological point of view: among the pharmacological effects studied, only 25% referred to anxiolytics or hypnotics.

These sparse pharmacological data found in the scientific literature concerning the native species in Brazil agree with that observed in a previous study conducted by Rodrigues and Carlini [414]. They developed a bibliographical survey conducted on Databases such as LILACS (Latin-American and Caribbean Literature in Health Studies) and MEDLINE (PUBMED), in order to verify the number of studies on plants (pharmacological and phytochemical) developed about South American plants in reference to the psycholeptic and psychoanaleptic effects. Results of this search showed that only 15 and 16 pharmacological studies, respectively, had been carried out to investigate the anxiolytic and hypnotic effects of plants in South America. A total of 863 studies and of 1,160 studies concerning anxiolytic and hypnotic plants, respectively, were recorded in the rest of the world.

These observations corroborate the idea that it is necessary to carry out more studies that will focus on plants with action/effect on the CNS since

this field of study is as yet extremely incipient. Even with so many studies worldwide, as cited above, up to the present time, it has not been possible to develop anxiolytic or hypnotic phytomedicines to substitute the allopathic medication prescribed by psychiatry.

ABBREVIATIONS

CNS = Central Nervous System
BDZs = Benzodiazepinies
EPM = Elevated Plus Maze
GABA = Gama Amino Butiric Acid

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BIOACTIVE NATURAL PEPTIDES

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ABSTRACT: Bioactive natural peptides are ubiquitous in all life kingdoms. They are often characterized by short amino acid sequences and they are found either free or encrypted in proteins thus requiring enzymatic hydrolysis for their release. Matrix-assisted laser desorption/ionization (MALDI) MS and electrospray ionization (ESI) MS techniques have been used for peptide identification and determination of post-translational modifications, directly from body fluids, organs, tissue samples or single-cells. Peptide structures have also been studied by CD and NMR spectroscopy. Antimicrobial peptides (AMPs) synthesized by microorganisms and multicellular organisms can have linear, cyclic or open-ended cyclic structures with one or more disulfide bridges. They exhibit α-helical conformations, amphipathic β-hairpin-like β-sheet, β-sheet and α-helix/β-sheet mixed folds. Some of them, in addition to containing hydrophobic amino acid residues, are rich in proline, histidine, arginine or lysine. AMPs exhibit two main modes of action, one involving an intracellular target, and another the interaction with the cytoplasmic membrane from microorganisms. Membrane-active peptides that include hormones, signal sequences and lytic agents, interact electrostatically with the cellular external membrane and eventually partition into the hydrophobic lipid bilayer where they express their activity. Counterparts of several human endogenous peptides of pharmacological and immunobiological importance are found also in other animal species and they have become the lead for development of new drugs. An increasing number of them display direct or immune-stimulated antitumor activities. Multifunctional peptides have also been recognized in food sources. Owing to the wide reactivity of endogenous peptides generated in cells, appropriate oligopeptide restricted peptidases either give rise to bioactive shorter peptides or contribute to the complete degradation of oligopeptides.

Finally, based on their original structures, several of them have been engineered to produce peptide derivatives characterized by peculiar aggregation resistance and/or increased biological activity. Considering their broad distribution and spectra of action, it is expected that large-scale peptidomics together with sequenced genomes should significantly increase the recognition of new bioactive natural peptides.

INTRODUCTION

Bioactive natural peptides found in different vertebrate and invertebrate species mediate a number of physiological responses that lead to protection of the organism against infections and tumor development. Endogenous peptides may help to maintain the homeostasis or, if lacking or over expressed, contribute to harmful reactions in the host. Their amino acid sequences, isoforms and steric conformations have been investigated as well as the mechanisms of their bioactivity. As expected, the different peptides display a vast number of reactivities depending on the cell, tissue and organism investigated. They can exert antimicrobial and antitumor effects or mediate specific cellular responses; they can promote or inhibit angiogenesis; they may induce apoptosis or protect against it; they may participate in neuro and immunomodulatory networks and occur as isolated units or are integrated in larger proteins. Their half live depends on the experimental system and the presence of specific peptidases. Their recognition and analysis have been made possible by a series of methods of increased sensitivity and adaptability.

Presently we focus on antimicrobial and antitumor peptides, their structures and mechanisms of action. An overview of the modern methodology used is given based on the original *Drosophila melanogaster* model. The differential induction of genes encoding antimicrobial peptides (AMPs) as triggered by bacterial infections is well documented. Peptides and proteins can now be analyzed in complex samples and in minute amounts of body fluids, tissues, organs or single-cells.

Other AMPs from several different sources were selected for discussion of their modes of action considering their structures, membrane interactions and target cells as well as their effects on the

immune system. Antitumor peptides are reviewed to show the variety of sources, their direct effects on tumor cells, anti-angiogenic and apoptotic effects as well as their immune presentation to mediate immune cellular responses. Peptide processing by oligopeptidases seems to be a powerful way of regulating enzyme activity.

MASS SPECTROMETRY, A USEFUL TOOL IN THE DISCOVERY AND CHARACTERIZATION OF *DROSOPHILA* IMMUNE EFFECTORS

Insects are remarkably resistant to microbial infections. It is now thought that insects control infection by an array of innate immune reactions that include (i) phagocytosis and encapsulation by blood cells, (ii) proteolytic cascades leading to coagulation and melanisation, and (iii) secretion of large-spectrum potent antimicrobial activities [1,2]. In 1981, Hans Boman et al. have isolated the first antimicrobial substance from the hemolymph of bacteria-challenged giant cecropia (*Hyalophora cecropia*) diapausing pupae [3]. This substance, named cecropin, is a small cationic AMP. Since this first report, an impressive number of AMPs has been identified from ameboid protozoa, prokaryotes and eukaryotes [4-9]. These AMPs are for a large part listed in several available databases including Swissprot and TrEMBL (http://www.expasy.org/sprot/sprot-top.html), AMSDd (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm), APD (http://aps.unmc.edu/AP/main.html) and ANTIMIC (http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/), and PenBase (http://www.penbase.immunaqua.com/) that is exclusively dedicated to shrimp AMPs [10].

In the early 1980s, insect AMPs have been identified either through a biological activity-screening assay (antibacterial) leading to purification and characterization of the target molecule [3] or by cloning genes by homology [11]. At that time, mass spectrometry (MS) has already had a significant impact for AMP discovery and

structural characterization, thanks to the development of soft ionization procedures such as electrospray ionization (ESI) and later to matrix-assisted laser desorption/ionization (MALDI). Until the past 15 years, MS has been essentially used as a complementary methodology to cDNA cloning, liquid chromatography, Edman sequencing and polyacrylamide gel electrophoresis. MS has allowed definition of accurate molecular masses of isolated AMPs providing information on the presence of post-translational modifications and about the maturation events from mRNA to mature bioactive AMP. Most of the AMPs however have been isolated from large-size insects.

It is only in the early 1990s that the fruit-fly *Drosophila melanogaster* has emerged as an original and powerful model to study the evolutionary conserved genetic and molecular mechanisms operating in innate immunity. The *Drosophila* host defense is complex (cellular and humoral) and remarkably powerful. The hallmark of the *Drosophila* humoral response is the induced synthesis of AMPs [12]. AMPs are synthesized in the fat body, a functional equivalent of the mammalian liver, and released into the hemolymph (blood) where they act individually or synergistically to kill the infectious agent. During *Drosophila* host defense, a differential induction of AMP genes occurs after infections by various classes of microorganisms [13]. This has evidenced that the immune response of the fruit-fly is specific and can differentiate among different species of microorganisms. Such differentiation involves the activation of two different signaling pathways (Toll and Imd) that regulate the systemic antimicrobial response of *Drosophila* [14]. The Toll pathway is critical during natural/experimental fungal and Gram-positive bacterial infections while the Imd pathway is activated by infections with Gram-negative bacteria.

One major challenge in the 1990s was to develop up-to-date biochemical tools for the discovery, identification and characterization of new key players of the *Drosophila* immune defense reactions. To improve this discovery, the development of MS-based platforms have been established, thanks to the

inducibility of the *Drosophila* immune response, to the completion of its genome, and to improvements in the soft ionization MS as well as to 2D-SDS-PAGE. This has been operated by peptidomics following the strategy summarized in Figure (**1**) and in Figure (**2**) for proteomics studies that emerged as valuable approaches. In 1996, a differential analysis by MALDI-MS of *Drosophila* hemolymph collected from a single uninfected *vs* a bacteria-challenged individual [15] was initiated showing that it would shortly be possible to identify markers of the immune response of *Drosophila* in only a few individuals, a progress unthinkable in the early 1990s.

With the completion of the *Drosophila* genome in 2000 [16], several reports have discussed the use of peptidomics (molecules with a molecular mass below 15 kDa) and proteomics (molecules with a molecular mass greater than 15-20 kDa) in the study of *Drosophila* immunity. Peptidomics studies have been performed in the blood from a single individual (larvae or adult) as well as on pooled samples [15,17-22], and in tissue preparations [23,24] while proteomics investigations require batches of samples [25,26]. In addition to these studies on *Drosophila* immunity, since 2002 peptidomics-MS-based approaches have also been developed on *Drosophila* central nervous system (CNS) [27-31], and proteomics on *Drosophila* larval blood before clotting [32,33] or after clotting [34] as well as on male accessory glands [35], wing imaginal discs [36] and mitochondria [37].

The variety of MS-based approaches [see Fig. (**1**) and Fig. (**2**) for a general view of the strategies] that have been used to discover, identify and elucidate effectors of the *Drosophila* immune defense reactions is presently reviewed.Regardless of the approach, MS allowed the discovery and structural characterization of an unprecedented number of systemic immune effectors, as well as the detection of such effectors within tissues expressing a local immune response.

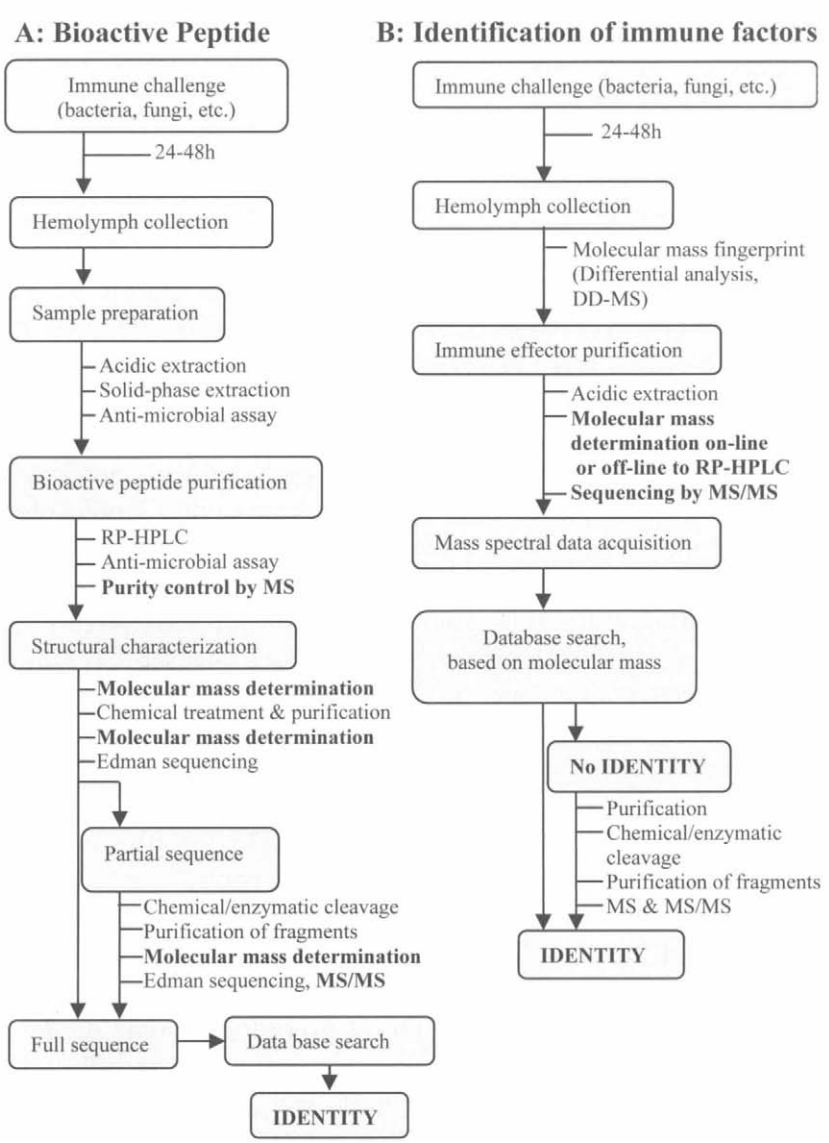


Fig. (1). Peptidomics strategies used to study *Drosophila* immunity. **(A)** Using antimicrobial assays (antibacterial and antifungal), the bioactive peptides were isolated from the blood of bacteria-challenged *Drosophila*. MS was used for molecular mass assignment, to identify post-translational modifications, and for primary structure elucidation; **(B)** Identification of peptidic immune effectors through differential display analysis (DD) by MALDI-MS and micro/nano RP-HPLC coupled (on-line) or not (off-line) to ESI-MS. When the HPLC was performed off-line to the mass spectrometer, fractions were individually analyzed by MALDI-MS. The identification and the structural characterization were performed either by molecular mass assignment and/or sequencing by ESI-MS/MS.

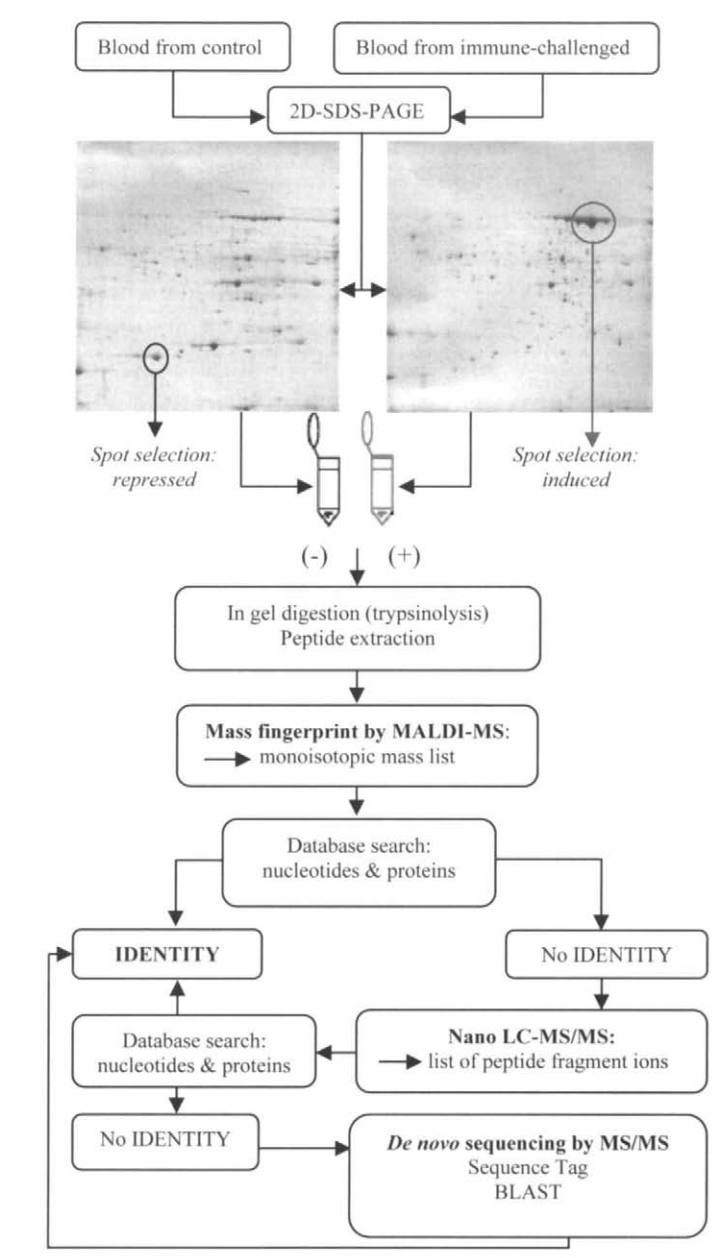


Fig. (2). Strategy for the identification of gel-separated (2D-SDS-PAGE) proteins that are part of the immune response of *Drosophila*. Blood from control and immune-challenged flies was collected and subjected to 2D-SDS-PAGE, immune-induced (+) or repressed (-) stained spots were excised, subjected to proteolysis (*e.g.* trypsin), and the peptides fragments analyzed by MS and/or MS/MS.

What is required to identify and characterize peptides and proteins?

Peptide and protein identification has historically been accomplished through sequencing by stepwise chemical degradation from the N-terminus to the C-terminus using the well-known Edman chemistry. Although a powerful technology, it requires a highly purified peptide/protein and a free amine group at the N-terminus (Edman chemistry is hindered by specific N-terminal modifications such as acetylation and cyclic glutamine or glutamate). It also has difficulties in reading a sequence of 40-50 residues, to identify post-translational modifications (glycosylation, phosphorylation, etc.) and such automated sequencers are proving too slow for the demands of the biotech revolution. Finally, purification was achieved either by liquid chromatography (LC) following detection by ultraviolet (UV) absorbance or fluorescence spectroscopy or by gel electrophoresis. Once purified by liquid chromatography, peptides or proteins were subjected either directly to Edman sequencing or to enzymatic cleavages (at least two different enzymes) for generating lower molecular mass fragments that following purification were individually subjected to Edman chemistry. When proteins were purified by gel electrophoresis, the proteins were electrotransferred and the band of interest was cut and directly subjected to Edman chemistry. In most cases of larger proteins, only partial information was obtained and cloning experiments were often required for final identification.

Gradually, over the past twenty years, mass spectrometers were interfaced with a number of protein chemistry assays to generate detectors providing superior information. With the increased performance and versatility of the instrumentation dedicated to the life sciences, new analytical strategies for peptide and protein identification and characterization have emerged in which MS and bioinformatic tools are key players. MS has an enormous impact on the capability for structural analysis of bio-molecules, thanks to the ability to create gas phase ions of the peptides and proteins to be analyzed. Peptides and proteins are often charged and polar, making

them difficult to volatilize into the ion source of the mass spectrometer. The development in the late 1980s of two new soft ionization procedures, ESI [38] and MALDI [39], has revolutionized the applicability of MS to polypeptide structural identification. Many types of mass spectrometers can be used for the characterization of peptides and proteins, but the majority of the experiments are performed with quadrupoles (Q) and time-of-flight (TOF) analyzers.

The different types of mass spectrometers provide a wealth of information going from simple molecular masses of intact components to an inference of the amino acid composition, sequence order, substitution site and nature of post-translational modifications, and tissue distribution of the molecule of interest [for reviews see [40-44]. For determining the molecular mass of a polypeptide, a single-stage mass spectrometer is appropriate whereas for analysis of structural features tandem MS (MS/MS) is required. In this latter case, after molecular mass measurement, specific ions are selected and then subjected to fragmentation through collision in a specific chamber (collision cell) supplied with an appropriate gas (*e.g.* argon). Instrument performance (resolution, sensitivity, mass accuracy) depends on the instrument type, the ionization method, and the scanning capabilities. No instrument offers all capabilities simultaneously and complementary mass spectrometers (ESI and MALDI) are often required for peptidomics and proteomics studies.

The efficacy of MS to characterize effectors of *Drosophila* immunity and *Phormia* (also referred in the literature as *Protophormia*) *terranovae* is illustrated by several examples. This will not follow a chronological order but is in accordance with the complexity of the MS analyses. MS allowed: (i) to define precise molecular masses, to identify post-translational modifications (N-terminal cyclisation, C-terminal amidation, disulfide bonds array, glycosylation), (ii) to determine primary structures of immune peptides (sequencing by MS/MS), (iii) to have mass fingerprints and to identify peptide effectors that are part of *Drosophila* immunity (molecular mass differential display by MALDI-MS),

(iv) to perform proteomics studies (2D-SDS-PAGE, MALDI-MS and MS/MS), and (v) to detect immune effectors within *Drosophila* tissues (MALDI-MS).

Measuring molecular masses to ascertain an identity and to evidence post-translational modifications.

Molecular mass measurement

Since the development in the late 1980s of ESI, MS has been frequently used for routinely measuring an accurate molecular mass of AMPs that have been isolated from large size insects and later on 1990, *Drosophila*. Lambert et al. [45] have used for the first time MS (ionization performed by fast atom bombardment with a cesium ion gun) for the characterization of two insect antibacterial peptides (insect defensins) with sequence homology to rabbit lung macrophage bacterial peptides. These two defensins were isolated through activity screening from immune blood of the large flesh-fly *P. terranovae* and their full sequence was determined by Edman sequencing. By comparing the monoisotopic molecular mass calculated from their primary structure and the ones measured by MS, both peptides were found to have six cysteine residues engaged in three intramolecular disulfide bridges and to carry no additional post-translational modification. Since this first report, MS has been routinely used for the characterization of AMPs from insects including *Drosophila*.

In 1990, when the isolation of inducible AMPs from adult *Drosophila* was started, molecular cloning studies have shown that challenged larvae or adults express genes encoding peptides homologous to cecropin [46] and diptericin [47] that had been initially identified in larger insects. The refinement of the analytical methods (mostly HPLC and MS) as well as the development of antibacterial assays in microplates enabled the isolation and identification of several AMPs from 30,000 *Drosophila* bodies (defensin, metchnikowins and drosocin). ESI-MS has clearly shown that an equal amount of two isoforms of metchnikowins (not resolved by HPLC, one residue difference) were present in the

Drosophila extract. Data from the screening of a cDNA library of bacteria-challenged *Drosophila* [48] and from the *Drosophila* genome [16] have confirmed the presence of two allelic forms originating from a single gene.

Determination of disulfide bonds

Taking advantage of the inducibility of the immune response in insects, Fehlbaum et al. [49] isolated and characterized from an extract of 2,000 *Drosophila* (HPLC-purification, Edman sequencing, and cDNA cloning), a 44-residue peptide containing eight cysteine residues engaged in four intramolecular disulfide bridges as confirmed by ESI-MS. This peptide (named drosomycin), which is produced in considerable amount upon a septic injury was found to have antifungal activity. In order to have adequate amounts of the peptide for studies on its mode of action, activity spectrum and 3D-structure analysis, recombinant drosomycin has been expressed in *Saccharomyces cerevisiae* [50]. Purification of the recombinant peptide was followed by antifungal assay. When subjected to MALDI-MS analysis, the bioactive fraction was found to contain four distinct molecular masses. One corresponded to the molecular mass of mature drosomycin (including the four intramolecular disulfide bridges) whereas the three others were N-terminally extended drosomycins as a result of incomplete maturation. In order to ascertain that the recombinant drosomycin was perfectly identical to the natural peptide (identical disulfide scaffold), the disulfide pairing was determined by subjecting both recombinant and native drosomycin to endoproteinase digestions. The products of the recombinant peptide proteolyses were purified by RP-HPLC and the eluted peaks subjected to MALDI-MS and Edman sequencing [see Figure (**3A**)]. The proteolysis products of native drosomycin were directly analyzed by MALDI-MS. Similar mass fingerprints have been observed, establishing that recombinant and native drosomycins were identical.

During the biological assay-based discovery program of AMPs from *Drosophila*, the group of Hoffmann in Strasbourg has reported in 1993 the isolation of drosocin, the first inducible insect AMP carrying an *O*-glycosylation [51]. This substitution was evidenced by molecular mass measurement. Drosocin is a 19-residue peptide that represents the prototype of the small-size proline-rich AMPs from insects. Chronologically, drosocin has been purified through a three-step HPLC purification procedure, and the pure bioactive peptide subjected to molecular mass measurement and Edman sequencing. A 19-residue sequence has been obtained with an unidentified amino acid at position 11. To solve the identity of this residue that could not be directly defined by deduction from the molecular mass measured by ESI-MS, a size-selected cDNA library prepared from bacteria-challenged larvae was screened. All the positive clones obtained were sequenced and the identity of residue 11 was shown to be Thr. Nevertheless, the molecular mass calculated from the primary structure deduced from the cDNA (2,199.6 Da) and that measured by ESI-MS (2,564.4 Da) on the native form were not in agreement suggesting the presence of a post-translational modification of 365 Da. As Thr-11 was found to be located within a putative consensus sequence for *O*-glycosylation (Pro-Thr/Ser-Xaa-Xaa-Pro), assuming that the mass difference observed could indeed reflect an *O*-glycosylation at Thr-11, drosocin was treated with anhydrous hydrogen fluoride (anHF) in order to hydrolyze the *O*-glycosidic bonds. Following ESI-MS analysis, the molecular mass of the native drosocin shifted to 2,199.6 Da, the molecular mass calculated from the cDNA deduced sequence [51]. Full characterization of the *O*-linked carbohydrate substitution was obtained following hydrochloride methanolysis, pertrimethylsilylation of the sugar moieties linked to Thr-11, and analysis of the mixture by gas chromatography MS and fragmentation with electron impact mode (GC-EI-MS). It was unambiguously established that Thr-11 of native drosocin is substituted by an *O*-linked N-acetylgalactosaminyl (GalNac) unit

linked to galactose (Gal). Interestingly, a series of different mono and disaccharide glycoforms of drosocin have been detected in the hemolymph of *Drosophila* while the peptide has never been isolated in a non-glycosylated form [15]. Surprisingly, a more complex glycoform (additional glycosylation of Ser-7) has been identified by MALDI-MS while screening for immune peptides that are under the control of the Imd pathway [20]. Although the presence of *O*-glycosylated AMPs has been evidenced in other insects (for review see [52]) by using MS, the biological role of the sugar remains an enigma. The mode of action of drosocin as well as other proline-rich peptides will be discussed in the next section.

One of the most complex problems involved in the analysis of glycopeptides is their heterogeneity in glycosylation. Post-translational modifications, which add sugar side-chains to peptides/proteins, not only involve an entirely different kind of chemistry to the analytical mixture, but also create a potential nightmare of complications in what would otherwise be a relatively "simple" amino acid-based MS spectrum. In general, a complete analysis of a glycopeptide requires the use of not only the modern soft ionization MS approaches but also GC-MS (for review see [53]). Such a combination of soft ionization- and GC-MS approaches has also been used for the full structural characterization of a 9 kDa glycopeptide, namely the diptericin from the flesh-fly *P. terranovae* [54,55].

The diptericin from *P. terranovae* is an anti-Gram-negative polypeptide isolated in 1988 from the blood of bacteria-challenged larvae [56] and its amino acid sequence was confirmed following cDNA-cloning [57]. Diptericins that are also present in *Drosophila* and other dipteran's species are characterized by an N-terminal proline-rich domain and glycine residues that are over represented in the central and C-terminal segments. The N-terminal domain that comprises 15 amino acid residues shows marked sequence similarity with drosocin and other insect proline-rich AMPs (for reviews see [4,12]). The proline-rich domain of diptericin contains a threonine residue (Thr-10) that is located within a consensus sequence for *O*-glycosylation. In the first report by Dimarcq et al.

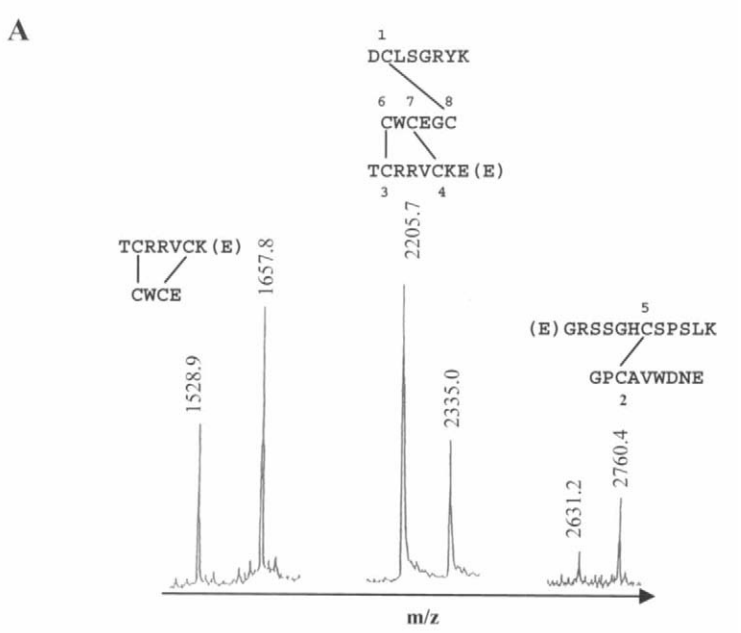
[56], the isolated diptericin had not been either subjected to MS measurement or evaluated for post-translational modification. Following the results obtained with drosocin, the structure of the *Phormia* diptericin was re-examined taking advantage of the MS development. A series of five closely related compounds (α to ε, according to [55]) has been isolated, following their activity against Gram-negative bacteria, and subjected to Edman sequencing and arginyl endopeptidase digestion. Edman sequencing of the native peptides and of the different proteolytic fragments has demonstrated that these five diptericin isoforms have the same amino acid sequence (82 residues) as the one deduced from the cDNA with threonines at positions 10 and 54 [56]. Nevertheless, these five compounds when subjected to ESI-MS analysis yielded different masses ranging from 8,895.8 Da (isoform ε) to 9,745.7 Da (isoform α) for the largest, with a predominant form (γ) at 9,423.3 Da [see Figure (**3B**)]. The differences observed between the calculated molecular masses and the measured ones corresponding to multiples of 203 and/or 162 Da were assumed to be N-acetylhexosamine and hexose, respectively. The hypothesis that *Phormia* diptericin is an *O*-glycopeptide was confirmed by deglycosylation since the anHF treatment released a peptide with an average molecular mass measured by ESI-MS (8,692.6 Da) identical to the calculated molecular mass of diptericin deduced from cDNA. The identification of the carbohydrate composition was performed following the procedure used for drosocin by subjecting the native glycopeptide to methanolysis and permetylsilylation, and the resulting carbohydrate derivatives examined by GC-EI-MS analysis. Only three carbohydrates with retention times and fragmentation spectra identical to GalNac, Gal and glucose (Glc) were observed. This carbohydrate composition is more complex than the one observed for drosocin that carries only GalNac and Gal [see Figure (**3C**)]. Finally, the analysis by LC-ESI-MS of a tryptic digest of the most abundant diptericin glycoform (diptericin γ) has allowed to precisely map the position and nature of the carbohydrates on substituted Thr-10 and -54 [see Fig. (**3C**)].

In addition, thanks to this LC-ESI-MS analysis and to the monoisotopic resolution obtained, the smallest fragment generated by trypsinolysis was found to correspond to the nine C-terminal residues of diptericin but as the molecular mass measured (990.6 Da) was lower by 1 Da than the calculated one (991.6 Da), the authors could established that *Phormia* diptericin in addition to being highly *O*-glycosylated at two different sites is also C-terminally amidated [55], as summarized in Fig. (**3C**). This last observation is consistent with the presence of a glycine codon at the C-terminus of the diptericin cDNA [57].

Uttenweiler-Joseph et al. [55] have also used MALDI-MS to define the origin of the carbohydrate heterogeneity by measuring the relative abundance of the different diptericin glycoforms after each step of purification, and directly in the hemolymph and the fat body (site of production of diptericin) of *P. terranovae* previously infected with bacteria. The authors used MALDI-MS rather than on-line LC-ESI-MS because of the high complexity expected either for the different purified fractions to analyze or the complex mixtures that hemolymph and fat body tissue could represent. Following optimization of the sample preparation for optimal mass mapping of the diptericin glycoforms, Uttenweiler-Joseph et al. have shown that the purification procedure was not responsible for the carbohydrate heterogeneity observed. However, as the authors could not detect any diptericin signals in the crude hemolymph and in the fat body tissue, they could not exclude the presence of a glycosidase activity in the hemolymph of *P. terranovae*.

Nevertheless, the authors observed that several molecular masses between 3-5 kDa were detected exclusively in the fat body tissue collected from bacteria-challenged larvae of this flesh-fly while others were only present in control flies [55]. Such approach evidenced the sensitivity and potency of MALDI-MS to perform peptide mass fingerprints directly on tissues or organs. Analyzing complex biological samples by MALDI-MS without any pre-treatment of the sample (*e.g.* solid-phase extraction, LC) has thus become a reality.

A



B

Diptericin	MMm (Da)	Mass excess (Da)	Carbohydrate type
dipt.	8,692.6	-	-
ε	8,895.8	203	GalNac
δ	9,099.6	2x203	2xGalNac
β	9,260.6	(2x203) + 162	2xGalNac, Gal
γ	9,423.3	(2x203) + (2x162)	2xGalNac, Gal, Gal/Glc
α	9,745.7	(2x203) + (4x162)	2xGalNac, 2xGal, 2xGal/Glc

C

MMm (Da)	MMc (Da)	Peptidic sequence & carbohydrate location
615.7	615.7	*1*DEKPK*5*
933.6	933.6	*41*VWTSDNGR*48*
990.6	991.6	*74*IGAGYSYNF**a***82*, C-terminal amidated fragment*
1289	1289	*29*DGFGVSVDAHQK*40*
1387	1387	*28*KDGFGVSVDAHQK*40*
2504	2139	*6*LILPTPAPPNLPQLVGGGGGNR*27* | **Gal-GalNac**
2631	2266	*6*LILPTPAPPNLPQLVGGGGGNRK*28* | **Gal-GalNac**
2919	2715	*49*HSIGVTPGYSQHLGGPYGNSRPDYR*73* | **GalNac**
3081	2715	*49*HSIGVTPGYSQHLGGPYGNSRPDYR*73* | **Gal-GalNac**

Fig. (3). **(A)** Determination of the cysteine pairing on recombinant drosomycin. The disulfide arrangement (C1-C8, C2-C5, C3-C6 and C4-C7) was defined by combining proteolyses, HPLC, Edman sequencing and MALDI-MS. **(B)** The five *O*-glycosylated diptericin isoforms (α to ε) from *P. terranovae* were evidenced by combining HPLC purification, Edman sequencing, ESI-MS for molecular mass assignment. **(C)** Identification of carbohydrates was performed by GC-EI-MS and determination of the glycosylated sites and of the C-terminal amidation on the glycoform γ by trypsinolysis and LC-ESI-MS. **GalNAc, Gal, Glc** and **a** = *N*-acetylgalactosamine, galactose, glucose and amidation, respectively. MMm and MMc mean molecular mass measured and molecular mass calculated, respectively.

Direct mass profiling of tissue samples

Drosophila model

As already mentioned, MALDI-MS is particularly well-adapted for complex mixtures as (i) it generates mostly monoprotonated ions while ESI-MS generates series of multi-charged ions, (ii) subpicomolar to low femtomolar concentrations can be rapidly and easily detected with good accuracy even on molecular species of the size of diptericin, and (iii) it tolerates impurities (salts, lipids or other additives) better than other MS methods [58]. As illustrated in Figure (**4A**) with the study of the diptericin heterogeneity in crude hemolymph and whole fat body of *P. terranovae*, even while

diptericins could not be detected, MALDI-MS evidenced the presence, upon bacteria-challenge, of a series of up- or down-regulated peptides in the mass range considered. This reveals the potentiality of MALDI-MS for the identification through a differential analysis (*e.g.* bacteria-infected *vs* control individuals) of markers of infections in insects and also for direct mass profiling from tissues.

Ferrandon et al. [23] have found in some non-experimentally infected *Drosophila*, using drosomycin-green fluorescence protein (drosomycin-GFP) reporter transgene, the expression of drosomycin-GFP in a variety of epithelial tissues (*e.g.* trachea, salivary glands and female sperm storage organs). To check that the drosomycin-GFP expression detected in the tracheal system corresponded to the actual synthesis of the endogenous drosomycin, fluorescent and non-fluorescent parts of the same tracheal trunks were directly subjected to MALDI-MS. The results obtained evidenced that endogenous drosomycin is exclusively expressed in the fluorescent section of the tracheal trunk [see Figure (**4B**)]. Moreover, Tzou et al. have detected, following MALDI-MS, endogenous drosocin and defensin peptides on dissected fluorescent tracheal trunks of drosocin-GFP larvae and on oral region of defensin-GFP larvae, respectively [24]. Not only AMPs were detected in tissue samples by MALDI-MS, as illustrated by the detection of pherokine-2 (phk-2) in *Drosophila* legs or ejaculatory bulb. Phk-2 is a 12.8 kDa molecule, initially detected in *Drosophila* hemolymph after a viral infection [19]. Using transgenic flies (phk-2-GFP), expression of phk-2 has been shown in several tissues including legs, wing veins, the reproductive and digestive tracts and the labellum. As shown in Figure (**4C**) endogenous phk-2 has been detected by direct MALDI-MS analysis of fluorescent legs and ejaculatory bulbs.

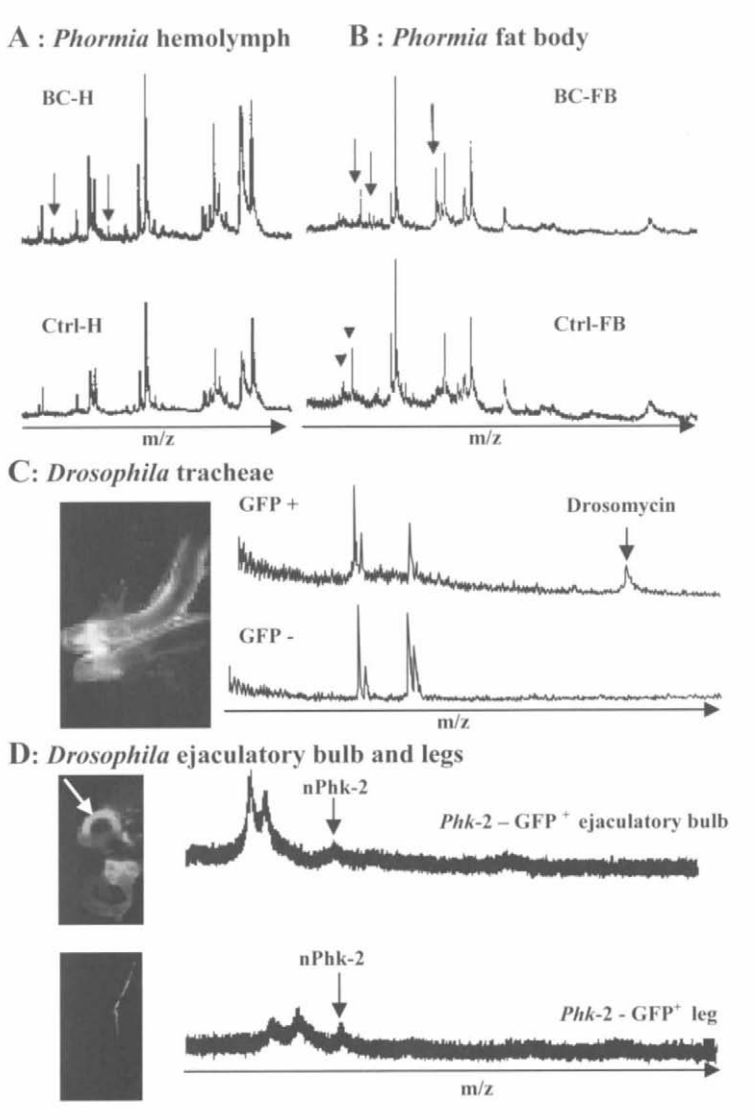


Fig. (4). (A) MALDI mass spectra of the hemolymph from bacteria-challenged (BC-H) *vs* control (Ctrl-H) larvae of *P. terranovae*. The arrows indicate immune-induced components. **(B)** MALDI mass spectra of the whole fat body from bacteria-challenged (BC-FB) *vs* control (Ctrl-FB) larvae of *P. terranovae*. The arrows indicate immune-induced components while the arrowheads marked repressed molecules. Detection by MALDI-MS of **(C)** endogenous drosomycin in a fluorescent trachea from larvae (GFP+) *vs* a non-fluorescent portion (GFP-) of the same trachea, and **(D)** of endogenous pherokine-2 directly in a dissected ejaculatory bulb and leg expressing *pherokine*-2-GFP.

Although several studies have been performed on peptide profiling on crude tissue preparations, MALDI-MS, nano-LC and ESI-MS/MS have also been used to study large-size single-cells such as mollusk neurons from the pulmonate fresh water snail *Lymnea stagnalis* [59-67] and from the sea-slug *Aplysia californica* [44,68-74]. As a result of the relatively large size of their neurons (20-500 μm) and because of the simplicity of their nervous system, these mollusks have been valuable model organisms to study learning, memory and behavior. MALDI-MS in combination with collision-induced dissociation and post-source decay led to the discovery of numerous peptide hormones in *L. stagnalis*. To mention only a few of them, a complex set of peptides encoded by the egg-laying prohormone (ELH) as well as cleavage products of the light-yellow cells peptide prohormones [59] have been identified. Peptide hormones involved in muscle [61] and cardiac modulation [62], and copulatory behavior [66], have also been evidenced by single-cell MALDI-MS peptide profiling. This practice has also allowed the identification of an impressive number of peptide hormones from *A. californica* (for review see [41]). In addition to all previously identified products of the ELH, MALDI-MS unambiguously determined novel proteolytic processing products of the ELH prohormone [68]. In 1999, Floyd et al. reported the intracellular processing of the insulin prohormone [69]. As for the study of *Drosophila* immunity, in addition to identification of a novel peptide, MALDI-MS has been used in *Aplysia* to study the regulation of peptides in response to stimuli or specific physiological conditions (for some examples see [68]) and for special peptide profiling from subcellular regions [74].

As highlighted in the reviews from Li et al. [71] and Hummon et al. [41], in addition to the mollusk single-cell models, MALDI-MS associated or not to nanoLC and on-line LC-ESI-MS/MS has been successfully applied to a wide variety of animal species from invertebrates to vertebrates.

Indeed, recent reports demonstrated the potential use of enzymatic digestion combined with LC-ESI-MS/MS directly on formalin-fixed paraffin-embedded tissues to identify tumor biomarkers (for a review see [68]). The ultimate hope would be to mobilize such powerful MS tools to routine clinical application for *in vitro* diagnosis assays.

Peptidomics

Regarding the results obtained on crude hemolymph and whole fat body of *P. terranovae*, Uttenweiler-Joseph and co-workers took advantage of the great potential of MALDI-MS to apply this methodology to the hemolymph of individual adult *Drosophila*. They performed a differential display MALDI-MS analysis of the blood of individual fruit-flies challenged or not with microorganisms. MALDI-MS allowed to establish the peptide profiles that served to obtain characteristic molecular mass fingerprints of *Drosophila* in different contexts of infections and mutations, as well as a time course of induction and degradation process of immune effectors, and to identify *Drosophila* immune-effectors [15,20,75,76].

If the performance of MALDI-MS to investigate complex mixtures is explained by its high sensitivity, its tolerance towards buffering solutions such as body fluids or tissues and an ionization process that chiefly produces single-charged ions, one of the most challenging aspect in MALDI-MS is sample preparation (for review see [42,77,78]). In fact, the choice of the matrix, the matrix to analyte ratio, and the procedure of sample preparation (co-crystalization of the matrix with the analyte) are the most critical parameters to carefully adjust when analyzing complex mixtures. Uttenweiler-Joseph and colleagues used a sandwich sample preparation, α-cyano-4-hydroxycinnamic acid (4HCCA) as matrix and a laser power adjusted to obtain the best signal to noise performance [15]. Typical mass spectra obtained in these conditions from the hemolymph of a 6h- and a 24h-bacteria-challenged *Drosophila vs* a control (unchallenged) fly are reported on Figure

(**5**). Following this molecular mass differential display, a series of 24 molecules (DIMs, for *Drosophila* immune-induced molecules) with molecular masses ranging from 1.5 to 10 kDa was induced experimentally.

Using this approach, kinetics of induction and degradation processes were conducted up to several weeks evidencing that most of these molecules were at their induction peaks after 24h and were almost totally degraded between two and three weeks [15,76]. Finally, MALDI-MS analysis of the hemolymph of *Drosophila* mutant altered in the Toll and Imd pathways has demonstrated that most of the DIMs are controlled by the Toll pathway with the exception of three DIMs that were found to be under the control of the Imd pathway [15,20]. Combining HPLC and MALDI-MS additional DIMs have been detected with some of these immune effectors corresponding to known *Drosophila* AMPs (metchnikowin, drosocin, drosomycin). Different methodologies have been applied to establish the primary structure of these DIMs: HPLC, N- and C-terminal sequencing either by Edman sequencing or carboxypeptidase digestion, proteolytic treatment, ESI-MS/MS, cDNA cloning and genome mapping [15,20,79].

Since 2002, on-line nanoscale LC-ESI-MS/MS was used for the analysis of the peptidome of *Drosophila* samples. This combination greatly improves the sensitivity of detection. Starting from only 50 larval *Drosophila* CNS, 28 peptides were isolated and sequenced in an on-line quadrupole time-of-flight mass spectrometer [27]. Later, two-dimensional capillary LC-ESI-MS/MS has enhanced the coverage of this peptidomics analysis with the identification of twenty additional peptides [31]. The CNS extract has been first fractionated onto a strong cation-exchange column then onto a reversed-phase column before ESI-MS/MS analysis. Recently this approach has been applied to *Drosophila* larvae hemolymph to identify new peptides induced by a septic injury [22]. Most of the identified molecules correspond to truncated forms or propeptides of known AMPs and DIMs [15,20,21], but two previously unknown peptide precursors, potentially involved in the innate immune response, have been also detected by this way.

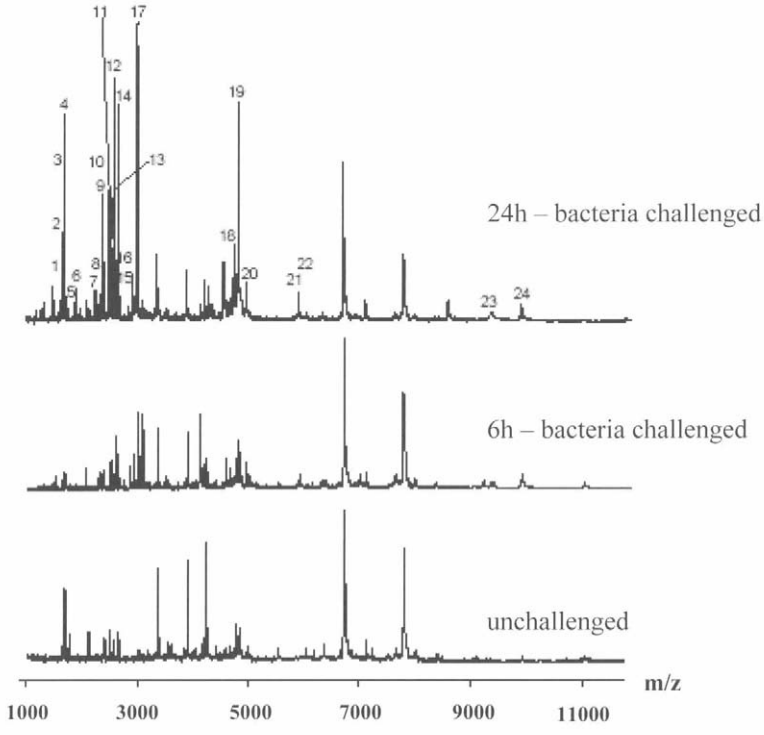


Fig. (5). Typical mass spectra from the *Drosophila* hemolymph (0.1 μL) collected from a single fly at 6h, 24h post challenge and from a control (unchallenged) fly. MALDI mass spectra were acquired with a sandwich sample preparation and α-cyano-4-hydroxycinnamic acid as matrix. Numbers (1-24) correspond to the *Drosophila* immune-induced molecules (DIMs).

Proteomics

Since 2003, several proteomic analyses have been performed on *Drosophila*, combining 2D gel electrophoresis and MS. 2D-SDS-PAGE is a highly resolving technique for arraying proteins by isoelectric point (first dimension, isoelectrofocusing, IEF) and molecular mass (second dimension, SDS-PAGE). It allows the separation of thousands proteins and can differentiate among post-translationally modified forms of a protein, *e.g.* differentially phosphorylated proteins. An increase in reproducibility resulted

from the introduction of immobilized pH-gradient first dimension gels. For protein identification, selected spots are excised and digested in gel with an enzyme, the most documented being trypsin, and the resulting peptides are analyzed using ESI-MS/MS or MALDI-MS. Data from the MS spectra of the generated peptides are unique (a kind of mass fingerprint) for each protein and allow its identification by consulting available databases [see Fig. (**2**)]. A weakness of 2D gel electrophoresis, however, is its inability to deal with certain classes of proteins, mostly the highly hydrophobic ones and those with isoelectric points at extreme values of the pH scale.

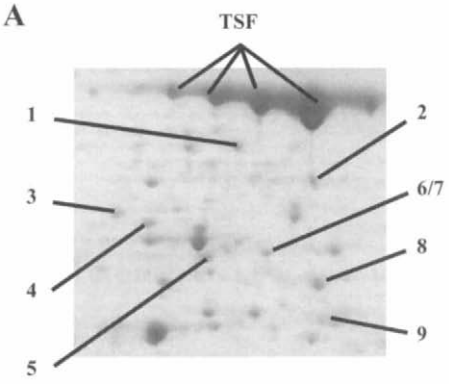
Well-resolved 2D gels were obtained from the hemolymph of 600 adult flies and 70 of the 160 Coomassie-detected spots had a 5-fold increased expression after fungal or bacterial challenge [25]. Similar differential strategies were used for larvae hemolymph after Coomassie detection [80] or labeling with different fluorescent dyes [81,82]. Regulated spots were analyzed using classical in-gel digestion and MALDI-MS analyses. Comparison of the measured masses of the tryptic peptides with the predicted masses from databases allowed identification of the protein, based on the completion of the *Drosophila* genome.

Taken together these studies identified proteins belonging to families likely related to the *Drosophila* immune response (serine proteases, serpins, Gram-negative binding protein-like, complement-like protein, prophenoloxydase-activating enzymes) but also new protein candidates implicated in iron metabolism, odorant-binding, phosphatidyl-ethanolamine binding, detoxification, heat shock response, and protein, lipid and carbohydrate metabolism. As an example, a member of the CLIP-domain containing protease family, CG9372, was highlighted through a differential proteomic analysis after fungal infection and may represent a new candidate involved in proteolytic cascades of *Drosophila* immune response [21].

Interestingly 2D electrophoresis/MS approach allowed the characterization of the cleavage products formed during the immune response [25,81]. Truncated forms of proteins are detected on 2D gels at observed molecular masses lower than the entire forms and

the comparison between mass fingerprints of the entire and truncated forms can be used for determination of the cleavage site. Figure (**6**) illustrates the cleavage of transferrin in *Drosophila* hemolymph after a fungal infection. Fig. (**6A**) shows the mass spectrum obtained from an induced spot [spot 4, on Fig. (**6B**)]. Monoisotopic masses measured from this spectrum clearly identified the transferrin protein but the sequence coverage lacks the cleaved C-terminal part. As shown in Fig. (**6C**) nine fragments of transferrin were characterized through MS fingerprinting.

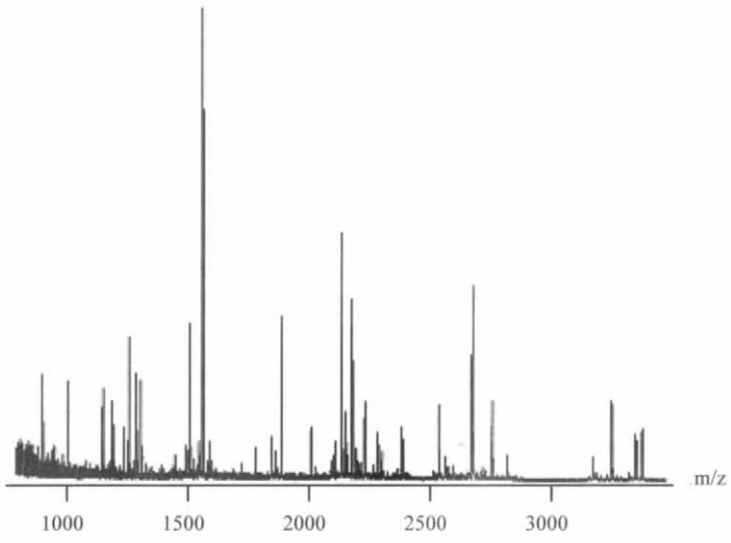
A



B

Spot number	Sequence coverage
TSF	36-637
1	36-555
2	211-602
3	97-637
4	36-456
5	36-538
6	128-610
7	36-488
8	36-456
9	60-355

C



D

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1   MMSPHKTHTW LPLAVAALLL ILGPQSSLAE EPIYRLCVPQ IYLAECQQLL ADPSEAGIRM
61  ECVAGRDRVD CLELIEQRKA DVLATEPEDM YIAYHRKNED YRVISEIRTQ QDKNAAFRYE
121 GIILVKKDSP IRTLQQLRGA KSCHTGFGRN VGYKIPITKL KNTHVLKVSA DPQISATERE
181 LKSLSEFFTQ SCLVGTYSTH PETDRLLKKK YANLCALCEK PEQCNYPDKF SGYDGAIRCL
241 DKGQGEVAFS KVQYIKKYFG LPGAGPDAPP AEGNPENFEY LCEDGTRRPV TGPACSWAQR
301 PWSGYISNEQ AVHNSEQLHQ LQSRLERFFA NGLQAQNKDA AAHLLIQPNA VYHSKDAAID
361 PKVYLERAGY KDVIERDGSA IRKIRLCAQN DDEFAKCQAL HQAAYARDAR PELECVQSTD
421 CVVALTKKEA DLTIVRATGY ADARSNQLQP IVYEQRAQDD VLVAVAAPGV TREALQKASI
481 KFNENCERSR AAAALLNKRR GLDACRVSSS DDGEVQIVPA SELEKHKDAQ LVCPSLERRP
541 VTDFRDCNVD VQLPRAIFIR SDTTSVEQET VKHLFSLISD KFGARGKLVD VFALFGEFQK
601 GKKNVYFNDK AVQLTTELKN EIQNEQIYTD LQCNANKIAK Q
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Fig. (6). **(A)** Selected area of a 2D map (range of pI 5-8, 11% SDS-PAGE) of proteins induced in the hemolymph of *Drosophila* following an infection with the filamentous fungus *Beauveria bassiana*. Up-regulated *Drosophila* transferrin (TSF) and induced labeled spots (1-9) were subjected to proteomics studies. **(B)** Spots 1-9 were identified by MALDI-MS fingerprint as fragments of TSF. **(C)** Molecular mass fingerprint by MALDI-MS in reflector mode of the products digestion mixture of spot 4. **(D)** Coverage of the TSF sequence using the mass table (monoisotopic values) generated in spectrum (C). The identified segments are in bold characters.

ANTIMICROBIAL PEPTIDES

Direct action of Antimicrobial Peptides on microorganisms

The great increase in the resistance of pathogenic microorganisms to conventional antibiotics has stimulated the search for new drugs to be used in the combat of infections [83]. One such group of antibiotic molecules is that of antimicrobial peptides (AMPs) [84]. The great advantage in the use of antibiotic peptides is the reduced capacity of treated microorganisms to acquire resistance [85], the high specificity in relation to the microbial target and low toxicity for eukaryotic cells [86].

The mechanisms of action of the antibiotic peptides are being actively investigated, but they remain still undefined for the great majority of them. AMPs exhibit two main modes of action on microorganisms, one involving an intracellular target, and another the interaction with the cytoplasmic membrane [84,87,88].

Short proline-rich peptides were isolated from insects and are induced upon bacterial infection. They show structural similarities with longer proline-rich AMPs from insects and mammals. For a complete review on the proline-rich antibacterial family see Otvos [52]. Three examples will be considered in this review: apidaecin [89], drosocin [51], and pyrrhocoricin [90]. Short proline-rich peptides have between 16 and 20 amino acid residues with the threonine residue *O*-linked to a carbohydrate chain in the mid-sequence of drosocin and pyrrhocoricin. However apidaecins lack the *O*-glycosylated threonine residue. The biological role of glycosylation is not completely elucidated. A synthetic drosocin variant with classical *O*-linked sugars were replaced by oxime-linked carbohydrates showed a similar antibacterial activity to the native glycopeptide [91]. Surprisingly, synthetic *O*-glycosylated pyrrhocoricin is less active than the synthetic non-glycosylated analogue [92]. These peptides kill with high efficiency essentially Gram-negative bacteria. It has been proposed that the proline residues may target a proline carrier in the course of intracellular uptake of this peptide [93].

The peptides belonging to the proline-rich family are organized in an N-terminal DnaK-binding domain and a C-terminal cell-penetrating fragment (*i.e.* delivery fragment). They cross the cell membrane without cell lysis and, once inside the cell, they bind to target biopolymers. It is proposed that pyrrhocoricin, drosocin and apidaecin kill bacteria by specifically binding to the 70 kDa bacterial heat shock protein DnaK that is responsible for providing protein folding assistance to cells. The N-terminal half of pyrrhocoricin binds to the hinge between helices D and E of DnaK, while the positively charged residues located at the C-terminus are needed for the peptide to enter the cytoplasm [94,95]. While pyrrhocoricin, drosocin and apidaecin act on the folding of already assembled proteins, apidaecin was also shown to inhibit protein synthesis [93]. In fact, apidaecin exerts its antibacterial activity through a five-step mechanism that involves (i) binding to an outer-

membrane component of *Escherichia coli*, (ii) invasion of the periplasmatic space, (iii) interaction with a receptor/docking molecule that may be bound to the inner membrane or otherwise associated, (iv) translocation to the interior of the cell and (v) binding to component(s) of the protein synthesis machinery.

The group of Otvos at Wistar Institute has been actively working on the development of pyrrhocoricin for therapeutic treatment. They have already shown that the peptide (i) is non-toxic both to eukaryotic cells and mice, (ii) has good *in vitro* activity against model bacterial strains and (iii), when administered intravenously *in vivo*, protected mice from a systemic *E. coli* challenge [96]. Several target-specific analogues including chimeras of drosocin-pyrrhocoricin have been synthesized and their antimicrobial properties tested. Interestingly, one of them, in which the pyrrhocoricin's DnaK-binding domain was linked to the drosocin's cell penetrating domain, showed improved *in vitro* antibacterial efficacy against *E. coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. Interestingly this chimera was also efficient against *Staphylococcus aureus*, a strain resistant to pyrrhocoricin and drosocin. In addition, the pyrrhocoricin-drosocin mixed dimers seem to exert their antimicrobial activities by two different mechanisms, membrane damage and interference on intracellular metabolism [97].

Among the long-chain proline-rich AMPs, diptericin that is carrying post-translational modifications (C-terminal amidation and two O-glycosylations, see for details the previous section of this chapter) has a controversial mode of action. It was shown to disrupt the bacterial membrane, but it is unlikely that it acts primarily as a pore-former, rather it is expected to target metabolic processes such as nucleic acid, protein, and cell wall synthesis [98].

Buforin II is a 21-amino acids peptide derived from buforin I with strong antimicrobial activity against a large number of microorganisms including bacteria and fungi. Buforin I, which is a histone H2A-derived peptide, was isolated from the stomach of the toad *Bufo bufo garagrizans*. Studies on the mode of action of buforin II showed that the rapid killing of bacteria is due to the

inhibition of cellular functions achieved by binding to DNA and RNA after crossing the cell membrane. Interestingly, although buforin II is structurally related to membrane-acting peptides, which are formed by linear amphipatic α-helices, it does not cause cell lyses [99,100]. Park et al. showed that the proline located between the two helices of buforin II, is responsible for the peptide ability to penetrate inside the cells [101]. The substitution of the proline-hinge for a leucine decreased significantly the antimicrobial activity of buforin II. Moreover, while analogs with the proline-hinge penetrated the cell membrane without permeabilization and accumulated in the cytoplasm, those devoid of the proline-hinge lost that property, destroying the cell membrane, like magainin, a well known membrane-permeabilizing AMP from frog skin [102]. Recently it was shown that buforin II, crosses the membrane through pore formation, similarly to magainin 2, but the pore life-time is very short, allowing peptide translocation without membrane permeabilization [103].

AMPs targeting the microbial membrane

In general, the AMPs that attack cell membranes are positively charged at physiological pH due to the presence of a high content of basic amino acids (Arg and Lys), and have also hydrophobic domains. The positive charges are responsible for initiating the interaction between the peptide and the negatively charged cell wall and phospholipid membrane of microorganisms, whereas the hydrophobic domains allow the peptides to penetrate the membrane. Different mechanisms have been described to account for the loss of membrane integrity, namely carpet or detergent-like, barrel-stave, and toroidal or wormhole, Figure (**7**) [for reviews see [87,104-106]. In all those mechanisms the AMPs undergo considerable conformational changes to interact and insert into the membrane. Primarily, they must expose their hydrophobic domain to the lipidic part of the membrane. This can be achieved either by adopting an amphipathic structure (monomeric peptides) or by forming oligomers. In this last case, the hydrophobic domains are exposed to

the lipid core of the membrane and the hydrophilic domains are either isolated in the lumen of the oligomer (barrel-stave mechanism, [104]) or exposed to the solution (carpet or detergent-like mechanism, [104]). Finally, in the case of the toroidal (i.e. wormhole, [107]) mechanism, layers of phospholipids bend from one membrane leaflet to the other in order to relieve the tension caused by the accumulation of peptides on the membrane surface. As a result, the peptides, that turn out to be located at the hydrophilic/hydrophobic membrane interface, are dragged with the lipid molecules and, together with the lipid head groups, end up lining the wall of the pore.

Nearly all AMPs described in the literature are membrane active. They can be grouped into two main groups, the cyclic peptides due to the presence of one or more disulfide bridges, and the linear peptides with a potential to form amphipathic structures.

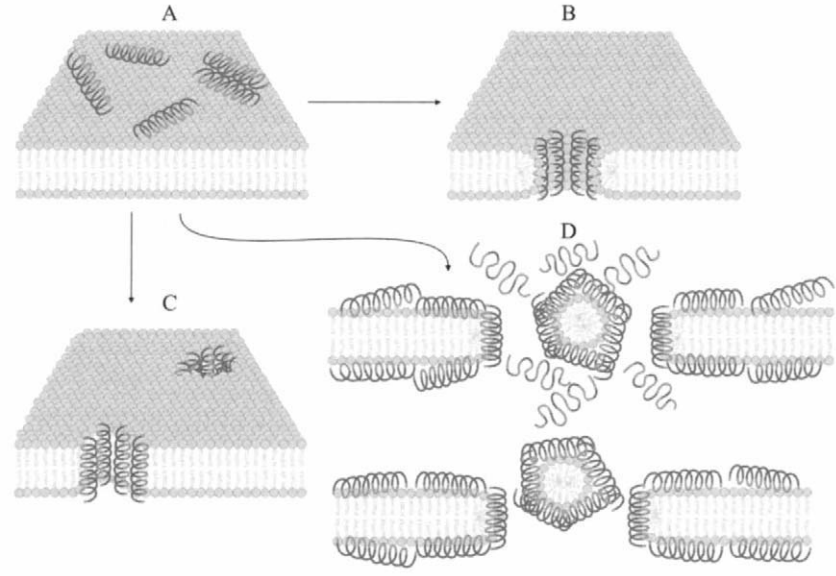

Fig. (7). Mechanisms for membrane permeabilization. **(A)** AMPs reach the membrane and bind to its surface with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent. Different mechanisms lead the loss of membrane integrity namely **(B)** toroidal or wormhole, **(C)** barrel-stave, and **(D)** carpet or detergent-like. Hydrophilic surface of the peptides are shown coloured in orange, hydrophobic surface of the peptides are shown in purple. Modified with permission from Nature Reviews Microbiology [105]. Copyright (2005) Macmillan Magazines Ltd.

Several cyclic peptides have between two and eight cysteine residues. They adopt a triple-stranded β-sheet structure (*e.g.* vertebrate defensins) or a β-hairpin-like structure (*e.g* thanatin, androctonin, gomesin, and tachyplesin from arthropods and protegrin from vertebrate) or a mixed α-helix/β-sheet conformation (*e.g.* invertebrate and plant defensins, including some vertebrate defensins). Several reviews have been published in the past years discussing the structure and the mode of action of cyclic AMPs from animals. The reader is referred to the reviews written by Powers and Hancock [108], Bulet et al. [4], Ganz [8], and Yount [88]. In this chapter, only cyclic peptides with a β-hairpin-like structure will be discussed.

Several peptides with a size ranging from 17 to 25 amino acid residues and usually one or two disulfide bridges adopt a β-hairpin-like structure. They include an amphipathic central area forming an antiparallel double-stranded β-sheet, and a number of basic residues, Arg and Lys, located at the extremities of the structure, Figure (**8**) In general, they have a significant antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, yeast, and fungi [109].

The hairpin-like structure has been conserved in the course of evolution, since it was found in many peptides isolated from various classes of arthropods, such as the primitive horseshoe crabs (tachyplesins [110,111] and polyphemusins [112,113]), arachnids (androctonin in scorpion [114] and gomesin in spider [115]) insect (thanatin [116]), in two classes of vertebrates, mammalian (protegrin [117,118], lactoferricin B [119] and hepcidins [120]) and fish (hepcidins [121]), and in plants (*Ib*-AMP1[122]).

Among the hairpin-like peptides only thanatin, isolated from the bug *Podisus maculiventris* [116], and lactoferricin B, a fragment resulting from the pepsin cleavage of the matured iron-binding protein lactoferrin/lactotransferrin present in milk and other exocrine secretions [119], have one disulfide bridge forming a well

defined β-sheet structure. Interestingly, the activity of thanatin against Gram-negative bacteria seems to occur through a stereospecific mechanism, while most of the hairpin-like peptides interact with the bacterial membrane in a non-specific manner [109]. In addition, the bactericidal and fungicidal activities of thanatin appear to be independent of membrane permeability [123], in contrast to the other hairpin-like peptides that will be discussed below. For the moment, the exact mechanism of action of thanatin on microorganisms remains unclear.

Contrasting to thanatin and lactoferricin B, tachyplesins [110,111] and polyphemusins [112,113], androctonin [114] from the scorpion *Androctonus australis*, gomesin from the spider *Acanthoscurria gomesiana* [115], protegrin from porcine leukocytes [117,118], and *Ib*-AMP1 from the plant *Impatiens balsamina* [122], have four cysteine residues in their sequences. They all fold into a double-stranded antiparallel β-sheet structure.

Finally, the hairpin-like peptides, hepcidins, isolated from human urine and liver [120], and from the gill of hybrid striped bass [121], are more complex, with eight cysteine residues forming four disulphide bridges. They form an unusual distorted β-sheet. Interestingly, besides their antimicrobial activity, hepcidins are the principal hormonal-regulators of iron homeostasis in humans [124].

Proton two-dimensional NMR (^{1}H 2D-NMR) and molecular dynamics calculations have shown that, in water, gomesin, which is highly cationic, folds in a well-resolved double-stranded antiparallel β-sheet where the strands are connected by a non-canonical β-turn. In addition, gomesin presents a hydrophobic face and two hydrophilic poles containing most of its positively charged amino acids, Figure (**9**) [115]. The comparison of gomesin, protegrin-1 and androctonin structures revealed several common features, especially between the first two peptides. Both have one β-sheet face characterized by a marked hydrophobicity, while the other face is hydrophilic and hydrophobic for gomesin and protegrin-1, respectively. Androctonin is clearly less hydrophobic than the other two peptides [109,115]. This feature may explain the lack of hemolytic activity of androctonin [125] and the moderate hemolytic

activity of protegrin 1 [126] and gomesin [127]. The main similarity between the three peptides is the conserved cationic amino acids located in the turn and at the N- or C-terminus [109,115].

These different properties may determine the peptide's mode of interaction leading to the disruption of the microbial membrane. Androctonin acts as a monomer in a carpet-like mechanism, in which the peptide remains parallel to its surface, leading to local perturbations of the bilayer, and finally to the disintegration of the membrane [128]; protegrin 1, instead, depending on the peptide:lipid (P/L) ratio may exist in two distinct states that give rise to different mechanisms of action [129-131]. At low P/L ratio, protegrin 1 adsorbs on the lipid head groups, lying parallel to the surface of the bilayer. At a higher ratio, the peptide molecules are reoriented perpendicular to the membrane and align to form toroidal pores, allowing ion efflux with dissipation of the membrane potential. A recent study, carried out using rotational-echo double resonance (REDOR) solid state NMR, supports this model. In fact, it is demonstrated that protegrin 1, in 1-palmitoyl-2oleyl-*sn*-glycero-3-phosphocholine (POPC) bilayers and at high P/L ratio, associates to form dimers where the protegrin 1 molecules are in a parallel fashion with their C-terminal strands acting as contact interface [132]. Concerning gomesin, due to its high structural similarity with protegrin 1, one may expect a similar mode of action. In fact, recent NMR data, obtained using paramagnetic probes, showed that the linear analogue of gomesin [D-$Thr^{2,6,11,15}$, Pro^{9}]-D-*Gm* (cysteine-2, -6, -11, -15 residues were replaced by threonine residues, glutamine-9 was replaced by L-proline, and the other amino acids by their D-isomers, *Gm* standing for gomesin), which has a conformation very similar to that of gomesin, is bound right under the surface of the sodium dodecyl sulfate (SDS) micelle, with the hydrophobic side chains of the β-sheet facing the centre of the micelle, while the basic residues located at the N- and C-termini and Thr-11, in the turn, have a more superficial position interacting with the negatively charged micelle surface (Daffre, S., unpublished data).

The role of the stereochemical configuration has been evaluated in several β-hairpin-like peptides. For example, D enantiomers proved to be as potent as the native molecules in the cases of androctonin [128], protegrin 1 [133], and gomesin [134], implying that the peptides do not act *via* a stereo-specific receptor.

Structure-activity relationship studies on gomesin have established that the removal of both disulfide bridges, either by substitution of the cysteines by serines or by acetamidomethyl-protected cysteines, resulted in a significant reduction in antimicrobial and hemolytic activities [127]. Moreover, while at least one of the disulfide bridges is needed for the maintenance of an expressive antimicrobial activity, both bridges are required for high stability in human serum. Similar results were found for protegrin [135] and tachyplesin [111]. Circular dichroism (CD) studies revealed that the linearization of gomesin produces analogues with a tendency to adopt an α-helical structure, while the single disulfide bridged peptides exhibit a conformation quite similar to that of native gomesin, irrespective of the disulfide bridge position. In summary, these results underline the fundamental role of the peptide structure for the preservation of the full activity of gomesin [127].

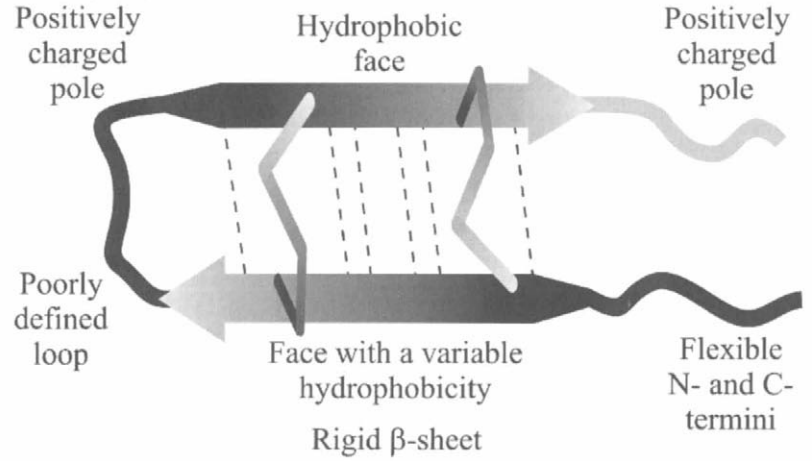

Fig. (8). Representation of the main structural characteristics of peptides with a β-hairpin fold. Peptides are formed by antiparallel two-strand β-sheet stabilized by several hydrogen bonds (hatched lines) and by disulphide bridges. Modified with permission from [109]. Copyright (2002) Research Signpost.

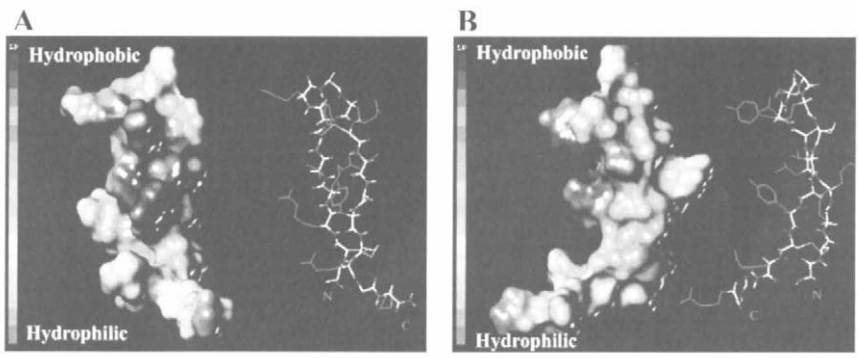

Fig. (9). Hydrophobic potentials on the two faces of the gomesin β-sheet [115]. The orientation of the peptide backbone is indicated on the right side at **(A)** and **(B)** images which were obtained by 180° rotation. Hydrophobicity increases from blue to brown while green is a colour halfway for intermediate potentials. These images were kindly provided by Dr Françoise Vovelle (CNRS, Orléans, France).

Linear Peptides

Linear AMPs have been found in invertebrates (arthropods and procordates) and vertebrates (fishes, frogs and mammalians). Usually, they are shorter than 40 residues in length, they are rich in basic residues and are C-terminally amidated [4,88,136]. Most of them have a tendency to change their conformation from unstructured in aqueous solution to an amphipathic α-helix upon interaction with an electronegative bacterial lipid membrane. For example, the amphibian peptide PGLa (member of the magainin family [102]) adopts an α-helical conformation in an environment composed by electronegative phosphatidylglycerol, while it has a random structure in phosphate buffer at physiological pH as well as in an environment composed of zwitteronic phosphatidylcholine and sphingomyelin that are characteristic of the erythrocyte membrane. These data suggest that PGLa targets bacteria owing to their negatively charged membrane [137]. In contrast, the amphipathic α-helix ovisporin maintains its helicity in the presence of both zwitterionic and anionic micelles. Apparently, the preservation of the ability to fold into an helical structure in zwitterionic environments may correlate with peptide toxicity

[138]. According to Yount et al. the alteration of peptide conformation can be part of a safety-mechanism that control peptide cytotoxicity by keeping peptides inactive prior to interaction with appropriate microbial targets [88].

It is well established that the amphipathic α-helical structure enables the peptides to interact with membrane bilayers, leading to the disruption of the target cell by different ways. Several AMPs including cecropins [139] and dermaseptins [140] may disturb the microbial membrane through carpet or detergent-like mechanisms. Investigations on magainin using surface plasmon resonance suggested that magainin uses the carpet mechanism for membrane permeabilization [141], whereas Huang's group employing oriented circular dichroism (OCD) detected toroidal pores depending on the quantity of peptide bound to the membrane [142]. More recently, two independent groups, Porcelli and co-workers [143] and Ramamoorthy and co-workers [144] used solution and solid state NMR as well as differential scanning calorimetry to study a magainin 2 variant when interacting with dodecylphosphocholine (DPC) micelles and with POPC or 1-palmitoyl-2oleyl-*sn*-glycero-3-phosphoglycerol (POPG) bilayers. Besides showing that the peptide forms antiparallel dimers that tend to be oriented nearly parallel to the bilayer surface, the results suggest that magainin adopts a mechanism of action (carpet or toroidal-type) that is influenced by the lipid system used [143,144]. Overall, these studies indicate that great care must be taken when studying the mechanism of action of these AMPs as both the choice of membrane mimicking systems and the methodologies used can generate different conclusions.

Although several studies on linear amphipathic α-helical AMPs showed the formation of transmembrane pores probably through the barrel-stave mechanism, only a few of them could be confirmed. Among them are pardaxin, alamethicin, and the helix α5 of the δ-endotoxin [87].

It has been reported that spatial separation of hydrophobic and positively charged residues on opposing faces along the α–helix is not essential for the antimicrobial activity of cationic α-helical peptides. Dermaseptins, which are characterized by a cytolytic

activity against a variety of pathogens, form a large family of cationic linear peptides produced by the skin of South American hylid frogs [136]. Among the different molecules studied along the years it is worth to compare dermaseptin B2 [145] and dermaseptin S9 [146]. Dermaseptin B2 is present in the skin of *Phyllomedusa bicolor* and is the most abundant member of the B family. Its structure, both in SDS micelles and in lipid bilayers, presents an amphipathic helix in the C-terminus and an N-terminal region characterized by a highly flexible conformation. The peptide tends to lye parallel to the membrane surface with the N-terminus that, maintaining its conformational flexibility, gives rise to a sequence of different states of interaction with the lipid surface expected to contribute to membrane destabilization. Dermaseptin S9 belongs to the S family and is found in the skin of the frog *P. sauvagei.* Differently from all the other members of the family that are cationic and form amphipathic helices, dermaseptin S9 is characterized by a central hydrophobic region flanked by polar and cationic N- and C-termini. On the one hand, CD and NMR studies show the peptide aggregates in water and partly in SDS micelles, and it folds into a monomeric non-amphipathic α-helical conformation in TFE. Interestingly, this potent AMP can penetrate deeply in the lipid core, demonstrating that lack of amphypathicity is not crucial for biological function. In summary, this comparison highlights the fact that the presence of either an amphipatic helix or cationic domains can promote the initial binding of the peptides on the membrane surface. Moreover, the results indicate that AMPs may contain in their sequence domains responsible for expressing specific tasks.

Cathelicidins form another major family of AMPs [147]. They are characterized by a highly conserved pro-domain and an extremely variable sequence in C-terminus. Interestingly, this C-terminal region turns out to be responsible for their antimicrobial activity. Among the many studied cathelicidins, fowlicidin-1 isolated from chicken (*Gallus gallus*) [148] is one of the most recent member identified and studied. Its structure determined in 50% TFE, present a helix-hinge-helix motif where the helical part is

primarily hydrophobic. Interestingly, Xiao et al. [148] recognizing that some AMPs, besides the antimicrobial activity may exhibit cytolytic and lipopolysaccharide-binding ability, were able to associate these various functions to specific region and secondary structure elements of fowlicidin-1, thus stressing the existence of functional domains also in a small peptide, Figure (**10**).

Another interesting cathelicidin member is PMAP-23 derived from pig myeloid cells. Though it exhibits a helix-hinge-helix fold as fowlicidin-1 it turns out to have a clear amphipathic nature. The study carried out by Yang et al. [149] aimed at understanding the mechanism by which the peptide expresses its antimicrobial activity. The proposed model suggests that the cationic N-terminal region is responsible for initial interaction with the negatively charged membrane surface allowing the formation of an amphipathic helix in that region. This initial step anchors the peptide to the membrane and subsequently the swiveling of the flexible central hinge allows the C-terminal portion of the peptide to interact with the membrane surface. Then, the peptide will acquire an helical fold and insert into the membrane, see Figure (**11**).

Peptide Hb33-61 [150] was isolated from the gut contents of the cattle tick, *Boophilus microplus*, and identified as an endogenous enzymatic cleavage product of bovine hemoglobin, encompassing the region 33-61 of the α-chain. Its amidated analogue (Hb33-61a) turned out to be active in micromolar concentrations against Gram-positive bacteria and fungi [150] and practically inactive against eukaryotic cells such as bovine erythrocytes (Daffre S, unpublished data). Using the fluorescent dyes SYTO 9 and propidium iodide it was observed that Hb33-61a promotes permeabilization of the membrane of *Micrococcus luteus* [151]. After this finding, several reports appeared describing the presence of hemoglobin fragments produced *in vivo* in animals and humans and possessing antimicrobial activity. In fact, it is now assumed that hemoglobin-derived fragments are components of the innate immune system acting against infections [152].

Hb33-61a has a specific affinity for negatively charged surfaces while it does not interact with zwitterionic micelles. The structure

of Hb33-61a, when bound to SDS micelles, presents two β-turns in the N-terminus and a β-turn followed by a non-amphipathic α-helical stretch in the C-terminus. These regions are joined by a five-residue loop containing a proline with a structure stabilized by side-chain interactions. Analysis of the molecular surface of Hb33-61a [see Figure (**12 A**)] [151] shows a hydrophobic patch along one side and of a polar strip extending over the peptide length on the other side. Despite this, the peptide does not possess a well-defined amphipathic nature. In addition, the peptide is mostly positively charged, thus justifying its specific affinity for negatively charged surface [see Fig. (**12 B**)] [151]. The study of the peptide localization in the SDS micelles reveals a certain similarity with cathelicidin PMAP-23. In fact, the positively charged C-terminal helix is embedded in the micelle while the N-terminal portion is close to the surface and the loop region fully exposed. Moreover, similarly to fowlicidin-1 and PMAP-23, these data suggest that the central hinge allows the N- and C-terminal segments of Hb33-61a to fluctuate independently. In particular, since H/D exchange NMR experiments indicate that the N-terminus is exposed to the solvent while the C-terminal region is protected, it seems that the hydrophobic helix is imbedded in the micelle acting as an anchor and that the N-terminus fluctuate over the membrane surface interacting with it as a hammer. The peptide caerin 1.1 [153] is another interesting example that supports the importance of a hinge connecting regions that hold elements of secondary structure as a feature important for the antimicrobial activity of peptides. Overall, it appears that microbicidal and cytolytic activity of AMPs may be associated to chemical and structural parameters such as: hydrophobicity, positive net charge, amphipathicity and helicity. A subtle balance of these features is responsible for the AMPs functionality. More than that it appears that AMPs possess a modular structure where each component acts in a synergistic manner with the others.

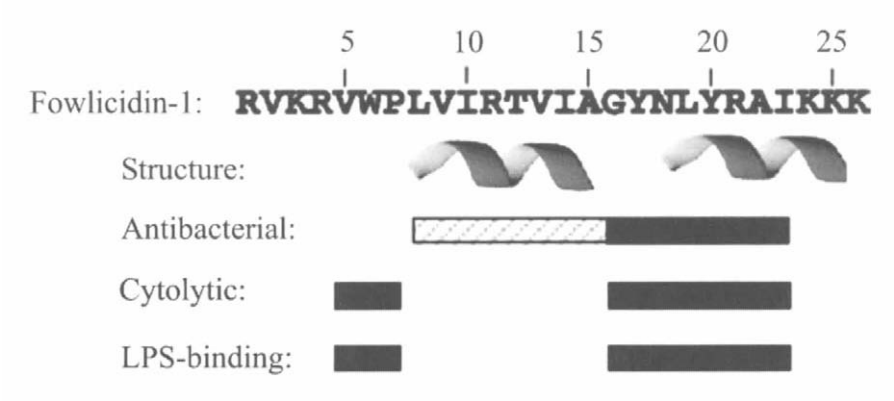

Fig. (10). Representation of the functional determinants of fowlicidin-1. The C-terminal α-helix (Gly16 – Ile 23) is required for the three activities of the peptide: antibacterial, cytolitic, and LPS-binding. The N-terminal unstructured region (Val5-Pro7) comprises the second determinant that is importantly implicated in citotoxicity and LPS-binding. The α-helix (Leu8-Ala15) also most likely enables the interaction of the C-terminal helix with lipid membranes. Reprinted with permission from [148]. Copyright (2006) Blackwell Publishing.

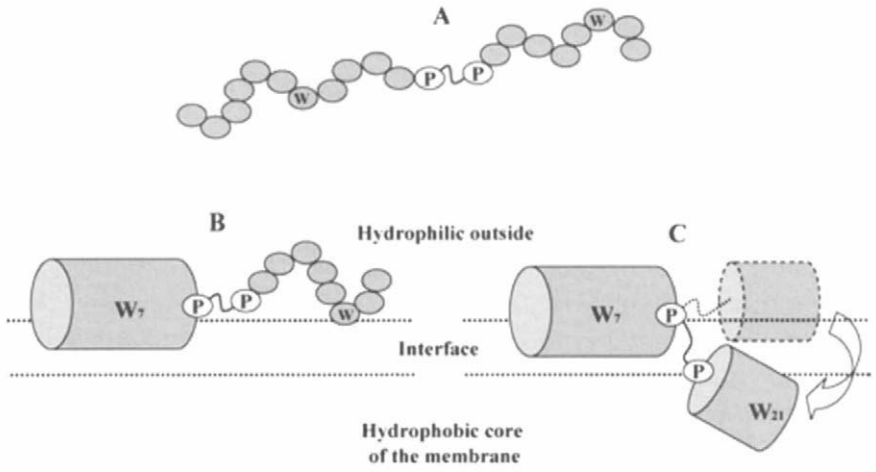

Fig. (11). Model for the interaction of PMAP-23 with a target cell membrane. **(A)** In aqueous buffer, PMAP-23 is unstructured. **(B)** The positively charged residues located at the N-terminal part of PMAP-23 are responsible for the initial binding to anionic membrane, which facilitate the N-terminal half to adopt an amphipathic α-helical structure. **(C)** The anchoring of Trp21 to the membrane interface induces formation of an α-helix at the C-terminus, after which bending of the flexible central hinge (PXXP sequence) permites the C-terminal α-helix to insert into the target cell membrane. Reprinted with permission from [149].Copyright (2006) American Chemical Society.

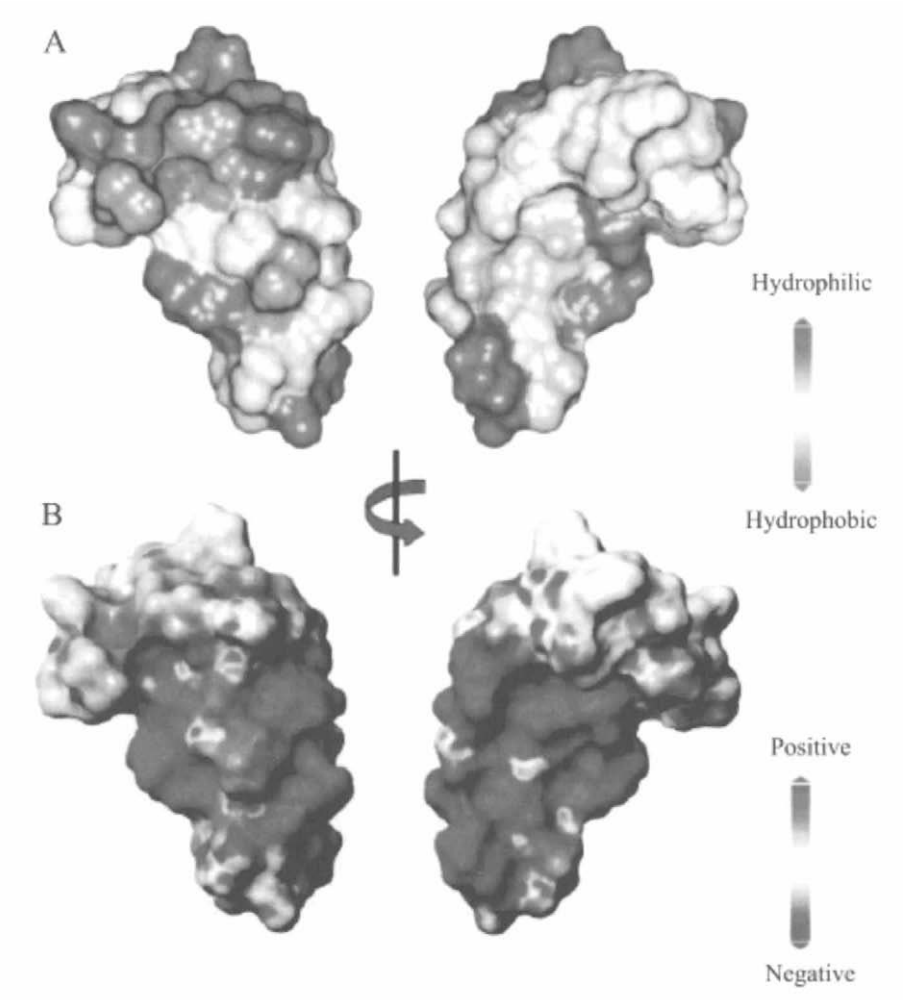

Fig. (12). (A) Hydrophobic/polar surface. **(B)** Potential surface describing the average positive and negative surface charge. The C-terminus is at the bottom. The two images on the right side of panels A and B are obtained by 180° rotation around the axis indicated in the center of the figure. Reprinted with permission from [151]. Copyright (2006) American Chemical Society.

AMP as modulators of the immune system

Apart from the overtly recognized AMPs which occur in high concentrations (*e.g.* α-defensins in neutrophil granules), other

peptides have reduced antimicrobial activity at a physiological salt concentration (*i.e.* 150 mM NaCl) [154], monovalent and divalent ions and serum [155]. They are also found in low concentrations (*e.g.* at mucosal surfaces), raising the question as to their role in the mammalian host. Recently, it has become evident that some AMPs can also act as immune modulators and that their production may depend on the host immune response to infections [9], involving inflammation [156-158] and vascular effects [159]. A suggestion has, therefore, been made to name these small molecules as "cationic host defense peptides" rather than "AMPs" [9].

Production of biologically active AMPs may be constitutive or inducible, and dependent on the species, tissue type, cellular lineages and/or differentiation stage of the cell [160,161]. A disturbance of tissue homeostasis (injury or inflammation) may induce their secretion. Human β-defensins (hBD) are upregulated in: (i) human monocytes [162], monocyte-derived-macrophages, and monocyte-derived-dendritic cells exposed to bacteria [163], lipopolysaccharide (LPS) or IFN-γ (hBD-1 and -2); (ii) keratinocytes stimulated with TNF-α, IL-1β, bacteria or IL-22 (hBD-2, -3 and -4) [164-166]; (iii) intestinal, uterine or airway epithelial cells stimulated with Toll-like receptor (TLR) agonists like LPS, peptidoglycan, CpG motifs (CpG oligodeoxynucleotides) and poly(I:C) (poly inosinic and cytidylic acid, a synthetic polymer that resembles the RNA of infectious viruses)[167-169].

Transcription factors involved in AMP regulation are also responsible for the transcription of inflammatory and immunity genes in mammals, suggesting that the expression of these peptides is coordinated with the expression of other factors of innate immunity and acute inflammation [147,170,171]. IL-1β- and LPS/peptidoglycan-induced hBD-2 syntheses in monocytes and intestinal epithelial cells, respectively, require the NF-κB transcription factor [167,172-174], and the expression of hBD-2,3 by IL-22-induced keratinocytes depends on STAT3 [166]. Secretion of hBD-2 by keratinocytes stimulated by IL-1β or culture supernatants of *Pseudomonas aeruginosa* requires the activation of transcription factors NF-κB (p50-p65) and AP-1 (activator protein-

1) [175]. These last factors also up-regulate hBD-2 secretion by the intestinal epithelial cell lineage Caco-2 stimulated with probiotic bacteria [176]. Although not yet demonstrated experimentally, mammalian cathelicidins may respond to inflammatory *stimuli*, since the 5' flanking sequences upstream of the peptide coding sequence have several potential consensus sequences for transcription factors involved in inflammatory response, such as NF-κB, NF-IL-6, acute-phase response factor and IFN-γ response elements [147,170,177].

Recent findings have established that AMPs stimulate a broad range of effects relevant to inflammation, innate immunity and adaptive immunity, interacting with innate immune cells (neutrophils and epithelial cells) and with cells that bridge the innate and adaptive immune system (monocytes, macrophages, dendritic cells) [158,178,179]. It has been demonstrated that mammalian host defense peptides may activate, inhibit or enhance cellular immune functions such as chemotaxis, apoptosis, gene transcription and cytokine production [178,180,181], eliminating microorganisms without direct killing.

Cathelicidins and defensins induce histamine release from mast cells [182-184]. Human BD-2, -3 and -4 and α-defensins recruit monocytes, T cells (memory and naïve) and immature dendritic cells [185-188] Cathelicidins (bovine, human, mouse and pig) are chemotactic for several subsets of peripheral blood cells *in vitro* [178,189] and *in vivo* [190]. For example, CRAMP (Cathelin-related antimicrobial peptide, the murine orthologue of human cathelicidin/LL-37), like LL-37, was chemotactic for human monocytes, neutrophils, macrophages, and for mouse peripheral blood leukocytes *in vitro* and *in vivo* [189]. These results suggest that host defense peptides recruit innate and adaptive immune cells for protective cellular and humoral responses to pathogens.

Cytokines may also be released after host defense peptide stimulation of several cells. LALF(31-52), a peptide derived from *L. polyphemus* anti-LPS factor, induced the release of a mixed Th1/Th2 cytokine profile (IFN-α, IFN-γ, IL-2 and IL-13) from human peripheral blood mononuclear cells [191], and increased

survival in mice after a lethal dose of *Pseudomonas aeruginosa*, with augmented mRNA synthesis of IL-2, IL-12 and IL-13 in spleen and liver [191,192]. Human keratinocytes stimulated by LL-37 produce a preferential Th1 cytokine profile, secreting IL-6, IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1β. LL-37 treatment of human monocyte-derived dendritic cells enhanced secretion of Th-1 type cytokines, IL-6, IL-12 and TNF-α, promoting Th1 responses *in vitro* [193]. The same cytokine profile was observed after treatment of bone marrow-derived mouse dendritic cells with BD-2 [194].

In addition to the stimulation of proinflammatory immune responses (induction of cytokines, chemokines and histamine release), some AMPs can also exert anti-inflammatory properties, protecting the host against an excessive inflammatory response, particularly after TLR engagement. The human cathelicidin LL-37 is a potent antisepsis agent inhibiting macrophage stimulation by bacterial components (such as LPS, lipoteichoic acid, and noncapped lipoarabinomannan), up-regulating the expression of MCP-1 (monocyte chemoattractant protein 1), IL-8, and chemokine receptors (CXCR-4, CCR2, IL-8RB) in whole human blood cells, and protecting mice against lethal endotoxemia [195]. Interestingly, while treatment of immune cells with LL-37 induced secretion of pro-inflammatory cytokines [193] this AMP prevented the release of proinflammatory cytokines by human peripheral blood mononuclear cells previously stimulated with LPS and other TLR2/4 and TLR9 agonists. The presence of LL-37 significantly reduced nuclear translocation of the transcription factor NF-κB after TLR engagement, suggesting that the peptide altered gene expression in part by acting directly on the TLR/NF-κB pathway [196]. The endotoxin-neutralizing activities of these AMPs suggest that cathelicidins may be important for homeostasis maintenance, particularly at commensal-rich regions of the gut [9].

Cathelicidins may also control tissue damage and inflammation, inhibiting the production of reactive oxygen species (proline-arginine-rich porcine cathelicidin, PR-39) or inducing apoptosis in activated lymphocytes (bovine antimicrobial peptide-28) [9].

Resolution of inflammatory processes is completed by tissue regeneration, and cathelicidins and defensins also promote cell proliferation [197,198], angiogenesis [159] and wound repair [199].

Some AMPs can also modify the adaptive immune response, mainly modulating dendritic cell (DC) functions and antigen-specific immune responses, enhancing aspects of adaptive immunity. Primary human monocyte-derived DCs (immature DC) treated with LL-37 had up-regulated endocytic capacity, enhanced phagocytic receptor expression and function. LL-37-treated DC preincubated with LPS (mature DC) has increased expression of co-stimulatory molecules (CD86) and enhanced Th1 cytokine secretion (IL-12), promoting Th1 responses *in vitro* (IFN-γ secretion by allogenic T cells) [193]. Mouse BD-2 stimulates DC maturation, upregulating the expression of co-stimulatory molecules (CD40, CD80 and CD86), major histocompatibility complex class II (MHC II) and CCR7, a chemokine receptor that regulates trafficking towards T cell-rich areas [194].

AMPs can act as adjuvants for adaptive immune responses, enhancing specific and protective responses. LL-37 [200], cathelin-related antimicrobial peptide (CRAMP) [189] and mouse BD-2 [201] enhanced antigen-specific humoral and cellular immune responses, and induced protective anti-tumor immunity in some conditions [200]. It has been suggested that even low doses of AMPs can influence immune responses, since LL-37 has a synergistic activity with granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-1β [155,196].

Multiple mechanisms are probably involved in immune-modulating effects of AMPs. A variety of receptors has been described for cathelicidins, however, only one biological function, that of LL-37-mediated chemotaxis of human peripheral blood leukocytes, is associated with a known receptor (formyl peptide receptor like 1), [187]. Human BD-2 recruits mast cells and LL-37 activates epithelial cells through at least 2 classes of receptors [183,202]. LL-37 transactivates epidermal growth factor receptor (EGFR) and promotes the release of IL-1β [203], and LL-37-induced maturation of LPS-primed monocytes requires the P2X(7)

receptor, a representative of the family of ligand-gated ion channels activated by ATP [204].

After receptor interaction, AMPs may activate mediators of the mitogen-activated protein-kinase signal transduction pathways [189,202,203,205], induce calcium (Ca^{2+}) mobilization [183,189], bind to SH3-domain-containing proteins [206,207], or inhibit LPS-induced NF–κB nuclear translocation [196,208].

AMPs may also be regulated by a preexisting immune response. Human bronchial epithelial cells were preincubated with Th2 cytokines and infected with *Pseudomonas aeruginosa*, resulting in a significant decrease in the antimicrobial activity of the cells and in suppressed mRNA levels of hBD-2 [209].

Recently it was demonstrated that transfection of tumor cells with an antitumor peptide induced a protective immune reponse. Inoculation of mice with murine BD-2-transfected leukemia cells enhanced cytotoxic T lymphocytes (CTL) and natural killer (NK) anti-tumor activity, with augmented IL-12 and IFN-γ production. Animals vaccinated with transfected cells were protected against a challenge with parental cells (50% protection) and the vaccination generated leukemia-specific memory CTL [210].

In conclusion, the activity of some AMPs on the mammalian immune system has been recently reported [178,194]. They show a broad range of effects, from immune protection to immune suppression, and although the same AMP may have apparently contradictory effects, results must be analyzed carefully, since they vary depending on the experimental conditions. For example, hBD and LL-37 are described as inducers of protective immune responses in several systems, being upregulated in cells involved in the first contact with pathogens (keratinocytes, intestinal, uterine or airway epithelial cells) and also chemoattracting/activating cells from the innate immune response that will act as effectors and as a bridge between the innate and adaptive immune response, stimulating specific cellular and humoral immunity. These AMPs induce the production of Th1-type cytokines, and interfere with cell proliferation, angiogenesis, and wound repair. They may act as adjuvants as well, since co-inoculation of these AMPs with antigens

and/or GM-CSF, or even transfection of tumor cells used for vaccination, enhanced a protective immune response. These results may suggest that treatment with hBD or LL-37 will always produce a protective immunity, inducing a proinflammatory response that might protect against pathogens and tumor cells. However, LL-37 acts likewise as an important anti-inflammatory agent, reducing the production of proinflammatory cytokines by human peripheral blood mononuclear cells previously stimulated with TLR2/4 and TLR9 agonists. Further, in a Th2-type environment, there was a significant decrease in the production of hBD-2 after stimulation with bacterial products. Although the anti-inflammatory activity of LL-37 has been related with homeostasis at commensal-rich regions of the gut, the general use of AMPs as immune modifiers must be preceded by a carefully evaluation of the host immunological and/or clinical condition.

ANTI-TUMOR PEPTIDES

Peptides targeting tumor cells

There are relatively few peptides that specifically interact with the target tissues and have anti-neoplastic properties. Shadidi and Sioud [211] listed 31 peptides from phage-display libraries that targeted surface immunoglobulin in lymphoid tumors and human multiple myeloma M protein as well as ligands on the cell surface of several cancers, among them prostate, head and neck squamous cell, glioma, neuroblastoma, human colorectal and breast cancer cells. Peptides targeting whole tumor cells and causing inhibition of spreading and proliferation may be used as potential drugs [211]. Other peptides bind to the cell surface and are able to internalize into cancer cells. Peptide TSPLNIHNGQKL bound to and was internalized into human head and neck squamous cancer cells [212]. Similarly, peptide VPWMEPAYQRFL was incorporated by a neuroblastoma cell line [213]. A series of 7-mer peptides with the

consensus motif LTVxPWx showed preferential binding and internalization into breast cancer cells [214].

These peptides could function as carriers for the specific delivery of therapeutics into cancer cells.

Identification of cell binding peptides by phage and cell display methodologies use the selection process known as panning. Other methods to select ligands on the outer surface of cells can use FACS for quantitative clone screening and analysis. A high-throughput quantitative screening was designed to isolate cell specific affinity reagents using fluorescent bacterial display peptide libraries coupled with FACS instrumentation. Fluorescent display libraries co-expressed GFP and used *Escherichia coli* to display peptides on the bacterial surface [215]. Live bacteria are used as fluorescent affinity probes. Quantitative screening using FACS identified for the first time unique peptide consensus groups binding to target cells. The consensus motifs of binding peptides to a human breast ductal carcinoma cell line (ZR-75-1) were selected using an OmpA 15-mer bacterial display library. Peptides identified using the bacterial display loop insertions retained their cell binding property (μM range) in the absence of the display scaffold [215].

Opioids act as antitumor agents *in vitro* and *in vivo* by decreasing cell proliferation in a dose dependent and reversible manner. Immunoreactive opioid peptides have been detected in neural and non-neural tumors and in 50% of metastatic breast tumors [216]. The antiproliferative effect of opioid receptor agonists have been investigated on the T47D human breast cancer cell line. Agonists ethylketocyclazocine, morphine, [D-Ala-2, D-Leu-5] enkephalin (Tyr-Gly-Gly-Phe-Xaa), [D-Ser-2, Leu-5] enkephalin-Thr-6 and etorphine inhibited cell proliferation in a dose dependent manner [217]. This effect was opposed by diprenorphine, an opioid receptor antagonist. The opioid receptors on the breast cell line were characterized as mainly belonging to the kappa-type (κ 1, 2 and 3), a few δ-opioid receptor sites and no μ-opioid receptors. μ-Acting opioids cross reacted with type-II somatostatin receptors. The antiproliferative activity of casomorphin peptides (generated by enzymatic degradation of alpha- and beta-casein) was also

investigated. Five casomorphins including morphiceptin inhibited growth of T47D human breast cancer cell line and acted as competitors to somatostatin receptors in the same cells [218] α_{S1}-Casomorphin, Tyr-Val-Pro-Phe-Pro, the most potent opioid inhibiting T47D cell proliferation, did not interact with somatostatin receptors [219]. Casomorphin peptides could, if well tolerated, be used as physiological agents in cancer chemotherapy.

Opioid agonists, active on κ-opioid receptors in T47D cell line decreased both the activity of NOS (nitric oxide synthase) and the release of NO_3^- in the medium; δ- and μ-acting opioid agonists showed no effect [220]. κ-Opioid receptors are rapidly internalized being found in the cytoplasm after 20 min exposure to the ligand. After removal of the agonist they can recycle to the plasma membrane. This could explain the rapid opioid action on NOS possibly involving a direct dissociation of the enzyme dimer. Although NO (nitric oxide) has many roles depending on concentration and cell type, it has been associated with tumor progression and metastasis [221]. It is suggested that inhibition of NOS and NO production by opioids, which could affect angiogenesis, may be related to the suppression by the latter of tumor metastasis. Moreover, endogenous opioids were shown to modulate *in vivo* angiogenesis: opioid growth factor ([Met-5]-enkephalin) has a receptor-mediated activity regulating angiogenesis in developing endothelial and mesenchymal vascular cells [222].

The involvement of milk protein-derived cytomodulatory peptides to determine the viability of cancer cells is a field of great interest. Commercial yoghurt starter cultures hydrolyse casein to produce bioactive peptides that control colon cell kinetics *in vitro*. Bioactive sequences of casein modulate cell viability in different human cell cultures. Peptides from an extract of Gouda cheese inhibited growth of leukemia cells even at 1 pmol/L [223]. They were able to induce apoptosis in the tumor cells. Cancer cells are more reactive to peptide-induced apoptosis than non-malignant cells [224]. Casein-derived peptides could have a role in the prevention of colon cancer by blocking proliferation of the epithelium and by

inducing apoptosis. Casomorphins and α_{S1}-casein exorphins inhibit human prostate cancer cell lines which express opioid and other membrane receptors [225]. The 50% inhibitory concentrations were in the picomolar range.

Since its discovery as an inhibitor of growth hormone release from the pituitary gland, somatostatin was shown to play a role in the regulation of a wide variety of functions in the brain, pituitary, pancreas, gastrointestinal tract, adrenals, thyroid, kidney and immune system. They include inhibition of endocrine and exocrine secretions and of intestinal motility, modulation of neurotransmission, motor and cognitive functions, absorption of nutrients and ions and vascular contractility. The peptide also controls the proliferation of normal and tumor cells as mediated by a family of G protein-coupled receptors (SSTR) which are widely distributed in normal and cancer cells. Antitumor activities include blockade of autocrine/paracrine growth-promoting hormone and growth factor production, inhibition of growth factor-mediated mitogenic signals and induction of apoptosis (review in [226]). Indirect antitumor effects include inhibition of growth-promoting hormone and growth factor secretion, and anti-angiogenic actions.

Recently, Mendoza et al. [227] focused on the molecular pathways governing apoptosis and summarized recent peptide-based approaches that target mdm-2, p53, NF-κB, ErbB2, MAPK, as well as Smac/DIABLO, IAP BIR domains, and Bcl-2 interaction domains, particularly BH3. A special attention was given to the anti-cancer effect of proteasome inhibitors (PI).

Adhesion molecules such as P-selectin, LFA-1, ECAM and ICAM-1 can be targets of PI. The synthetic peptide Bortezomib (Velcade ^{TM}) is a PI showing anti-cancer-effects by controlling the stability (reduced degradation/turn over) of proteins involved in the regulation of apoptosis, survival, adhesion, angiogenesis, tumor invasion and metastasis. It has also an anti-inflammatory effect due to inhibition of NF-κB and of adhesion molecules for leukocyte-endothelial cell interaction [227]. PIs prevent translocation of NF-κB to the nucleus because the inhibitory IκBα is not degraded as in a normal signaling process. Bortezomib is being used for treatment

of multiple myeloma and has also been investigated for use in the control of solid and hematological malignancies.

Cell delivery of therapeutic agents is a challenge to medicinal chemistry. Cationic peptides have been used based on their property to cross the cytoplasmic membrane and enter cells. Neutralization of the anionic membranes with cationic peptides was shown to induce a lamellar to inverted hexagonal phase transition resulting in membrane translocation through inverted micelle formation. Nuclear localization signal (NLS) sequences are cationic peptides that accumulate within cells when added exogenously. They could therefore carry other components including therapeutics to target cells [228]. The authors have evaluated NLS peptides derived from the transcription factors NF-κB, Oct-6, TFIIE-β, TCF1-α, SV40, HATF-3, and *C. elegans* SDC3 for cellular uptake and subcellular localization. The NLS sequences were found to target a wide range of cancerous cell types. The NLS of NF-κB (VQRKRQKLMP-NH_2) successfully delivered covalent adducts of proteins and oligonucleotides to MCF-7 cells. It seems then feasible, to combine the specific properties of peptides to improve drug delivery devices for oligonucleotides [229].

Protein kinases are components of the altered transformed cells that can be over-expressed, as in several tumors. Among relevant receptors EGFR and ErbB2 have been studied for peptide reactivity. Synthetic peptide WTGWCLNPEESTWGFCTGSF, deduced from EC-1 clone from a phage display random peptide library, bound to the extracellular domain of ErbB2, inhibited its phosphorylation and the proliferation of ErbB2-overexpressing breast cancer cells [230]. Peptide KDI-1,Trx-VFGVSWVVGFWCQMHRRLVC-Trx, from a random peptide library integrated into the thioredoxin scaffold protein, interacted with the intracellular domain of EGFR, interfered with STAT 3 activation and inhibited the growth of tumor cells [231].

A small peptide based on amino acids 143-153 of the c-Jun N-terminal kinase (JNK)-binding domain of JIP-1 inhibited JNK activity. Peptide TI-JIP: RP-KRPTTLNLF, resembles the kinase-interaction motif KIM [(K/R)(2-3)X(1-6)(L/I)X(L/I)], which is

common to upstream activators, downstream substrates, phosphatases, and scaffold proteins in MAPK cascades [232]. TI-JIP competes with c-Jun but shows non-competitive inhibition in relation to ATP. Analysis of other KIM-based peptides indicates that TI-JIP is a unique inhibitor of JNK activity.

Cancer cells frequently have mutated p53 and in consequence become resistant to apoptosis induced by chemo- and radiotherapy. Three types of peptide-based therapy could reactivate the p53 functional phenotype: stabilization of mutated p53, disruption of the allosteric p53 regulation and blocking the interaction between p53 and regulatory protein mdm-2. Peptide CDB3, REDEDEIEW, derived from a p53 binding protein, bound to p53 core domain and stabilized it *in vitro*. NMR studies showed that CDB3 bound to p53 at the edge of the DNA binding site, partly overlapping it. Possibly, the peptide could chaperone destabilized p53 mutants keeping them in a native conformation. For instance, CDB3 restored specific DNA binding activity in the structural mutant I195T to almost wild-type levels [233]. The wild-type conformation of several p53 mutants was equally rescued by CDB3, with activation of p53 target genes.

The C-terminal domain of p53 acts as an allosteric regulator of p53. It harbors a tetramerization domain plus a region that regulates specific DNA binding by the core domain and binds single-stranded DNA ends. A synthetic 22-mer peptide 46, corresponding to the carboxy-terminal amino acid residues 361-382 of p53, GSRAHSSHLKSKKGQSTSRHKK, partially restored the transcriptional transactivating function in at least some p53 mutants, while induced p53-dependent-apoptosis in tumor cell lines with mutant or wild-type p53 [234]. These results raise the possibility of developing drugs that restore the tumor suppressor function of mutant p53 proteins, and could selectively eliminate tumor cells.

Three peptides from the mdm-2 binding domain of human p53, residues 12-26 (PPLSQETFSDLWKLL), residues 12-20, and 17-26 were synthesized and attached at the carboxyl termini to the penetratin sequence, KKWKMRRNQFWVKVQRG. All three peptides were cytotoxic to human cancer cells in culture but not to

normal cells, and an unrelated peptide attached to the same penetratin sequence had no effect.

These peptides were cytotoxic in p53-null cancer cells or in those having mutant or normal p53 [235]. In another study, a GST fusion peptide with the sequence MPRFMDYWEGLN was introduced into osteosarcoma cells that overexpressed mdm-2, and in other cell lines that expressed mdm-2 and p53 but were transformed by the HPV16 E6 oncogene [236]. This peptide also induced apoptosis in p53-containing cells, but not in cells with homozygous deletions of p53.

The Bcl-2 family of proteins include anti- and pro-apoptotic members. Peptides of BH3 domains of pro-apoptotic BAX and BAK proteins induced apoptosis and mitochondrial alterations including swelling and cytochrome c release. The BH3 domain of BAK, GQVGRQLAIIGDDINR, fused to antennapedia protein internalization domain induced apoptosis in HeLa cells, preventable by over-expression of Bcl-X_L [237]. Ant-BAK BH3, however, was still able to re-sensitize these cells to Fas-induced apoptosis. A peptide of pro-apoptotic BAD (BH3 domain: NLWAAQRYGRELRRMSDEFEGSFKGL) fused to decanoic acid and cell-permeable moiety (cpm)-1285 was internalized in HL-60 tumor cells, bound Bcl-2 and induced apoptosis [238]. When tagged with polyarginine, BAD BH3 and BID BH3 (EDIIRNIARHLAQVGDSMDR) acted to synergistically kill Jurkat cells [239]. Another advance in the area was to assure the α-helix conformation of the BH3 domain of the BID protein by introducing a hydrocarbon-staple,that rendered it protease resistant and cell permeable. The construction was able to bind to Bcl-2 and induce apoptosis of human leukemia cells [240].

Members of the IAP family inhibit caspases 3, 7 and 9 and interact with inhibitory Smac/DIABLO protein that is released from the mitochondria during apoptosis. They are over-expressed in some cancers. Peptide inhibitors of IAPs were then designed based on the Smac/DIABLO protein. The N-terminal sequence (AVPI) of Smac/DIABLO reacts with BIR3 of X-linked IAP (XIAP) and its N-terminal heptapetide promote procaspase-3 activation [241]. The

heptapeptide fused with the protein transduction domain of Tat protein sensitized neuroblastoma and melanoma cells to apoptosis induced by TNF-related TRAIL or doxorubicin. The peptide also increased the anti-tumor effect of TRAIL in an implanted malignant glioma [242]. The N-terminal tetrapeptide sensitized Jurkat cells to TRAIL induced apoptosis, reinforcing the fact that Smac/DIABLO derived peptides are not active unless followed by an additional apoptotic signal [243].

The antimicrobial peptide database (APD) has 18 entries of antitumor peptides. The database can be assessed at the URL: http://aps.unmc.edu/AP/main.html. Of these, seven are aurein peptides from the Australian bell frogs *Litoria aurea* and *Litoria raniformis*. These peptides are classified in three groups according with their sequences. The more active antitumor peptides are aurein 1.2 (GLFDIIKKIAESF-NH_2), aurein 3.2 (GLFDIVKKIAGHIASSI-NH_2) and aurein 3.3 (GLFDIVKKIAGHIVSSI-NH_2). They were active against the majority of 60 human tumor cell lines tested with LC_{50} values in the 10^{-5}-10^{-4} M range. NMR of aurein 1.2 showed it to have a solution structure of an amphipathic α-helix with well-defined hydrophilic and hydrophobic regions [244].

A peptide (DP1), comprised of a protein transduction domain fused to an antimicrobial peptide (AMP) KLAKLAKKLAKLAK, triggered apoptosis in murine fibrosarcoma (MCA205) and human head and neck tumor cell lines *in vitro*. It also induced tumor apoptosis and reduction of tumor volume (MCA205) by direct intratumor injection [245].

The α-helical peptide P18 (KWKLFKKIPKFLHLAKKF-NH_2) designed from the cecropin A (1-8)-magainin 2 (1-12) hybrid has a strong tumoricidal activity without being hemolytic. There was a positive correlation between α-helicity on lipid membranes and hemolytic and antitumor activity of peptides. N-terminal deleted analogs (N-1, N-2 and N-3) and Leu-substituted analogs (N-3L and N-4L) of P18 also showed a potent antitumor activity against human transformed tumor cells with little or no toxicity against normal fibroblasts (NIH 3T3 cells). Peptides with helix-bend-helix structure may serve as candidates for antibacterial and antitumor

activity [246]. Magainin 2, a 23-residues peptide from the African clawed frog (GIGKFLHSAKKFGKAFVGEIMNS) exerts cytotoxic and antiproliferative activities by pore formation in bladder cancer cells. It has no effect on normal murine or human fibroblasts, therefore may offer a novel therapeutic strategy in the treatment of bladder cancer [247].

Short derivatives of the lactoferrin model peptide L12, PAWRKAFRWAKRMLKKAA, were designed to elucidate the structural basis for their antitumor activity. Three tumor cell lines were included in the study. A strong correlation was observed between antitumor activity and net positive charge, since a net charge close to +7 was essential for a high antitumor activity. In order to increase the antitumor activity of one of the peptides with a net charge less than +7, the hydrophobicity had to be increased by adding a bulky Trp residue. None of the peptides were hemolytic. Peptides showed a 7-fold selectivity for tumor cells compared with fibroblasts [248]. The cytotoxic effect of the AMP, lactoferricin B (FKCRRWQWRMKKLGAPSITCVRRAF) was tested in a panel of human neuroblastoma cell lines. The peptide induced rapid destabilization of the cytoplasmic membrane of target cells and formation of membrane blebs. Depolarization of the mitochondria membranes and irreversible changes in the mitochondria morphology were also evident. In fact, the peptide co-localized with mitochondria and treated neuroblastoma cells induced cleavage by caspase-6, -7 and -9 followed by cell death. Caspase inhibitors were unable to reverse the cytotoxic effect of lactoferricin B. Treatment of established neuroblastoma xenografts with the peptide resulted in significant tumor inhibition [249].

Antagonists of growth hormone-releasing hormone (GHRH) inhibit proliferation of various human cancers. Derivatization with fatty acids could enhance their clinical efficacy. Zarandi et al. [250] synthesized a series of antagonists of GHRH(1-29)NH(2) acylated at the N terminus with monocarboxylic or alpha, omega-dicarboxylic acids containing six to sixteen carbon atoms. The peptides were analogs of the potent antagonists JV-1-36, JV-1-38, and JV-1-65 with phenylacetyl group at their N terminus. The new

analogs, MZ-J-7-46 and MZ-J-7-30, more effectively inhibited GHRH-induced GH release *in vitro* than their parent compound JV-1-36. All antagonists acylated with fatty acids with 8-14 carbon atoms inhibited the proliferation of MiaPaCa-2 human pancreatic cancer cells *in vitro* better than JV-1-36 or JV-1-65. MZ-J-7-114 significantly suppressed the growth of PC-3 human prostate cancers xenografted into *nude* mice. These results suggest that these GHRH antagonists might be effective in the treatment of various cancers [250].

Inhibition of tumor growth by angiogenesis-targeting peptides

Development of tumor cells *in vivo* depends critically on angiogenesis. The search then, for anti-angiogenic agents is of great relevance. The primary sequence of anti-angiogenic peptides usually shows an abundance of hydrophobic and cationic residues. The β-amyloid derived peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ are formed by the cleavage at the NH_2-terminus of Aβ within amyloid precursor protein (APP) by β-secretase (s) and at the C-terminus of Aβ within APP by γ-gamma-secretase (s). The latter comprises a molecular complex of four integral membrane proteins. In the major regulated secretory pathway the cysteine protease cathepsin B represents the β-secretase giving rise to 95% of Aβ. The minor constitutive secretory pathway produces 5% of Aβ and the active β-secretase is the aspartyl protease BACE 1 [251,252]. Aβ peptide, mainly $A\beta_{1-42}$, induces cell death in brain regions responsible for memory as one of the main manifestations of Alzheimer's disease (AD). It has further been shown that it inhibits angiogenesis in *ex vivo* and *in vivo* systems [253].

$A\beta_{1-40}$, DAEFRHDSGYEVHHQKLVFFAQDVGSNKGAIIGL MVGGVV (Dutch mutation of Gln^{22}) and $A\beta_{1-42}$, DAEFRHDSGY EVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA, form β-sheet aggregates that deposit on senile plaques and vessels in AD. Several other peptides with a β-sheet conformation are also anti-angiogenic. The angiogenesis inhibitor endostatin [254], platelet factor-4 [255],

TNF-α [256] and bactericidal-permeability-increasing (BPI) protein [257], all form anti-parallel β-sheets. Aβ at low doses inhibits the formation of capillaries by human brain endothelial cells growing on Matrigel whereas at high doses it causes capillary degeneration [253]. Since angiogenesis is required for tumor growth, the effect of Aβ on human glioblastoma (U87MG) and lung adenocarcinoma (A549) was tested by intra-tumor injection. Aβ inhibited growth and vascularization of xenografts of both tumors in *nude* mice. Vascularization of the tumors was evaluated by CD31 immunostaining: $A\beta_{1\text{-}40}$ reduced vascular density in glioblastoma by 50%. The intraperitoneal injection of human $A\beta_{1\text{-}40}$ to treat *nude* mice implanted with adenocarcinoma cells was followed by rapid diffusion of the peptide into the blood circulation. The peptide administered at 50 mg Ab/kg of body weight remained detectable in the blood for 72 h. The average size of tumors in control mice injected with vehicle or a scrambled peptide, was 289 ± 44 mm^3, whereas injection of $A\beta_{1\text{-}40}$ rendered tumors of 95 ± 18 mm^3 [253].

Tumor angiogenesis requires angiogenic mediators that stimulate host vascular endothelial cell mitogenesis and chemotaxis. The vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis, its expression in growing tumors being up-regulated by hypoxia, growth factors and oncogenes. Anti-VEGF monoclonal antibodies inhibit tumor growth and metastasis in experimental animal systems. In one such experiment, human rhabdomyosarcoma, glioblastoma multiforme and leiomyosarcoma cell lines were implanted in *nude* mice. Treatment with a monoclonal antibody specific for VEGF inhibited growth of tumors, and caused a decrease in the density of vessels [258]. More recently, avastin, a humanized monoclonal antibody to VEGF has shown promising results in phase III clinical trials. This antibody is aimed at treating relapsed metastatic colorectal cancer, and in combination with platinum-based chemotherapy, as first-line treatment of advanced non-small cell lung cancer and metastatic breast cancer. Peptides, however, are alternative low-cost agents that can be used to inhibit binding of VEGF to its endothelial cell receptors.

Blocking the interaction of VEGF and its receptor, KDR/Flk-1 (a kinase domain receptor) led to the regression of murine and human tumors. Peptide K237 (HTMYYHHYQHHL), isolated from a phage-display peptide library, bound to KDR with high affinity thus interfering with VEGF-KDR interaction [259]. It was able to inhibit growth and metastasis of a breast carcinoma cell line in SCID mice, thus suggesting a potential application in the treatment of a variety of cancers.

Another peptide (F56, WHSDMEWWYLLG), also identified from a phage display library, specifically bound to VEGF and abolished VEGF binding to Flk-1 receptor *in vitro*. This peptide was also able to inhibit tumor growth and metastases [260].

Other peptides identified by phage-display libraries and targeting the vasculature of various tumors have been listed by Shadidi and Sioud [211]. In addition to VEGF and VEGFR, peptides targeted known markers of sprouting endothelial cells and angiogenic tumor vasculature, such as the $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins, matrix metalloproteases MMP-2, -9 and -11, aminopeptidases N and P and NG2 proteoglycan. The specificity of peptide homing to normal and tumor tissues, and the eventual antitumor effect of these peptides alone or coupled to anticancer drugs should be considered to define the selective cytotoxicity of the peptide as such or as a drug-carrier. A phage expressing a cyclic nonapeptide, CPGPEGAGC, was found to home to the blood vessels of normal breast tissue with a 100-fold selectivity over nontargeted phage [261]. The phage also bound to the vasculature of hyperplastic and malignant lesions in transgenic breast cancer mice suggesting a common target that was identified as the GPI-anchored membrane-bound aminopeptidase P. The enzyme is expressed on the surface of vascular endothelial cells in various tissues, on lymphoid cells, and on the brush-border membrane in the intestine and in kidney tubules. The highly selective homing of the CPGPEGAGC peptide to breast vasculature, however, might be explained by the occurrence of different isoforms of aminopeptidase P [261]. The peptide might specifically recognize a particular isoform of aminopeptidase P.

A few other anti-angiogenic peptides have been studied and their anti-tumor properties evaluated *in vivo*. The C-terminal segment of platelet factor 4, PF-4^{47-70} conserves a potent anti-angiogenic activity *in vitro* and *in vivo*. By modifying the PF-4 peptide to contain the sequence ELR (or DLR) replacing the DLQ motif a new reagent was obtained with much increased anti-angiogenic activity. Established intracranial glioma in *nude* mice was treated with PF-4^{47-70}DLR with a significant reduction in tumor size [262].

The amino acid sequence 79-93 of VEGF is that involved in the interaction with VEGFR2. Arg-82, Lys-84 and His-86 are key residues in this interaction. The sequence is within a β-sheet structure built with β5 and β6 antiparallel strands linked by a type II β-turn. Zilberberg et al. [263] synthesized a 17-amino acid cyclic peptide (cyclo-VEGI) that inhibited binding of VEGF to its receptor in a dose-dependent manner. Cyclo-VEGI (*D*FPQIMRIKPHQGQHIGE) but not the linear control (PQIMRIKPHQGQHIGE) competed for receptor binding. The addition of 2, 2, 2-trifluoroethanol to an aqueous solution of cyclo-VEGI stabilized helical conformations in the 1-8 domain. This was unexpected because β-sheet structures and random coil conformations are those observed in macrocyclic peptides. In cyclo-VEGI, Pro-2 induces helix formation and Pro-9 breaks the 1-8 helical domain.

Cyclo-VEGI inhibited MAP-kinase activation in endothelial cells stimulated by VEGF and also endothelial cell migration, essential for angiogenesis. In a model of established human intracranial glioma in *nude* mice, cyclo-VEGI administration caused a significant reduction in tumor size (70%). Although the peptide did not affect the tumor proliferation index it decreased microvessel density which was simultaneous to an increase in the apoptotic index [263]. Further, syngeneic intracranial GL 261 tumors in Balb/c mice were also inhibited by the peptide [264].

Angiostatin and endostatin are polypeptide fragments of larger proteins, of 57 kDa and 20 kDa, respectively. Angiostatin derives from plasminogen by autoproteolysis and is a potent inhibitor of angiogenesis [265]. Administration of human angiostatin induced

and sustained dormancy of primary human carcinomas in mice [266]. Angiostatin encloses 3 to 5 contiguous Krinkle modules, and each module has two small beta sheets and three disulfide bonds. The anti-angiogenic effect of angiostatin seems to involve inhibition of endothelial cell migration, proliferation and induction of apoptosis. Administration of recombinant human angiostatin in combination with paclitaxel and carboplatin resulted in a high disease control rate in patients with advanced non-small-cell lung cancer [267].

Angiostatin binds to several proteins including angiomotin and endothelial cell surface ATP synthase and also to integrins, annexin II, C-met receptor, NG2-proteoglycans, chondroitin sulfate proteoglycans and CD26. Its mechanism of action is therefore rather complex and has been addressed mainly with specific antibodies and gene therapy experiments.

Endostatin or endostatin-like collagen XVIII fragments arise by proteolytic enzymes, including cathepsin L and matrix metalloproteases. They cleave peptide bonds at a protease-sensitive hinge region of the C-terminal domain. The processing of collagen XVIII to endostatin may represent a control mechanism for regulation of angiogenesis. The crystal structure of endostatin has been determined at 1.5 Å resolution. It shows a compact fold distantly related to the C-type lectin carbohydrate recognition domain and the hyaluronan-binding Link module. Endostatin has a high affinity for heparin due to a patch formed by 11 arginine residues. This polypeptide may inhibit angiogenesis by binding to the heparan sulphate proteoglycans involved in growth factor signaling [254]. The presence of zinc as a constituent of endostatin suggests that it may be required either for activation of endostatin from its precursor and/or for the anti-angiogenic activity. In the case of a structural role of Zn in endostatin, it could still influence its activity by stabilizing the tertiary structure or directly assuming that the N-terminal loop around Zn is involved in activity. It could lead to endostatin dimer formation or interact with a target protein. Mutation of the arginine, phenylalanine, and glutamine residues, which project from the N-terminal loop to form the dimer might

address this hypothesis [268]. Binding of endostatin to heparin and heparan sulfate required divalent cations. Addition of $ZnCl_2$ to endostatin increased in 40% its binding to heparan sulfate and also stimulated its antiproliferative effect on endothelial cells [269].

A number of antitumor experiments have explored the anti-angiogenic properties of endostatin. Many of them involved gene therapy and long-term infusion. In one of these, adenovirus-mediated human endostatin gene was successfully used to express endogenous endostatin *in vitro* and *in vivo*, and significantly inhibited the growth of BEL-7402 liver tumor xenografts in *nude* mice [270].

The first angiogenesis inhibitors for cancer have been approved by the F.D.A. in the USA and in 28 other countries, including China [271]. Most of them are monotherapies that block VEGF. The least toxic angiogenesis inhibitors are Caplostatin and endostatin. Endostatin inhibited 65 tumor types and modified 12% of the human genome to downregulate pathological angiogenesis. The angiogenic response *in vivo* depends on the genetic background of the host. It is worth noticing that several types of angiogenesis inhibitors showed a biphasic, U-shaped curve of efficacy.

Cadherins are glycoproteins that mediate Ca-dependent, homophilic cell-cell adhesion. They are involved in the regulation of cell motility, proliferation and apoptosis. Type I cadherins have a highly conserved sequence at the homophilic binding site containing His-Ala-Val (HAV). Peptides containing the HAV motif inhibit cadherin-mediated responses such as cell aggregation, compaction and neurite outgrowth. A cyclic peptide, N-Ac-CHAVC-NH_2 disturbed endothelial cell interactions resulting in apoptotic cell death [272]. The addition, however, of bFGF to the peptide-treated endothelial cell cultures blocked the apoptotic effect. It was suggested that cadherin-mediated signaling is essential for viability of confluent endothelial cells which can be disturbed by the cyclic-N-Ac-CHAVC-NH_2 peptide [272].

The key integrin involved in angiogenesis is the $\alpha_v\beta_3$-integrin (vitronectin receptor). It mediates adhesion of endothelial cells to the extracellular matrix and their migration involving the RGD

(Arg-Gly-Asp) motif. The screening of phage display libraries provided peptides containing the RGD sequence that recognized tumor vessels in mice. A cyclopentapeptide c(RGDFV) with either D-Phe or D-Val residues were 20- to more than 100-fold better inhibitor of cell adhesion to vitronectin and/or laminin P1 fragment as compared to the linear variant. NMR studies of the two most active cyclic peptides showed an all-trans conformation with a beta II' and gamma turn [273]. The cyclopeptide inhibited tumor neovascularization in tumors implanted in chick embryos [274] by inducing apoptosis of angiogenic blood vessels. Addition of RGD to a mutant human endostatin was also found to increase the antitumor activity of endostatin [275]. The combined protective effect of anti-angiogenic and cytotoxic therapy should always be tried because tumors contain both endothelial and neoplastic cells. A derivative of the cyclopeptide c(RGDFV) is currently in phase I/II clinical trials for treatment of glioblastoma multiforme (quoted in [227]).

Peptides eliciting anti-tumor immune responses

Immunosurveillance and destruction of cancer cells depend on the host immune system, as suggested by recent studies providing direct evidence to the role of cells of the immune system in the inhibition of spontaneous tumor growth [276]. T-cell-mediated antitumor immunity was demonstrated in murine tumor models [277-279] as well as in human tumors [280-282]. It is widely accepted that $CD4^+$ and $CD8^+$ T cells are the major components of this response [283-285], although other cells play important roles in the immunosurveillance against cancer, as the natural killer (NK), NK1.1 T (NKT) and $\gamma\delta$ T cells [286-288].

Much attention has been paid to the role of $CD8^+$ T cells in the immunotherapy of cancer due to the cytotoxic property of these cells, and a large number of MHC class I-restricted tumor antigens have been identified using tumor-reactive $CD8^+$ T cells from patients peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes (TILs) [283,289,290].

Clinical studies using molecularly defined MHC class I-restricted antigens and peptides are being conducted in several institutions, and antigen-specific T-cell responses are detected after vaccination. However, the overall immune responses elicited by vaccination with $CD8^+$ T-cell peptides are weak and transient. Recently, several studies are demonstrating that to enhance antitumor immunity and eradicate tumor cells in patients, it is necessary to induce both $CD4^+$ and $CD8^+$ T-cell responses [285,291].

$CD8^+$ tumor-specific cytotoxic T lymphocytes (CTLs) are primed and activated by specific tumor peptides. All antigenic peptides described correspond to a unique fragment of about 8-10 amino acids, presented by major histocompatibility complex (MHC) class I molecules at the cell surface. The turnover of most cell proteins occurs via the ubiquitin-proteasome pathway, and it is responsible for the generation of the great majority of MHC class I-restricted T-cell peptides. Peptides ranging 4-20 residues are generated by proteasome degradation of proteins, some of them with appropriate C-termini for MHC binding. In case of longer N-terminal extensions, aminopeptidases present in the cytosol and endoplasmic reticulum may trim the peptide to generate octa- or nonapeptides [292]. A significant proportion of these peptides appear to result from the degradation of newly synthesized proteins, presumably misfolded or erroneous, and have been called "defective ribosomal products" [293]. However, successful presentation by MHC requires that the peptide escape the destruction by cytosolic peptidases. One mechanism that modify this process and enhance the generation of class I-presented peptides is the immunomodulation by IFN-γ, generating the "immunoproteasome" by inducing three β-subunits (LMP2, LMP7, and MECL-1) that replace homologous subunits in newly formed proteasomes [294]. Immunoproteasomes cleave peptides after hydrophobic and basic residues at enhanced rates, increasing the production of peptides capable of binding to class I molecules. IFN-γ also induces PA28, an activator of peptide hydrolysis by the 20S proteasome, as well as leucine aminopeptidase, which can trim the

N termini of longer peptides to generate the final antigenic peptide. In addition, IFN-γ reduces the content of thimet endooligopeptidase (TOP), which together with aminopeptidases seem to destroy many of the cytosolic peptides, limiting their supply for MHC presentation [295]. The proteasome exhibits three distinct peptidase activities. One is termed "chymotrypsin-like activity," because it preferentially hydrolyzes peptides after hydrophobic residues, another is the "trypsin-like" activity that cleaves peptides after basic residues, and a third promotes hydrolysis after acidic residues. The chymotrypsin-like activity is rate limiting in protein degradation [292].

Dendritic cells (DCs) can present tumor peptides via a pathway called cross-presentation: antigens derived from tumor cells are captured by DCs, and are processed for presentation by MHC class I molecules. Antigens are transferred from tumor cells as intact proteins or proteasome substrates, not as peptides [296,297].

Tumor cells can also present by themselves new MHC class I-restricted peptides by cross-presentation, a feature believed to be unique of dendritic cells. A peptide derived from secreted matrix metalloproteinase-2 (MMP-2) is presented by melanoma cells, and the presentation is dependent on the enzyme secretion in the extracellular space, followed by uptake of exogenous MMP-2 through endocytosis mediated by integrin $\alpha_V\beta_3$. The process involves transfer of MMP-2 from the endocytic compartment to the cytosol and processing by the proteasome, because presentation of this peptide was blocked by proteasome inhibitors [298].

Several technologies are used for identification and characterization of novel tumor-associated antigens: (i) molecular cloning of single antigens by screening tumor-derived cDNA libraries with autologous tumor-specific T lymphocytes, (ii) reverse immunology (epitope prediction performed by software based on known HLA-binding motifs), (iii) biochemical methods (elution and fractionation of peptides naturally expressed on tumor cells in the context of HLA molecules by chromatography and mass spectrometry), and (iv) DNA microarray technology [comparison of gene expression profiles in tumor tissues and normal counterparts

using different techniques, like RDA (representational difference analysis), DD (differential display), SSH (suppression subtractive hybridization) and SAGE (serial analysis of gene expression)] [299].

A comprehensive list of all available tumor-associated antigens, their $CD8^+$ and $CD4^+$ T cell epitopes and HLA I and II restriction, respectively, was published by Novellino et al [299], containing all classes of tumor-specific antigens: cancer-testis antigens (expressed by normal spermatocytes and/or spermatogonia of testis), differentiation antigens (expressed in tumors and the normal tissue from which the tumor arose), overexpressed tumor-associated antigens (TAAs, widely occurring, generally with lower expression in normal tissue), unique and shared TAAs (arise from point mutation of normal genes).

Alternative mechanisms are involved in generation and modulation of T-cell epitopes

Identification of T-cell epitopes has revealed several alternative mechanisms by which MHC class I-restricted peptides are generated for T-cell recognition. The processing of these epitopes can be modulated at transcription/splicing, translation and protein degradation levels.

Transcriptional/splicing control involves the generation of T-cell epitopes from an intron of an incompletely spliced mRNA, or aberrant mRNA. Incompletely spliced forms of gp100 [300] and TRP-2 RNA [301] were identified, both containing epitopes presented by MHC class I molecules to T cells. Guilloux et al. [302] identified a cryptic promoter present within one of the introns of the Gnt-V (N-acetylglucosaminyl-transferase V) gene, responsible for the generation of an aberrant transcript of 74 amino acids containing a T-cell epitope.

Recently, it was described the generation of CTL epitopes by post-translational protein splicing, a process previously observed only in plants and unicellular organisms. Hanada et al. [303] demonstrated that the NTYASPRFK peptide, recognized by a CTL clone isolated from human CTLs infiltrating a renal cell carcinoma,

is generated by protein splicing from fibroblast growth factor 5 (FGF-5) protein most likely by reverse proteolysis. This nonapeptide is formed by the fusion of two non-contiguous fragments present in the native protein. The dependence of antigen presentation on proteasome and TAP, and the independence of leader sequences to originate polypeptides suggest that the splicing occurs in the cytosol. It has not been established whether protein splicing in human cells occurs only during protein degradation or is used to generate mature stable substrates. A second spliced peptide recognized by CTLs on human tumor cells [304], derived from 2 noncontiguous fragments in melanocyte differentiation protein gp100. The incubation of an unspliced precursor peptide with purified proteasomes resulted in the production of the spliced peptide *in vitro*. Despite the remarkably low efficiency of the splicing reaction, it has the potential to increase significantly the diversity of antigenic peptides presented by MHC class I molecules [293].

Gene products translated from cDNA in alternative reading frames can generate novel T-cell epitopes. An antigenic peptide, recognized by a patient CTL clone, was derived from the third open-reading frame (ORF3) of TRP-1/gp75 [305]. Another example is the T-cell epitope from the alternative gene product encoded by ORF2 of NY-ESO-1 protein [306]). CTLs for both ORF1 and ORF2 of this protein have been detected in melanoma patients with MHC/peptide tetramer [307]. In addition, another T cell epitope was identified in NY-ESO-1 ORF2 [308,309], this same epitope was identified in CAMEL, translated in an alternative open reading frame from LAGE-1, a protein that shares 94% nucleotide and 87% amino acid homology to NY-ESO-1 [310]. Four naturally processed $CD4^+$ T cell epitopes were also identified on NY-ESO-1 ORF2/CAMEL [311]. The mechanism by which an alternative ORF is translated remains unclear, as well as the biological significance of these gene products [312].

Some T-cell epitopes are generated only by the immunoproteasome, while others are destroyed by it, suggesting a proteolytic control of epitope generation. T-cell peptides presented

by MHC class I molecules are generated from the degradation of intracellular molecules by the 20S proteasome [292]. The IFN-γ-induced immunoproteasome may change the cleavage profiles of antigenic peptides [294,313], and there are several examples of T-cell peptides modulated by the immunoproteasome [314-316]. Chapiro et al. [317], on comparing the degradation of antigenic peptides by standard proteasome and immunoproteasome, suggested that the observed differential processing and generation of peptides mainly results from differences in the efficiency of internal cleavages by both proteasome types. The immunoproteasome may destroy some antigenic peptides, rather than favor their production, due to its higher chymotrypsin-like activity (for example, peptide gp100 209-217 ITDQVPSFV and tyrosinase 369-377 YMDGTMSQV, which are cleaved after an internal hydrophobic residue), while the proteasome cleaves the peptide MAGE-C2 336-344 ALKDVEERV after an acidic residue.

Results from clinical trials indicate that inducing therapeutic T cells against tumor antigens has been difficult, and one explanation for this hyporesponsiveness of the human immune system to many tumor antigens could be that they are normal, nonmutated "self"-proteins, expressed on normal tissues as well as on tumor cells. Studies indicate that substituting favorable key peptide residues to enhance the affinity of MHC-peptides or the stability of the T-cell receptor of a specific T-cell for MHC-peptide complexes was correlated with improved T-cell responses and antitumor activity, both *in vitro* and *in vivo*. Also, immunization with orthologous gene products increased avidity of orthologous peptide for host MHC molecules relative to weak avidity of the cognate self peptide [318-325].

Several studies have shown that tumor-specific $CD4^+$ T cells play a central role in initiating and maintaining protective immune responses against cancer, and the lack of these T cells in any vaccine strategy could be the cause for weak and transitory immune responses [285,291,326,327]. There are, however, few effective methods for identifying MHC class II-restricted tumor antigens that can stimulate $CD4^+$ T helper cells, and in addition to that, the

majority of tumor peptide epitopes binding to class II HLA are not promiscuous and react in the context of a given class II molecule [282,291,299]. A comprehensive list of all available tumor-specific $CD4^+$ T cell epitopes was published by Novellino et al. [299].

An alternative is the use of MHC class II-restricted peptides from tumor unrelated proteins. Casares et al. [328] demonstrated that a tumor-unrelated but strong Th1 (T helper type 1) peptide could induce protective responses against tumors. Immunization of mice with a tumor-specific T $CD4^+$ helper peptide from CT26 tumor cell lineage or a tumor-unrelated strong Th1 peptide from ovalbumin, in combination with a tumor-specific $CD8^+$ T cell epitope, protected the same percentage of animals against a challenge with tumor cells. The presence of Th0 peptides in immunization mixtures may diminish the efficiency of Th1 peptides. The same effect was observed by other groups, using different tumor models [326,329-331].

An efficient $CD4^+$ T cell helper peptide for antitumor immunization must bind to several HLA molecules to be considered for human vaccination of genetically distinct populations, since different epitopes are recognized by T cells from individuals displaying distinct major histocompatibility complex molecules. However, promiscuous peptides are not frequently identified in tumor-related or tumor-unrelated proteins. P10 peptide is one example of a promiscuous MHC class II-restricted epitope. It is a pentadecapeptide (p181-195) identified in gp43, a major secreted glycoprotein from *Paracoccidioides brasiliensis*, a pathogenic fungus causing a prevalent systemic mycosis in Latin America. Immunization of mice with P10 in Freund Adjuvant induced peptide-specific $CD4^+$ T cells of Th1 type, secreting IFN-γ, and the immunized animals were protected against a challenge with viable fungi. The HTLAIR inner core of P10 is the essential domain of the epitope, with various flanking regions possible [332]. Iwai et al [333] used the TEPITOPE algorithm to select T cell epitopes in the gp43 sequence that would most likely bind multiple HLA-DR molecules, and tested the recognition of peptides by T cells from sensitized individuals. P10 was recognized by 53% of previously

treated-paracoccidiodomycosys patients, and this peptide bound to 9 most frequent HLA-DR molecules. The strong induction of IFN-γ-secreting $CD4^+$ T cells and the promiscuous binding to multiple HLA-DR molecules make P10 peptide a possible candidate for human vaccination.

Besides peptides that are presented by MHC molecules to prime/activate T cells, there is another family of peptides responsible for immune response modulation and rejection of tumor cells. Alloferon 1 (HGVSGHGQHGVHG), a peptide isolated from the hemolymph of experimentally infected insect *Calliphora vicina*, showed an antiviral and antitumor activity in mice. Although immunomodulatory properties of the peptide remain to be clarified in detail, it is suggested that alloferon 1 has a significant capacity to stimulate NK cell activity and IFN synthesis in murine and human models [334].

Immunomodulatory properties are described for other peptides, and although not all have been tested for a direct antitumor activity, some of them or derivatives might potentially be active. Representatives of these peptides are listed on Table 1.

Table 1: Immunomodulatory peptides

Peptide	Sequence	Origin	Immune activity	Reference
pYR	YLKGCWT KSYPPKPC FSR	Skin secretion of dusky gopher frog (*Rana sevosa*)	Histamine release from rat peritoneal mast cells.	[335]
CEL-1000	DGQEEKA GVVSTGLI GGG	L.E.A.P.S.™ technology	Promotes a Th1 response upon challenge by infectious agents; rejection of allogeneic tumor.	[336]
α-Melanocyte Stimulating Hormone (αMSH)	SYSMEHFR WGKPV	Pro-opiomelanocortin (POMC)-derived neuroimmuno-modulatory peptide	Inhibits the production and activity of NO and pro-inflammatory cytokines such as TNFα, IL-1, IL-6, and IL-8.	[337],[338]
pLR	LVRGCWT KSYPPKPC FVR	Skin extract of the Northern Leopard frog (*Rana pipiens*)	Histamine-liberating peptides.	[339]

RDP58	NH2-arg-norleucine(nle)-nle-arg-nle-nle-nle-gly-tyr-CONH2	Synthetic, rational design peptide	Inhibit production of Th1 cytokines, such as TNF-α, IFN-γ, IL-2, IL-6, and IL-12. Disrupt formation of the TRAF6-MyD88-IRAK complex responsible for activating crucial signal transduction pathways (p38MAPK, JNK, and IKK) involved in inflammation.	[340],[341] [342],[343] [344]
Allotrap 1258	RDP58 D-isomer	HLA-derived peptide sequences, novel immunomodulatory peptides were developed by computer-aided rational design	Inhibited production of TNF. Partially inhibited production of IFN and IL-12, but not IL-1, IL-2, IL-4, IL-6, or IL-10. Prolong allograft survival in rodents.	[345],[346]
Thymosin-alpha1 (Zadaxin, SciClone Pharmaceutical)	SDAAVDTSSEITTKDLKEKKEVVEEAEN	Prothymosin-alpha derived peptide, thymus	Enhanced amount of IL-1, TNF, ROI, NO, GM-CSF, IFN-γ and IL-2, IL-2 receptors in mitogen-stimulated PBL, maturation of T cells and bone marrow progenitor cells to NK cytolytic effectors, maturation of $CD34^+$ stem cells into $CD3^+4^+$ cells by inducing increased IL-7 synthesis. Thymosin α1 used in combination with cytokines and chemotherapy for treatment of cancer.	[347]
Tuftsin	TKPR	IgG-derived peptide	Binding to specific receptors. Present on virtually all phagocytic cells.	[348]

Peptide trimming and destruction by amino- and oligopeptidases

Immunepeptides are generated from the vast majority of cellular proteins and are transported to the cell surface after binding to MHC class I molecules. Binding involves recognition of terminal α-amino and carboxy groups of amino acids as well as their side chains on peptides of uniform length (8 to 9 residues). To generate those peptides two proteolytic systems were phylogenetically selected. Most cellular proteins are degraded by the ubiquitin-proteasome pathway [349]. Ubiquitin recognizes de ε amino group of lysines in the polypeptide substrate and is followed by additional binding of other ubiquitin molecules (four or more). The chain of ubiquitin molecules tags the protein for degradation by the 26S proteasome. In the 20S core of the proteasome six peptidase sites cleave the protein into a large number of oligopeptides. This step of polypeptide processing by proteasomes is a key step in the generation of immune peptides [350]. In fact, proteasome inhibitors block the production of MHC class-I immunepeptides from proteins and peptides that are extended at the C-terminus by even one amino acid [295]. Apparently, proteasomes are required for generation of the C-terminus of MHC class-I epitopes, because N-extended epitopes (as many as 20 amino acids at the N-terminus) are not affected by proteasome inhibitors although they have to be trimmed before MHC class-I presentation. Proteasome cleaving of protein substrates yields a range of peptides from 2 to 25 amino acids [351], but the actual generation of MHC-I-binding peptides is inefficient. For instance, most of the ovalbumin degraded by proteasomes (94%) does not give rise to the H-2K^b SIINFEKL epitope. The yield is better in immunoproteasomes stimulated by IFN-γ: 11% production of SIINFEKL and N-extended precursors as opposed to 89% epitope destruction [295,352]. It appears that *in vitro* as well as *in vivo* most of the MHC class-I presented peptides was initially produced as N-extended precursors. The latter were identified bound to heat shock proteins [353].

Proteasomes rather than cytosolic carboxy-peptidases act to trim the C-terminal amino acids to conform the peptide to the proper size for MHC class-I presentation. Presentation from N-extended precursors is inhibited by acetylation of the terminal α-amino group at the N-terminus [354], which prevents the peptide to be cleaved by aminopeptidases (*e.g.* leucine aminopeptidase) but not by proteasomes or endopeptidases. The TAP system transports peptides to the ER including both mature epitopes and longer precursors. It seems then that the peptides to be presented by MHC class-I can arise from N-extended precursors both in the cytosol and in the endoplasmic reticulum (ER). This assertion has been experimentally confirmed [355,356].

Secreted or membrane proteins have N-terminal signal peptides that are transported into the ER through the transmembrane SEC61 complex. The signal peptide is then removed by a signal peptidase. TAP-negative cell lines may present peptides that arise from signal peptidase activity. Another Zn-containing metallopeptidase in the ER called ERAP1 is active against 9-16-residue-long substrates, and converts N-extended peptides into presentable peptides of 8 to 9 residues. By using small interfering RNA (siRNA) to lower the expression of ERAP1 the presentation of N-extended SIINFEKL was significantly reduced, suggesting that in HeLa cells and mouse fibroblast cells ERAP1 is the predominant ER peptide-trimming enzymatic activity [357]. When induced by IFN-γ an arginine aminopeptidase called ERAP2, homologous to ERAP1, is also produced which preferentially cleaves basic dipeptides [358].

Several peptidases can trim antigenic peptides in the cytosol. As mentioned above a leucine aminopeptidase is expressed in greater quantities when stimulated by IFN-γ. Puromycin-sensitive aminopeptidase and bleomycin hydrolase are constitutively expressed but are not induced by IFN-γ. Tripeptidyl peptidase II (TPPII), is a large multimeric complex that removes 3 residues at a time from the N-terminus of oligopeptides more than 15 residues in length [359]. Silencing of TPPII with siRNA blocks the presentation of a peptide from HIV Nef protein [360]. Further investigation is needed to establish the relative importance of

peptide trimming in the cytosol in comparison with that in the ER [295] to generate mature epitopes to be presented by different alleles of MHC class-I.

The enzyme in cell extracts that is able to cleave most antigenic peptides is the thimet endooligopeptidase [EC 3.4.24.15 or TOP] [361]. Primarily cytosolic, this enzyme may also be expressed as a membrane-associated form [362]. TOP and neurolysin activities in melanoma cells were found in the culture medium and tumor cell membrane in addition to their cytosolic expression (Paschoalin, T. and Travassos, L.R., unpublished results). Different roles have been attributed to TOP particularly in the neuropeptide metabolism [363,364].

That TOP destroys antigenic peptides and limits the extent of MHC class-I antigen presentation was shown by York et al. [365] and by Kim et al. [366]. Cells overexpressing TOP had reduced class I presentation of antigenic peptides but not of peptides generated in the ER or endosomes. On the contrary, inhibition of TOP expression by siRNA enhanced presentation of antigenic peptides. The related enzyme neurolysin (EC 3.4.24.16) is found in the cytosol and mitochondria but has a 100-fold lower affinity than TOP for the Cpp-AAF-pAb inhibitor [367]. Neurolysin may have a partial contribution to the remaining TOP-independent peptide hydrolysis in cell extracts [361]. If the degradation of longer proteasome products (15-25 residues) seems to be carried out by TPPII, that of peptides ranging from 8 to 17 residues is mainly a characteristic of TOP endoproteolytic activity. Aside from TOP and neurolysin, aminopeptidases can also substantially participate in the destruction of some antigenic peptides. Overexpression of leucine aminopeptidase reduces the supply of peptides to be presented by MHC class-I [368].

In conclusion, a kinetic competition exists between proteolytic destruction of peptides aimed at antigen presentation, proteolytic trimming, protection by chaperones and successful binding to TAP so that a small fraction of precursors or mature peptides escape destruction and become candidates for presentation by MHC-I [295].

Substrates, regulation and properties of TOP and neurolysin

The "thermolysin-like" family of Zn metalloendopeptidases belonging to Clan MA [369] hydrolyze peptide substrates of fewer than 40 amino acids. Members of the Clan participate in the metabolism of endothelial cell-derived vasoactive peptides including neutral endopeptidase (NEP, EC 3.4.24.11, neprilysin), angiotensin-converting enzyme (ACE, EC 3.4.15.1), endothelin-converting enzyme (ECE, EC 3.4.24.71), endopeptidase EC 3.4.24.15 (EP24.15, thimet oligopeptidase, TOP) and endopeptidase EC3.4.24.16 (EP24.16, neurolysin). Endopeptidases EP24.15 (TOP) and EP24.16 (neurolysin), presently classified in the metallopeptidase M3 family (MEROPS Peptidase Database) are 75- to 77 kDa enzymes containing the HEXXH motif, hydrolyze short peptides (5 to 17 amino acids) and were initially detected in rat brain homogenates [370,371]. They show 65% identity in their primary sequences [372]. They may share the same cleavage sites on bioactive and synthetic peptides but depending on the peptide sequence they sometimes have different sites of cleavage. The coordination of a Zn ion within the catalytic site of these enzymes involves the HEXXH motif, a distantly located carboxyl group and water. The histidine residues coordinate directly to Zn whereas glutamate in the HEXXH motif coordinates weakly to Zn via an activated water molecule. There is an absolute requirement for enzyme activity of both the HEXXH motif and a glutamate (Glu-502), 25 residues distant from it [373]. Both endopeptidases are primarily cytosolic but there are secreted and membrane-associated forms [362,373,374]. They may therefore hydrolyze peptides extracellularly [375]. Typically, EP24.15, with a broad distribution in brain, pituitary and gonads, cleaves neuropeptides such as neurotensin, bradykinin and gonadotropin-releasing hormone (GnRH).

Using the mouse hypothalamic neuronal GT1-7 cells line Jeske et al. [376] showed that EP24.15 occurs in lipid rafts in the plasma membrane and is localized to the exofacial leaflet of lipid rafts.

Biotinylated TOP is released into the cell medium in a constitutive-like manner.

Recombinant TOP and neurolysin activities were compared using seven series of peptides based on Abz-GFSPFRQ-EDDnp, an internally quenched fluorogenic substrate [377]. Most of the peptides were hydrolyzed at the bond corresponding to P(4)-F(5) in the reference substrate. Others were cleaved at this bond or at F(2)-S(3). The best substrates for TOP had at P(1), Phe, Ala or Arg and for neurolysin, Asn or Arg.

Fluorescent peptides derived from neurotensin (pELYENKPRRPYIL) were also synthesized and tested as substrates for neurolysin, TOP and neprilysin (NEP). The internally quenched fluorescent peptide Abz-LYENKPRRPYILQ-EDDnp, replacing pE (pyroglutamate) by Abz and adding Q-EDDnp at the C-terminus and several other shorter peptides were used as substrates of the oligopeptidases. Neurolysin and TOP cleaved the substrates at P-Y, or Y-I, or R-R bonds. NEP cleaved P-Y or Y-I bonds. Substrates Abz-NKPRRPQ-EDDnp and Abz-NKPRAPQ-EDDnp were specific for neurolysin [378]. Concentrations as low as 1 pM of neurolysin could be detected using these substrates.

The broad specificity of neurolysin has been attributed to flexible loops in the peptidase that carry the substrate binding region. A loop at 600-612 has a high average temperature factor and a high content of Gly residues. Modeling neurotensin in the active site of neurolysin showed that Tyr-606 (conserved in TOP sequence) is the probable residue reacting with the P_1 side chain of the substrate [379]. Site directed mutagenesis of Tyr-613 or Tyr-612 in neurolysin or TOP, respectively, to either Phe or Ala markedly reduced the activity of both enzymes. It seems then that both hydroxyl group and the aromatic ring at this position are important for the catalytic activity of the oligopeptidases [380].

Natural substrates were also searched for EP 24.15 and EP 24.16 using catalytically inactive forms of the enzymes without precluding their peptide binding activity. Endogenous peptides present in crude extracts from rat tissues bound to the oligopeptidases and some of them were sequenced by electrospray

ionization tandem mass spectrometry. Based on these sequences, the corresponding synthetic peptides were obtained and shown to bind to both oligopeptidases and ACE. One of these peptides, derived from the α_1 chain of hemoglobin had the sequence PVNFKFLSH, caused a dose-dependent hypotension in rats starting at 0.001 μg/kg, and was called hemopressin [381]. The relative hydrolyses of some endogenous peptides by oligopeptidases and ACE are shown in Table 2. Both neurolysin and TOP cleaved the newly identified rat brain peptides at the same sites as indicated: PVNF-K-F-LSH; VVYPW-T-Q-RY; LVVYP-W-T-Q-RY [381].

Regulation of TOP activity involves thiol activation [382]. An additional regulatory mechanism could be phosphorylation which has been shown to modulate proteasomes and caspase activities. The amino acid sequence of TOP contains PKA, CKII, and PKC consensus phosphorylation motifs [383]. The role of TOP phosphorylation on neuropeptide hydrolysis was addressed by Tullai et al. [384]. Only protein kinase A (PKA) was able to phosphorylate EP24.15 at Ser-644. A 7-fold increase in both Km and kcat for GnRH was observed upon oligopeptidase phosphorylation by PKA. In contrast, phosphorylation of peptides that are degraded by TOP, neurolysin and ACE leads to reduced cleavage suggesting a way of intracellular regulation of peptide degradation [385].

Following the phosphorylation of EP24.15 at Ser-644 there is an increased interaction with 14-3-3 epsilon, an isoform of the family of ubiquitous phosphoserine/threonine-scaffold proteins that play a role in cell signaling and are involved in exocytosis [386]. Over-expression of 14-3-3 epsilon in human embryonic kidney cells almost doubled the secretion of TOP stimulated by A23187. These data suggest that the interaction of TOP with 14-3-3 epsilon is important for the stimulated secretion of the oligopeptidase. Other examples of co-localization illustrate the potentiality of oligopeptidases in the modulation of cell signaling. Western blotting of crude subcellular fractions and lipid rafts of cultured rat trigeminal ganglia showed a similar expression of EP24.15/16 and type-2 bradykinin receptor (B2R). Treatment of primary cultures

with inhibitors of the oligopeptidases led to a 1,000-fold increase in B2R sensitivity to bradykinin, measured by inositol phosphate accumulation, as well as 31.1% potentiation of BK (bradykinin) inhibition of protein kinase B (Akt) activity [387]. EP24.15 was also co-immunoprecipitated with angiotensin II type 1 receptor (AT1) and B2R in rat kidney brush border and basolateral membranes. Furthermore, EP-24.15 associated with the receptors after their internalization suggesting a mechanism for endosomal disposition of ligand that could influence receptor recycling [388].

The modulation of BK induced effects by a number of metallopeptidases has been studied by various research groups. Bradykinin is readily degraded by ACE, NEP, aminopeptidase P, carboxypeptidases, and particularly by oligopeptidases TOP and neurolysin. Inhibitors of ACE and NEP exert their hypotensive and cardioprotective effects by enhancing BK activity. Hypotensive responses to BK are also potentiated by an inhibitor of EP24.15 and EP24.16 [389]. Recombinant EP24.15 inhibited BK-induced proliferation of cultured endothelial cells *in vitro* and melanoma growth *in vivo* probably by reducing kinin stimulated angiogenesis (Paschoalin, T. and Travassos, L.R., unpublished results). Peptide YPVEPFTE derived from casein showed a competitive inhibitory effect *in vitro* on ACE and TOP and potentiated the activity of bradykinin on isolated guinea pig ileum [390].

Norman et al. [391] compared the role of EP24.15 and 24.16 on the vasoactive peptide metabolism in endothelial cells and found that both peptidases are present in EC (transformed hybrid HUVEC cell line EA.hy926, or ovine aortic EC). EP24.16, however, was significantly more active in cytosolic and membrane preparations and intact cells than EP24.15. Neurolysin activity in VECs may, therefore, play an important role in the metabolism of BK and other circulating peptides. Recently, it was shown that bovine aortic endothelial cells submitted to cyclic strain regulates the mRNA expression of both TOP and neurolysin [392].

Table 2. Relative hydrolysis of endogenous peptides by oligopeptidases and angiotensin-converting enzyme (ACE)*

Peptides	Relative hydrolysis ratio (%)		
	EP24.15	EP24.16	ACE
Bradykinin	100	100	100
Dinorphin A_{1-13}	<0.01	<0.01	8.49
Angiotensin I	33.5	38.7	172.26
PVNFKFLSH	140	152.24	1146.19
VVYPWTQRY	10.62	1.9	138.89

*Partial data from [381]. PVNFKLSH corresponds to hemopressin.

Pz-peptidases

Pz-peptidases were named after the substrate Pz (4-phenylazobenzyloxycarbonyl)-Pro-Leu-Gly-Pro-D-Arg (Pz-PLGPR) containing the collagen sequence -Gly-Pro-Xaa-. Bacterial collagenases, many of them metalloproteases, cleave the substrate at the Leu-Gly bond (review in [393]). The Pz-peptidases are inert to collagens and similar substrates but hydrolyze collagen-derived peptides *in vivo*. Apart from Pz-peptide, Pz-peptidases can use BK and neurotensin as substrates with the following cleavage sites: RPPGF-SPFR and pELTGNKPR-RPYIL, respectively. Properties of Pz-peptidases are: (i) metallooligopeptidases; (ii) Zn-chelating HEXXH motif; (iii) hydrolyze oligopeptides but not protein substrates. They have been described in eukaryotes, found to differ from collagenases though using the same site for peptide hydrolysis, and were identified as thimet oligopeptidase EP24.15 [394]. They are similar to the bacterial Pz-peptidases except for the modulation of mammalian Pz-peptidases by protein kinase A [384].

The oligopeptidase activities in *Escherichia coli* very similar to those of mammalian TOP and neurolysin were investigated to identify the specific enzymes involved considering the translated genome of *E. coli* K12 [395]. Bacterial metalloproteases of the M3A subfamily, and particularly OpdA (oligopeptidase A) and Dcp

(dipeptidyl peptidase) were those sharing multiple similarities in their amino acid sequences with EP24.15 and EP24.16. They had a conserved Zn-binding catalytic site (HEFGH) and were inhibited by JA-2 inhibitor. Bacterial OpdA had a thimet-like oligopeptidase-like activity seen in neurotensin degradation and Dcp was the most likely enzyme degrading the C-terminal fragment of neurotensin. The recombinant EP24.15 and the bacterial enzyme could be differentiated using the Abz-GFSPFRQ-EDDnp substrate. With Gln at the C-terminus of the fluorogenic-quenched peptide the scissile bond shifts from Phe-Ser to Pro-Phe for EP24.15 and EP24.16 but not for *E. coli* recombinant OpdA that cleaves both GFSPFR and GFSPFRQ at the Phe-Ser bond [395].

PERSPECTIVES

As shown through the variety of examples presented in this chapter, the challenges of using peptidomics, proteomics, and MS in biomarker identification and peptide and protein discovery programs are limitless. Nevertheless, the most promising developments in MS were the advent of MS equipments and affiliated technologies (including bioinformatics) that allowed researchers to identify and characterize peptides and proteins in complex samples such as minute amounts of body fluids, tissues, organs or single-cells in a way that was not possible ten years ago. Many new and imaginative strategies are now available to researchers for the rapid discovery of biomarkers for various disorders. Nevertheless, while the investments in MS equipments yielded large numbers of validated biomarkers with high sensitivity and specificity, the sample collection and handling remains a source of high variability difficult to control for routine clinical applications. There is no doubt that in the coming years, in addition to being a choice procedure for peptidome and proteome characterization, MS-based-methodologies will be essential for high throughput programs to understand the physiological process, thanks to their sensitivity, resolution, direct and rapid detection of biomolecules in complex samples

The discovery of new bioactive peptides and the association with genomics, expression of recombinant molecules and chemical synthesis raise the opportunity of having a great number of molecular species in a large quantity that will demand other methodologies, such as crystallography and NMR to determine their 3D structure. The great increase in the resistance of pathogenic microorganisms to conventional antibiotics has stimulated the research development of new drugs to fight against infections. One promising antibiotic class is that of AMPs. The structure-activity relationship studies of AMPs have allowed finding molecules with high specificity for pathogens, low toxicity for eukaryotic cells and elevated serum stability. The development of new techniques for understanding the mechanism of action of AMPs is in progress. This will allow the identification of new intracellular targets of AMPs and expansion of our knowledge beyond the presently known membrane acting peptides.

Accumulating evidences suggest that AMPs, or cationic host defense peptides, may eliminate pathogens not only by direct killing, but also by interfering with the immune system of the infected host. For the few examples described recently, however, the same peptide can exert pro- or anti-inflammatory effects, depending on the host immunological environment. They may be protective or non-protective agents against infections in different experimental models. Thorough understanding of how AMPs can modulate and/or have their effects modulated by the host immune system, and how the peptide structure may influence this effect will allow the development of better and more specific immune adjuvants for induction of specific immune responses. There is an urgent demand for immune adjuvants in immunotherapy, particularly in human vaccination protocols.

In the case of antitumor peptides, it is clear that the numerous binding and internalization experiments associated with cytotoxicity of several tumor cell lines must be followed by *in vivo* experiments to select those peptides that are relevant in oncotherapy. With the advanced knowledge on the mechanisms of apoptosis of tumor cells, there is a perspective that the mechanisms of action of several

antitumor peptides be understood in great detail. The generation and metabolism of native peptides controlling angiogenesis and tumor development are essential aspects to be pursued using new methods of molecular cell biology. One should not miss the main objective of working with antitumor peptides which aims in a long term to use them as such, with adjuvants or carriers, or expressed in plasmids or viruses, for animal and human vaccination. Specific peptides inducing antitumor cellular immune response have been described but in many cases a robust immunity is not obtained due to immune tolerance. Modified and orthologous peptides have shown to effectively improve the immune response and this is a field of great progress. The use of MHC class II-restricted peptides from tumor unrelated proteins for generation of $CD4^+$ T cells may also improve the generation of stronger anti-tumor responses.

The role of oligopeptidases has been discussed in different biological systems but it is far from being completely understood. Membrane expression of protein clusters with enzymatic activity and cell-cell interactions are biological systems to be explored for a better knowledge of oligopeptidase distribution and function.

ABBREVIATIONS

AMP	=	Antimicrobial Peptide
MS	=	Mass Spectrometry
RP-HPLC	=	Reversed Phase-High Performance Liquid Chromatography
Da	=	Daltons
ESI	=	Electrospray Ionization
MALDI	=	Matrix-Assisted Laser Desorption/Ionization
TOF	=	Time-Of-Flight
GC	=	Gas Chromatography
EI	=	Electron impact
LC	=	Liquid chromatography
anHF	=	Anhydrous Hydrogen Fluoride
Imd	=	Immune Deficiency
CNS	=	Central Nervous System

2D-SDS-PAGE	=	Two-Dimensional-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
GFP	=	Green Fluorescens Protein
ELH	=	Egg-Laying Hormone
DIMs	=	*Drosophila* immune-induced molecules
NMR	=	Nuclear Magnetic Ressonance
SDS	=	Sodium Dodecyl Sulfate
CD	=	Circular Dichroism
OCD	=	Oriented Circular Dichroism
TFE	=	2,2,2-trifluoroethanol
BD	=	β-defensin
DCs	=	Dendritic cells
LPS	=	Lipopolysaccharide
CTL	=	Cytotoxic T lymphocyte
NK	=	Natural killer cells
FACS	=	Fluorescent activated cell sorting
VEGF	=	Vascular endothelial growth factor
MHC	=	Major histocompatibility complex
HLA	=	Human leukocyte antigen
EC	=	Endothelial cells
HUVEC	=	Human umbilical vein endothelial cell

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CYCLIC LIPOPEPTIDE ANTIBIOTICS

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Abstract: Numerous natural products have been discovered, mainly from microorganisms, algae and plants, that often exhibit interesting and useful biological activities. Of these, cyclic lipopeptide antibiotics form the biggest group and provide several clinically important drugs. Cyclic lipopeptide antibiotics consist of acyl side chains and peptides of various kinds, some including unusual amino acids. These characteristics confer a wide variety of biological activities and structural diversity on cyclic lipopeptide antibiotics. It is also interesting and noteworthy that the structural similarities of cyclic lipopeptides do not always reflect any similarity in their biological activities. The present review describes the chemistry, biological activities and pharmacology of natural cyclic lipopeptides.

INTRODUCTION

Many kinds of bioactive natural products have been discovered, mainly from the actinomycetes, fungi, bacteria, algae and plants. Among these, the peptide antibiotics are one of the biggest groups. The peptide antibiotics, as their name suggests, contain various kinds of amino acids, including a wide range of unusual amino acids, such as D-, *O*-methyl, *N*-methyl and β-amino acids. Many cyclic lipopeptide antibiotics, which have acyl side chains have been developed as chemotherapeutic agents, and some have been developed for agricultural and veterinary use. The cyclic lipopeptide antibiotics show weak or no sensitivity against plant or animal peptidases and often lose their biological activity when the cyclic structure is cleaved. These facts indicate that the cyclic three-dimensional structures, along with the presence of unusual amino acids, play an important role in biological activity. In cyclic lipopeptide antibiotics, the structures of the side chains greatly influence the biological activity both *in vitro* and *in vivo*. Daptomycin and micafungin are the best examples, and have been developed as antimicrobial and antifungal agents by optimization of the structure of their side chains.

Recent studies on the mechanism of biosynthesis of cyclic lipopeptide antibiotics have proved that these are systematically synthesized by the multifunctional proteins, non-ribosomal peptide synthetase (NRPS).

Cyclic lipopeptide antibiotics can be classified structurally into several groups. The enormous structural diversity in this group of antibiotics leads

to wide variation in biological activity. The present review describes the structural characteristics, chemistry, biological activity and pharmacology of natural cyclic lipopeptides.

1. LIPOPEPTIDES FROM SOIL-ASSOCIATED BACTERIA

1.1 Leucine-rich lipopeptides

Leucine-rich cyclic lipopeptides are mainly produced by gram-positive *Bacillus* spp. and gram-negative *Pseudomonas fluorescens*. Their structural similarity is not always reflected in their biological activity, and they exhibit some diversity in this regard.

Surfactin

Surfactin was isolated by Arima *et al.* (1968) as an inhibitor of fibrin clot formation, from *Bacillus subtilis*[1]. Rational drug design, based on surfactin structure, has been carried out by site-directed mutagenesis of the selectivity-conferring coding region of the NRPS adenylation domain in the surfactin biosynthesis gene cluster. A novel surfactin, containing asparagine substituted for aspartic acid has been produced[2].

Surfactin inhibits LPS signal transduction by direct interaction with LPS followed by the resulting suppression of the interaction of lipid A with an LPS-binding protein[3].

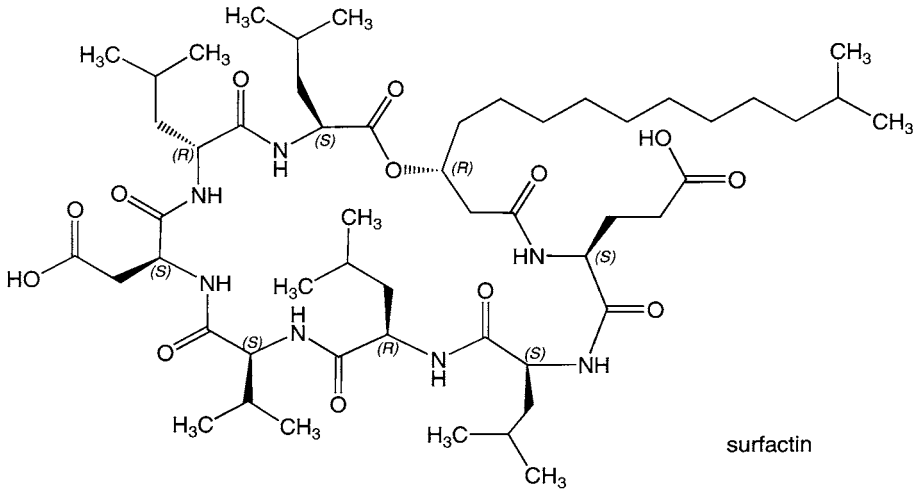


Fig. 1 Structure of surfactin.

Tensin and Amphisin

Tensin, a non-ribosomal lipoundecapeptide was discovered in 2000 by Henriksen *et al.* from *Pseudomonas fluorescens*[4]. Direct microscopic observations of its antagonistic activity against the plant pathogenic micro fungus *Rhizoctonia solani* suggest that tensin may affect the fungi in a different manner than viscosinamide[5], which has Ca^{++} channel-forming properties[6]. Amphisin was discovered by Sorensen in 2001 from *Pseudomonas* sp. DSS7, and is closely related to tensin in structure, which differs in only one constitutive amino acid residue, aspartic acid substituted for glutamic acid[7].

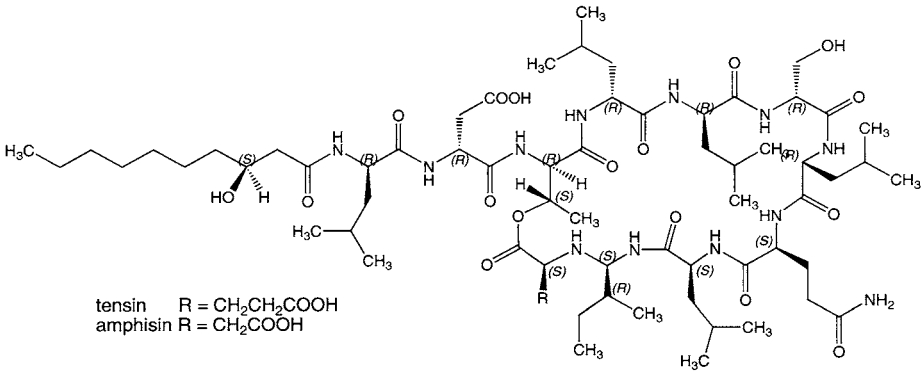


Fig. 2 Structure of tensin and amphisin

Pholipeptin

Pholipeptin was discovered by Ui *et al.* in 1997 as a novel phosphatidyl inositol specific phospholipase C (PI-PLC) inhibitor from *Pseudomonas* sp. Pholipeptin is composed of five residues of D-leucine, two residues of D-serine, D- and L-aspartic acid, L-threonine, L-isoleucine and 3-hydroxydecanoic acid. Pholipeptin inhibits PI-PLC at 7.8μg/ml (IC_{50}) in a non-competitive manner[8]. Absolute configurations of the constituent amino acids were determined by using chiral HPLC without the racemic mixture of aspartic acid. The stereo chemistry of the two aspartic acids was distinguished by biosynthetic incorporation of ^{13}C-L-aspartic acid[9].

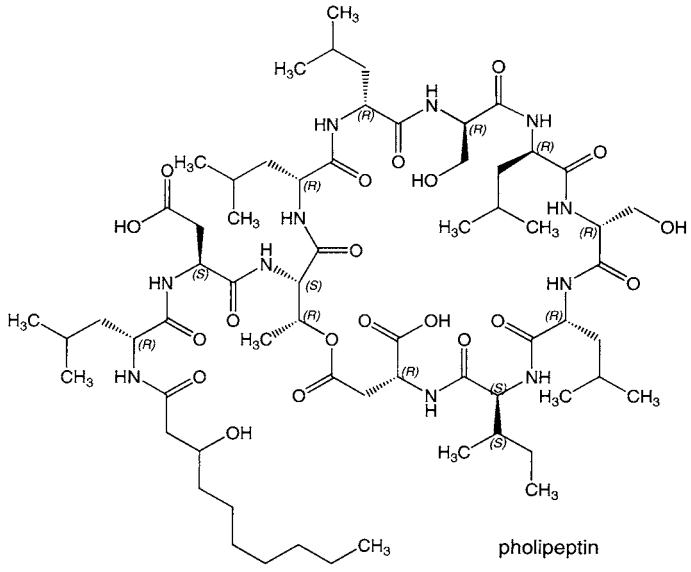


Fig. 3 Structure of pholipeptin

Halobacillins

Halobacillin was isolated by Trischman *et al.* in 1994 as a cytotoxic cyclic peptide from marine *Bacillus* sp[10]. Isohalobacillins, congeners of halobacillin, were also isolated as inhibitors of cholesteryl ester formation in macrophages, from a culture of *Bacillus* sp. A1238, by Hasumi *et al.* in 1995[11].

N-4909, identical to a subcomponent of isohalobacillin, was discovered from *Bacillus* sp. No. 4691 by Hiramoto *et al.* in 1996 as a stimulator of apolipoprotein E (apo E) secretion in human hepatoma Hep G2 cells[12].

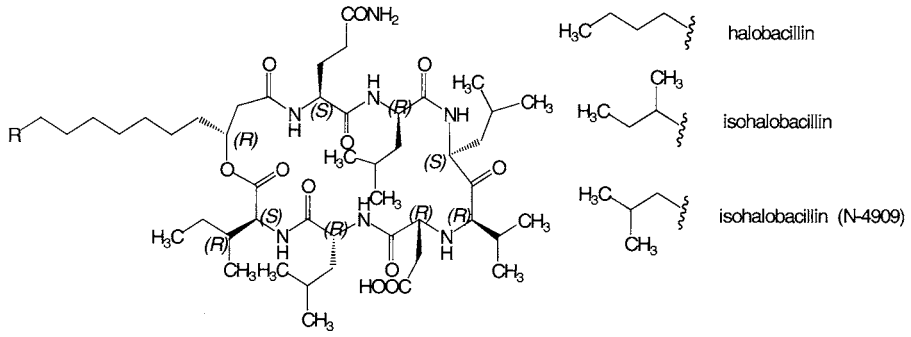


Fig. 4 Structure of halobacillins

Pumilacidins

Pumiacidins A, B, C, D, E, F and G (Fig. 5) were isolated by Naruse *et al.* in 1990 from *Bacillus pumilus*. Pumilacidins show no inhibitory activity against bacteria and fungi at 1,000μg/ml by paper-disk assay. These compounds inhibit herpes virus type1 and H^+, K^+-ATPase, and demonstrate anti-ulcer activity in rats[13].

Table 1 . Inhibitory effect of pumilacidins A and B and reference compounds on the hog gastric H^+, K^+-ATPase

compound	IC_{50} (μg/ml)
pumilacidin A	2.7
pumilacidin B	3.2
colistin	>200
amphomycin	>200
aspartocin	147
polypeptin	26.7
omeprazole	12.3
SCH 28080	11.5

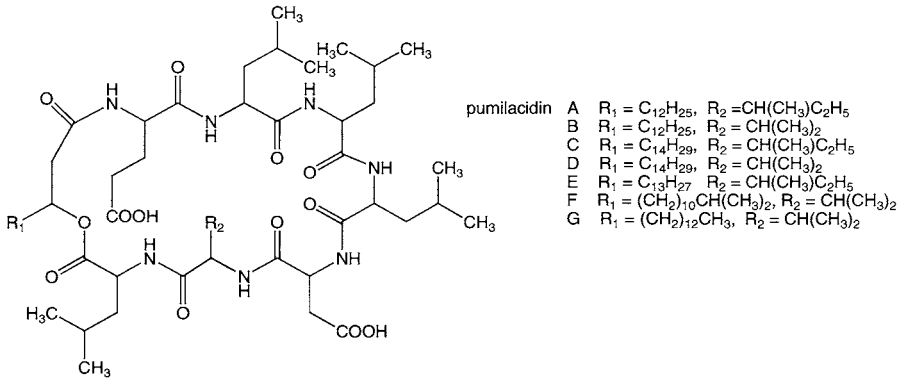


Fig. 5 Structure of pumilacidins

Viscosinamide

Viscosinamide was isolated by Nielsen *et al.* in 1998 as a novel antifungal antibiotic from the metabolites of *Pseudomonas fluorescens* DR54, and this strain was previously known to show antagonistic properties against plant pathogenic *Pythium ultimum* and *Rhizoctonia solani* both *in vitro* and *in planta*[14]. This viscosinamide-producing strain was isolated from the sugar beet rhizosphere[15].

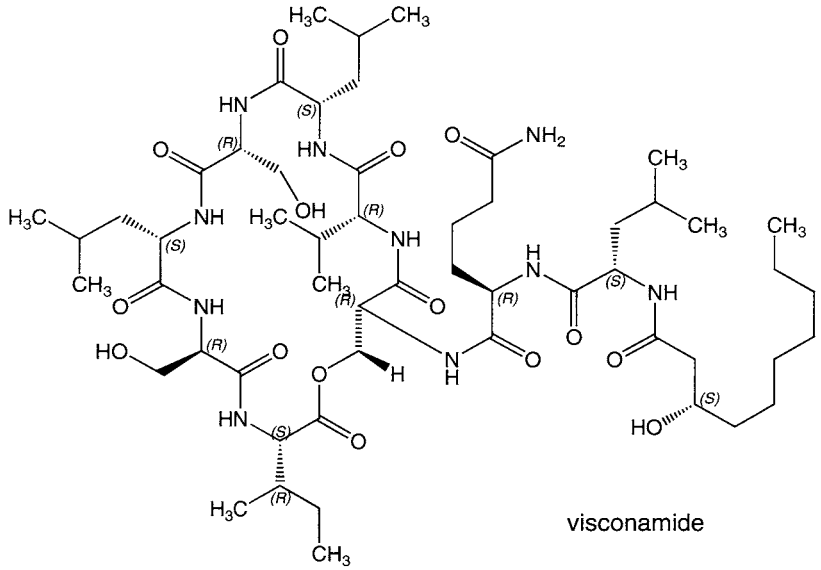


Fig. 6 Structure of viscosinamide

1.2 Asx-, Glx-rich lipopeptides

Iturins, mycosubtillins, bacillomycins and bacillopeptins

This class of antibiotics belonging to the iturin group, which includes iturins, mycosubtillins, bacillomycins and bacillopeptins is characterized by a beta-amino fatty acid moiety linked to the circular heptapeptide, and all of these are produced by *Bacillus* spp. They have a common macrocyclic structure composed of seven amino acids, Asx-Tyr-Asx-(Ser, Glx or Pro)-(Pro or Glu)-(Ser or Asn)-(Thr or Asn), in an LDDLLDL configuration sequence, and closed by a beta-amino acid linkage. Asx means asparagine or aspartic acid, and Glx means glutamine or glutamic acid. This class of antibiotics exhibits antifungal activity.

Iturins

Iturins were isolated by Delcambe in 1965 from *B. subtilis*, which exhibit strong antifungal activity, while iturin C has no antifungal activity[16,17]. Antimicrobial activity of iturin A (Fig. 7) was strongly reduced by the presence of $MgCl_2$, and the effect of this compound on the incorporation of radio active thymidine, uracil, isoleucine and alanine was non-specific. These remarks suggest that the primary target of iturin A is the cytoplasmic membrane[18].

Mycosubtillins

Mycosubtilins were isolated by Peypoux *et al.* in 1976 as antifungal metabolites from *B. subtilis*. The constituent amino acids of mycosubtilin D are the same as those of iturin A, but the peptide sequences differ[19] (Fig. 7).

Bacillomycins

Bacillomycins (Fig. 8) were discovered as antifungal compounds by Peypoux *et al.* in 1980 and Eshita *et al.* in 1995 from *B. subtilis*[20,21]. In bacillomycin L congeners, the antifungal activity increases directly with increases lipophilicity, and their antifungal activity is less potent than that of clinically available polyene antibiotics amphotericin B and nystatin[21].

Bacillopeptins

Bacillopeptins A, B and C (Fig. 8) were isolated by Kajimura *et al.* in 1995 from *B. subtilis* FR-2 obtained from the rhizosphere of garlic suffering from basal rot caused by *Fusarium oxysporum*. Bacillopeptin C, having the longest side chain among the bacillopeptins, shows both antifungal and anti-yeast activity, whereas bacillomycins A and B are inactive against gram-positive and gram-negative bacteria and fungi at 100μg/ml[22] (Table 2).

Table 2. Antifungal and anti-yeast activities of bacillopeptins A, B and C

test organism	MIC (μg/ml)		
	A	B	C
Candida albicans IFO 1594	>100	>100	50
Saccharomyces cerevisiae HUT 7099	>100	>100	25
Fusarium oxysporum HF 8801	>100	>100	25
F. oxysporum HF 8835	>100	>100	12.5
Aspergillus niger HUT 2016	>100	>100	6.25
A. oryzae IFO 4214	>100	>100	12.5
Penicillium thomii	>100	>100	12.5

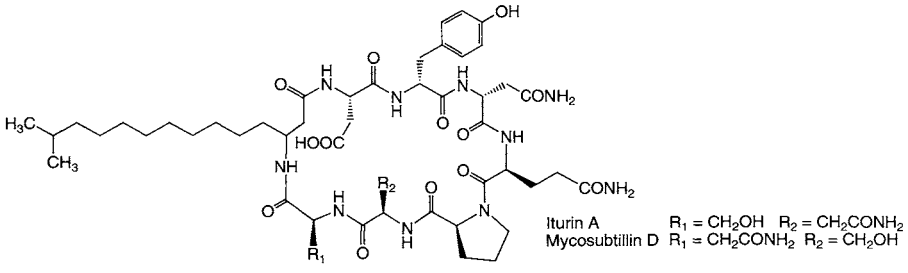
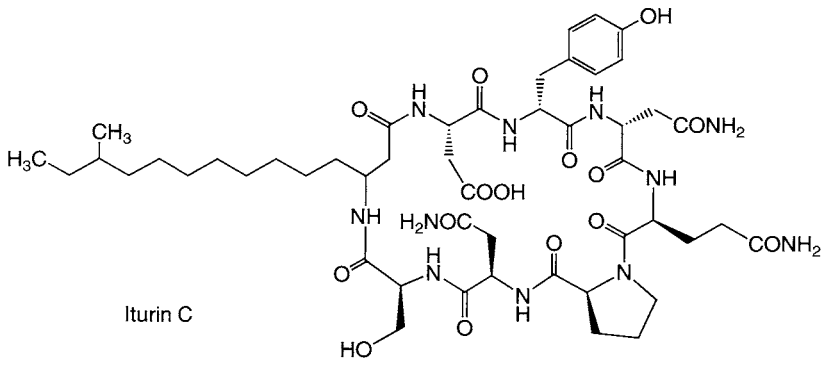
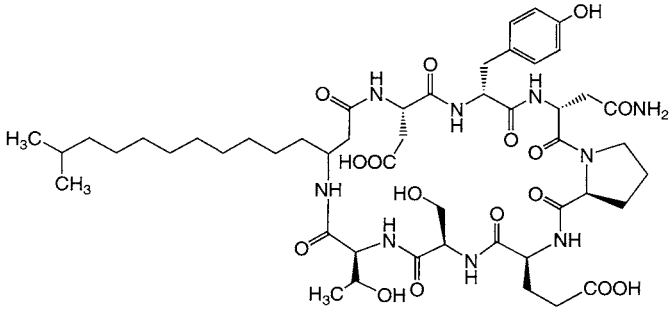
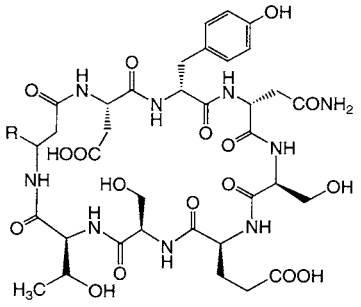


Fig. 7 Structure of iturin A and mycosubtillin D

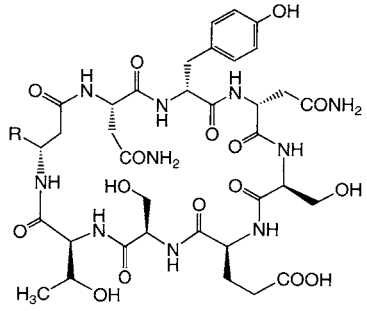






Bacillopeptins

A	R = $-CH_2(CH_2)_8CH_2CH_3$
B	R = $-CH_2(CH_2)_8CH(CH_3)_2$
C	R = $-CH_2(CH_2)_8CH_2CH(CH_3)_2$



Bacillomycin Lc

#0	R = $-(CH_2)_{10}CH_3$
#1	R = $-(CH_2)_8CH(CH_3)CH_2CH_3$
#2	R = $-(CH_2)_9CH_2CH(CH_3)_2$
#3	R = $-(CH_2)_{10}CH_2CH(CH_3)_2$
#4	R = $-(CH_2)_{14}CH_3$

Fig. 8 Structures of iturin C, bacillomycins and bacillopeptins

Plipastatins and Fengycins

Plipastatins

Plipastatins were discovered as phospholipase A_2 inhibitors from *B. cereus* BMG302-fF67 by Umezawa *et al.* in 1986. All plipastatins inhibit phospholipase A_2, phospholipase C and phospholipase D at a concentration of $1.3\text{-}3.5\text{x}10^{-6}$M. Plipastatins exhibit no antimicrobial activity even at 100μg/ml, except for activity against *Corynebacterium bovis* 1810 (MIC, 6.25μg/ml). Intraperitoneal injection of 0.1mg/mouse of plipastatin A1 or B1 slightly suppresses delayed hypersensitivity; T/C are 49 and 58%, respectively. The acute toxicities (LD_{50} values) of plipastatin A1 in mice are 250-500mg/kg (i.p.) and 200~400mg/kg (i.v.)[23,24,25]. In plipastatins, the hydroxyl group of tyrosine is involved in lactone linkage. The absolute structures of the constituent fatty acids are inferred from their optical rotations[24].

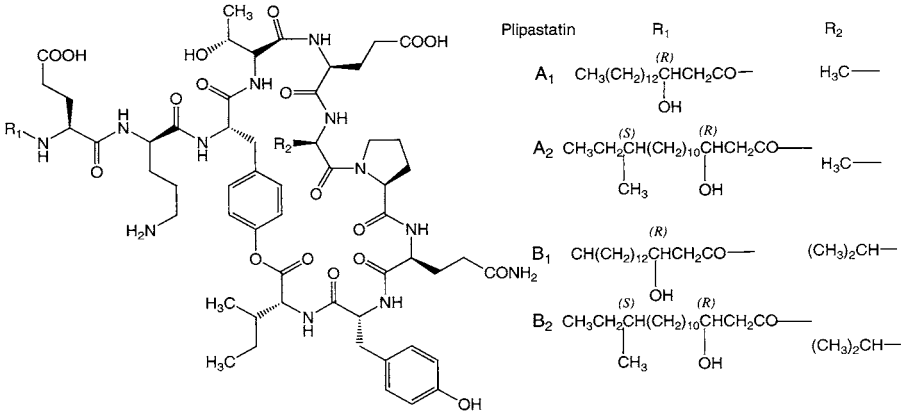


Fig. 9 Structures of plipastatins

Fengycins

Fengycins, closely related to the plipastatins in structure, were discovered by Vanttanakom *et al.* in 1986 as antifungal antibiotics from *Bacillus subtilis* strain FG-29-3. Fengycin A_1 and A_2 are composed of D-Ala, L-Ile, L-Pro, D-allo-Thr, D-Tyr, L-Tyr, D-Orn and 3 L-Glu, whereas in fengycin B_1 and B_2 the D-Ala is replaced by D-Val. Fengycin exhibits potent antifungal activity against *Pyricularia oryzae* (MIC 1.0μg/ml), *Conidiobolus coronatus* (3.16μg/ml) and *Curvularia lunata* (3.16μg/ml), while it shows no inhibition against *Aspergillus, Trichophyton* species and yeast even at 1000μg/ml. The antifungal activity of fengycin against *Paecilomyces spp.* was antagonized by the presence of cholesterol, ergosterol, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine[26].

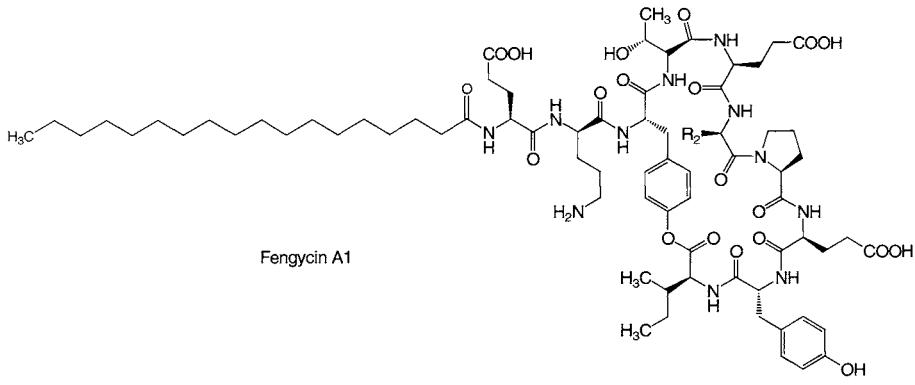


Fig. 10 Structure of fengycin A1

Lipopeptins and Neopeptins

Lipopeptins A, B and C (Fig. 11), discovered by Tsuda *et al.* in 1980 from *Streptomyces* sp. No. AC-69, show weak antifungal activities against phytopathogenic fungi, *Piricularia oryzae* and *Colletotrichum lagenarium* (MIC, 150μg/ml)[27].

Neopeptins A, B and C (Fig. 11) were discovered by Ubukata *et al.* in 1986 from *Streptomyces* sp. The structures of neopeptins are related to those of the lipopeptins, and are characterized by the existence of unusual amino acids. Every neopeptin exhibits growth inhibition against the pathogenic fungi, *Cochliobolus miyabeanus*, *Pyricularia oryzae* and *Glomerella cingulata*, and enzyme inhibition of mannnoprotein synthase (ID_{50}, 300μg/ml) and beta-1,3-glucan synthetase (ID_{50}, 150-350μg/ml) from *Saccharomyces cerevisiae*[28].

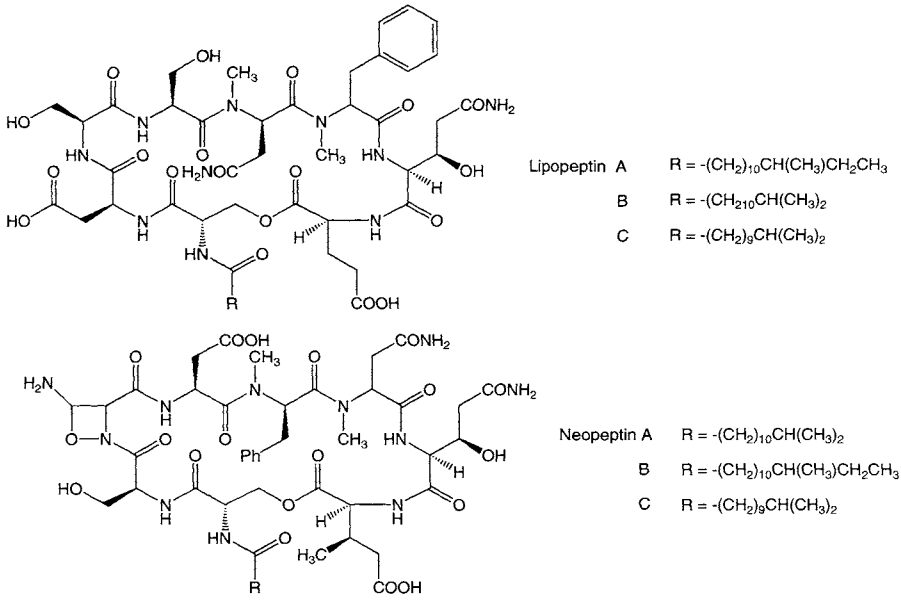
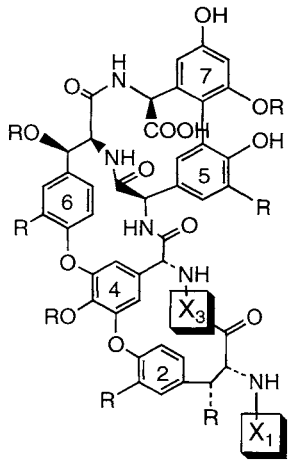


Fig. 11 Structures of lipopeptins and neopeptins

1.3 Glycopeptides

This class of antibiotics contains clinically important agents, such as vancomycin and teicoplanin. Glycopeptide antibiotics exhibit potent antimicrobial activity against gram-positive bacteria, especially for methicillin-resistant *Staphylococcus aureus* (MRSA), which is mediated via inhibition of the transglycosylation step in the later stage of peptidoglycan synthesis. Meanwhile, utilization of avoparcin, closely related to vancomycin in structure, as a veterinary growth promoter has brought about the emergence of vancomycin-resistant *Enterococcus* spp.(VRE). These compounds are produced by the Actinomycetales. Glycopeptides consist of seven amino acids, as shown below. The aromatic amino acids between numbers 2 and 4 and between numbers 4 and 6 are bound via diphenylether bonds, while the aromatic amino acid between numbers 5 and 7 is bound by s C-C bond.



group	X_1	X_3
vancomycin	Leucine	Asparagine
ristocetin	*p*-OH-Phg	*m*-diOH-Phg
avoparcin	*p*-OH-Phg	*p*-OH-Phg

p-OH-Phg: *para*-hydroxyphenylglycine,
m-diOH-Phg: meta-dihydroxyphenylglycine

Fig. 12 The common structural nucleus of glycopeptides

Vancomycin

Vancomycin, discovered by McCormick *et al.* in 1955, is one of the most important antimicrobial agent that shows potent antibacterial activity against gram-positive bacteria especially MRSA, and is produced by the soil bacterium *Amycolatopsis orientalis*[29,30,31]. Vancomycin is used clinically as the last resort for MRSA treatment. Nowadays, MRSA is commonly isolated all over the world and shows resistance to many other clinically important antibiotics[32]. Due to the long-term use of avoparcin as an animal growth enhancer, VRE have come to be frequently isolated from food animals and humans[33,34,35]. Recently, three strains of high-level vancomycin-resistant *S. aureus* (VRSA) have been clinically isolated, and all strains contain the oxacillin resistance gene *mecA* and the transferable vancomycin resistance determinant *vanA*[36,37,38]. The *vanA* determinants might have been transferred to *S. aureus* from *vanA*-containing enterococci.

Teicoplanin (teichomycin)

Teicoplanins (Fig. 14), discovered by Parenti *et al.*, show potent antibacterial activity against gram-positive bacteria especially MRSA, and are produced by the soil bacterium *Actinoplanes teichomyceticus*[39,40,41]. Teicoplanins are also used for MRSA treatment as well as vancomycin. The gene cluster encoding biosynthesis of teicoplanin was cloned from *A. teichomyceticus*, and was assigned roles in teicoplanin biosynthesis, export, resistance and regulation from the identified 49 putative ORFs within an 89 kbp genetic locus[42].

Avoparcin

In animals, antibiotics are used not only for therapy and prevention of bacterial infectious diseases, but also in animal feed to act as growth promoters. Supplementing animal feed with antimicrobial agents to enhance their growth has been a common practice, and avoparcin has been used in this way. Avoparcin is a natural glycopeptide antibiotic, structurally related to vancomycin, produced by the Actinomycetales (Fig. 13). Avoparcin is known for cross-resistance to vancomycin and teicoplanin. Acculmulating evidence indicates that the use of avoparcin in animal husbandry creates a potential resevoir of transferable *vanA*-mediated glycopeptide resistance in enterococci[33, 34, 43]. Emergence of glycopeptide-resistant bacteria is a social issue since vancomycin has been the last resort for MRSA treatment.

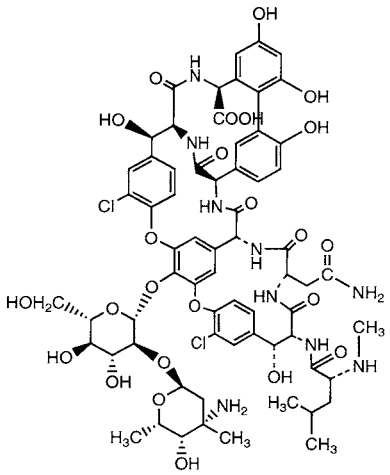
Dalbavancin, oritavancin and telavancin

Dalbavancin, oritavancin and telavancin are a new class of semi-synthetic glycopeptide antibiotics and are now under clinical trial. These antibiotics have important side chains and exhibit potent activity against gram-positive bacteria, including MRSA and VRE (Fig. 14, Tables 3,4).

Dalbavancin is the novel semi-synthetic derivative of the natural glycopeptide A-40926, closely related to teicoplanin in structure, with a long half-life and excellent antimicrobial activity[44,45,46].

Oritavancin is a semi-synthetic derivative of vancomycin, and differs from the parent compound by the presence of an additional 4-*epi*-vancosamine and a *p*-chlorodiphenyl side chain to the additional vancosamine. These modifications confer unusual pharmacodynamic and pharmacokinetic properties on oritavancin. Oritavancin exhibits rapid and concentration-dependent bactericidal activity against gram-positive bacteria in association with a prolonged postantibiotic effect[47,48].

Telavancin is a new semisynthetic glycopeptide antibiotic with rapid bactericidal activity for gram-positive bacteria, which is mediated by a pair of biochemically distinct mechanisms: inhibition of bacterial membrane phospholipids synthesis and inhibition of bacterial cell wall synthesis[49,50,51,52].



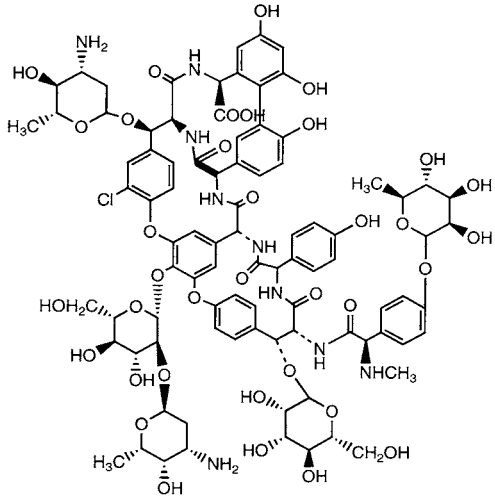
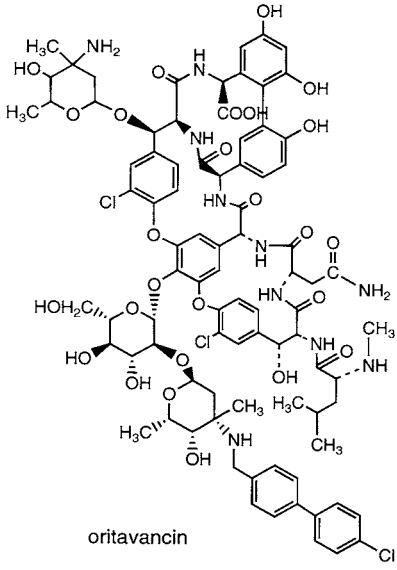
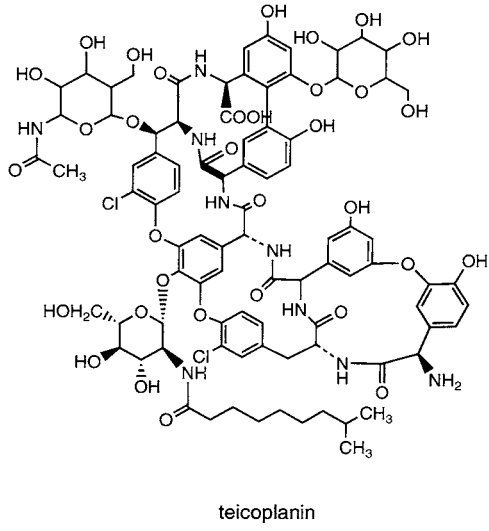


Fig. 13 Structures of vancomycin and avoparcin





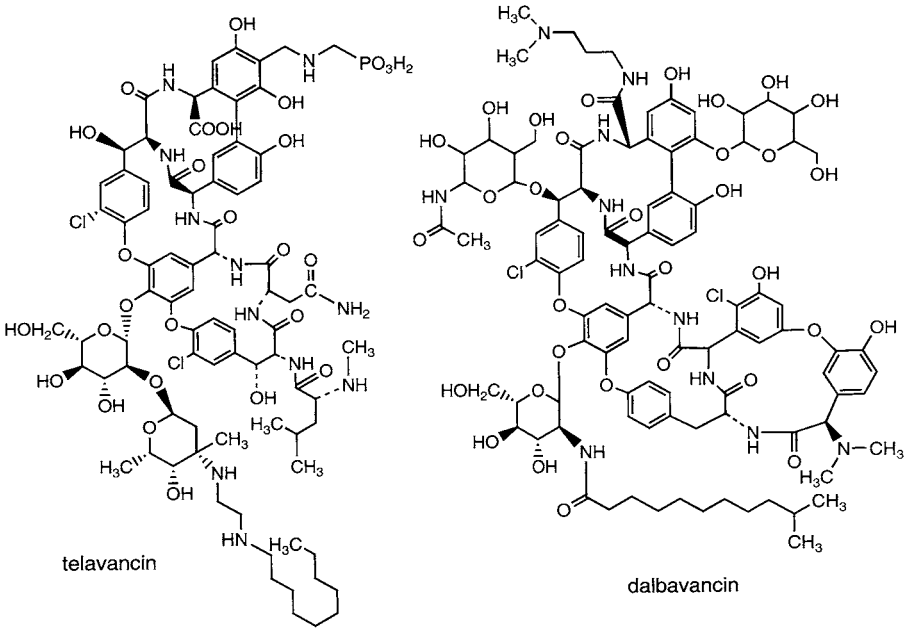


Fig. 14 Structures of oritavancin, teicoplanin, telavancin and dalbavancin

Table 3. Antimicrobial activities of telavancin

test organism	MIC (μg/ml) range			
	Telavancin	Vancomycin	Linezolid	Nafcillin
Staphylococcus aureus (MRSA) n=4	0.5~2.0	1.0~2.0	2.0~4.0	32~64
Staphylococcus aureus (MSSA) n=4	0.5~1.0	1.0	2.0~4.0	0.25
Staphylococcus epidermidis (MRSE) n=1	1.0	2.0	2.0	4.0
Staphylococcus epidermidis (MSSE) n=1	1.0	2.0	4.0	0.25
Streptococcus pneumoniae (PRSP) n=2	0.062	0.25	0.5~1.0	4.0
Streptococcus pneumoniae (PSSP) n=2	0.062	0.5	1.0~2.0	<0.031
Enterococcus spp. (VRE) n=1	4.0	>64	2	4.0

Table 4. Antimicrobial activities of dalbavancin and related compounds

test organism	MIC (μg/ml) range					
	Dalbavancin	Vancomycin	Teicoplanin	Linezolid	Oritavancin	Daptomycin
Staphylococcus aureus MSSA (n=43)	<0.015~0.125	0.5~1.0	0.125~2.0	1.0~4.0	0.25~4.0	0.125~0.5
Staphylococcus aureus MRSA (n=29)	<0.015~0.125	0.25~2.0	0.125~1.0	1.0~4.0	0.25~4.0	0.125~0.5
Coagulase-negative staphylococci methicillin-S (n=38)	<0.015~0.06	0.5~2.0	0.25~16.0	1.0~4.0	0.5~2.0	0.125~1.0
Coagulase-negative staphylococci methicillin-R (n=36)	<0.015~0.25	1.0~2.0	0.5~>16.0	1.0~2.0	1.0~4.0	0.25~1.0

1.4 Piperazic acid-containing lipopeptides

Cyclic lipopeptides containing piperazic acid show potent toxicity against tumor cells. This class of compounds shows potent antibacterial activity against gram-positive bacteria. The following five cyclic lipopeptides are all hexadepsipeptides and are produced by *Streptomyces* spp.

IC101

IC101 was discovered by Ueno *et al.* in 1993 from *Streptomyces albulus*, as an inhibitor of cell adhesion to components of extracellular matrix, fibronectin, laminin and collagen type IV(Fig. 15). IC101 shows potent cytotoxicity against tumor cells and strong antibacterial activity against gram-positive bacteria, including MRSA at concentrations of lower than 0.78μg/ml, but not against gram-negative bacteria, *Mycobacterium* spp. and fungi[53].

L-156,602 (PD-124,966)

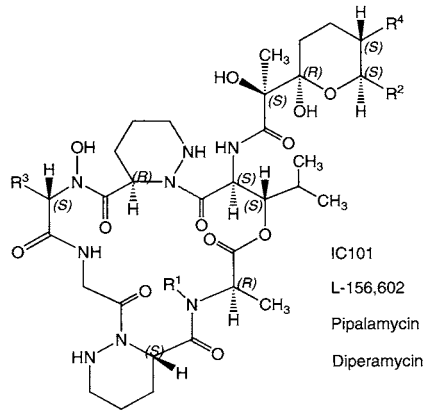
L-156,602 was discovered by Hurley *et al.* in 1986 and Hensens *et al.* in 1991 as a competitive inhibitor for binding of anaphylatoxin C5a to its receptor on human polymorphonuclear leukocytes[54] (Fig.15). L-156,602 shows strong cytotoxicity against tumor cells and antibacterial activity against gram-positive bacteria[55].

Pipalamycin

Pipalamycin was discovered by Uchihata *et al.* in 2002 as an apoptosis inducer in apoptosis-resistant human pancreatic adenocarcinoma AsPC-1 cells from *Streptomyces* sp. (Fig. 15). In this apoptotic pathway, both caspase-3 and -9 are activated. Pipalamycin shows strong cytotoxicity against tumor cells and antibacterial activity against gram-positive bacteria[56].

Diperamycin

Diperamycin was discovered by Matsumoto *et al.* in 1998 and is produced by *Streptomyces griseoaurantiacus* MK393-AF2 (Fig. 15). This compound exhibits potent antibacterial activity against gram-positive bacteria including MRSA (MIC, 0.1-0.39μg/ml), and shows the strong growth inhibitory activity against various tumor cell lines (IC_{50}, 0.009-0.098μg/ml)[57].



IC101 R^1=OH, R^2=C_2H_5, R^3=CH_3, R^4=$CH_2CH_2CH(CH_3)_2$

L-156,602 R^1=OH, R^2=CH_3 R^3=CH_3, R^4=$CH_2CH(CH_3)CH_2CH_3$

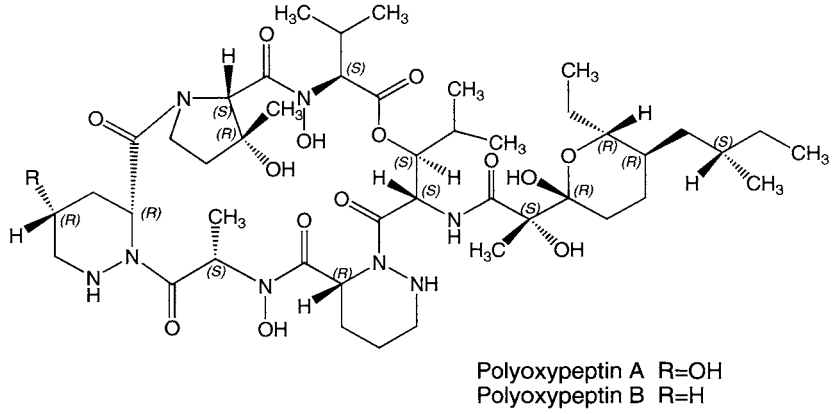
Pipalamycin R^1=H, R^2=C_2H_5, R^3=CH_3, R^4=$CH_2CH_2CH(CH_3)_2$

Diperamycin R^1=OH, R^2=CH_3, R^3=CH_2OCH_3, R^4=$CH_2CH_2CH_2CH_2CH_2CH_3$

Fig. 15 Structures of IC101, L-156,602, pipalamycin and diperamycin

Polyoxypeptins

Polyoxypeptins A and B were discovered by Umezawa *et al.* in 1998 from Streptomyces sp. and were also isolated as apoptosis inducers in apoptosis-resistant human pancreatic adenocarcinoma AsPC-1 cells[58,59]. Polyoxypeptin contains the novel amino acid, (*2S, 3R*)-3-hydroxy-3-methylproline[59].



Polyoxypeptin A R=OH
Polyoxypeptin B R=H

Fig. 16 Structure of polyoxypeptins

1.5 Calcium-dependent lipopeptides

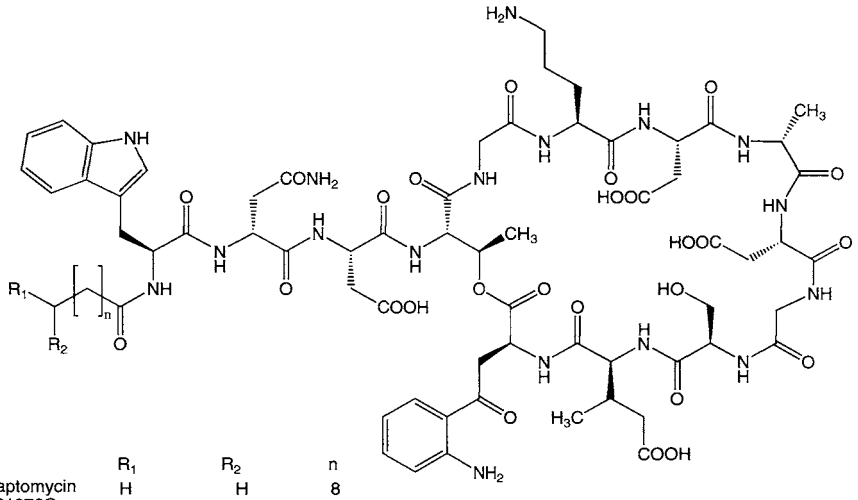
The antimicrobial activity of this class of antibiotics depends on the presence of calcium ions. This group of antibiotics contains the clinically important antimicrobial agent, daptomycin (cubicin).

Daptomycin

Daptomycin is a semi synthetic derivative of A21978Cs (LY146032), produced by the soil actinomycete *Streptomyces roseosporus,* which were discovered by Eliopoulos *et al.* in 1985, and shows more potent antimicrobial activity *in vitro* against gram-positive bacteria, including MRSA (Table 4), GISA (glycopeptide intermediate resistant-*S. aureus*) and VRE, than vancomycin, linezolid (zyvox) and quinupristin-dalfopristin (synercid)[60]. A21978Cs are the lipopeptide antibiotics containing an unusual amino acid, kynurenine and a fatty acid chain at the amino terminus of the peptide moiety. The presence of kynurenine is essential for the antimicrobial activity of daptomycin[61]. The *in vitro* antimicrobial activity of daptomycin is enhanced as the concentration of calcium ions in the test medium is increased[62,63]. Daptomycin inhibits the biosynthesis of the bacterial peptidoglycan[64] and lipoteichoic acid[65,66], and is responsible for its mode of action for membrane depolarization[67,68]. Daptomycin is now clinically available as an intravenous formulation in the USA.

Rational drug design by genetic engineering of A21978C-producing *Strep. roseosporus* has been accomplished. As a result, substantial amounts of nine daptomycin analogs, in which one or two amino acids are substitutied in their cyclic peptide nucleus, are readily obtained by fermentation, and all compounds show antibacterial activity against gram-positive pathogens[69].

A recent study shows that recombinant *Streptomyces lividans* TK23 and TK64 strains also produce the A21978C complex when a 128 kbp region of cloned *Strep. roseosporus* DNA containing the daptomycin biosynthetic gene cluster is inserted site-specifically in the varphiC31 attB site[70].



	R_1	R_2	n
Daptomycin	H	H	8
A21978C_1	C_2H_5	CH_3	6
A21978C_2	CH_3	CH_3	8
A21978C_3	C_2H_5	CH_3	8

Fig. 17 Structure of daptomycin and A21978Cs

CDAs (calcium-dependent antibiotics)

CDAs 1b, 2a, 2b, 3a, 3b, 4a and 4b are acidic lipopeptides comprising an *N*-terminal 2,3-epoxyhexanoyl fatty acid side chain and several nonproteinogenic amino acid residues, were isolated from *Streptomyces coelicolor* A3(2)[71]. The complete genome sequence of the CDA-producing *Strep. coelicolor* A3(2) has been uncovered. The total genome size of this strain is 8.667 Mbp, including more than 20 clusters coding for known or predicted secondary metabolites such as CDAs, actinorhodin, desferrioxamines, geosmin and prodiginines[72].

Deletion of a putative 4-hydroxymandelic acid synthase-encoding gene (*hma*S) located in the CDA biosynthetic gene cluster, abolished CDA production. Meanwhile, CDAs production was re-established in this Δ*hma*S mutant when 4-hydoroxymandelate, 4-hydroxyphenylglyoxylate or 5-hydroxyphenylglytcine was exogenously supplied. Feeding analogs of these precursors to the mutant resulted in the directed biosynthesis of the novel lipopeptides, CDA2d, 2fa and 2fb with modified arylglycine residues[71].

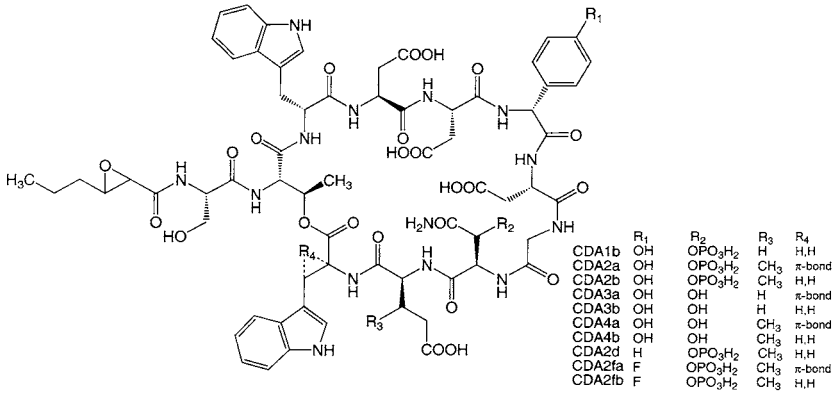


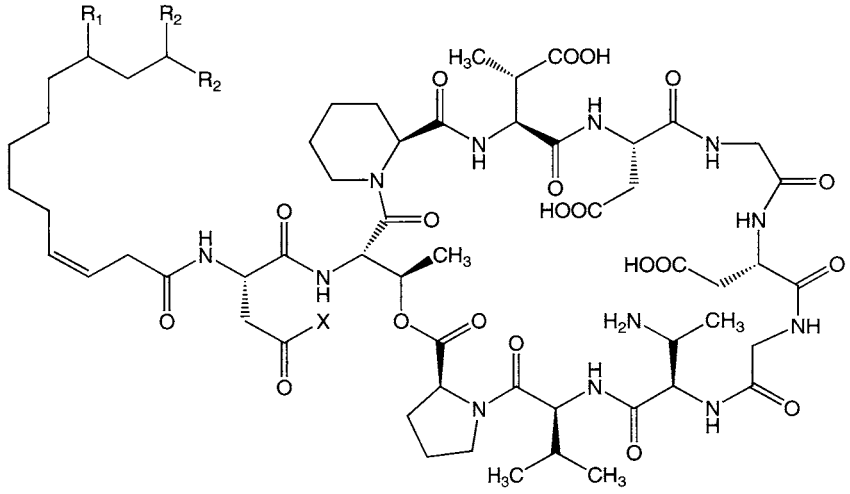
Fig. 18 Structure of CDA

Friulimicins and HMR 1043

Friulimicins A, B, C and D, discovered by Aretz *et al.* in 2000, were isolated from the rare actinomycete, *Actinoplanes friuliensis,* in the screening for new antibiotics with activity against multidrug-resistant gram-positive bacteria such as MRSA and VRE[73,74]. Friulimicins exhibit peptidoglycan synthesis activity[74]. Friulimicins contain the unusual amino acids, diamino butylic acid, pipecolinic acid and methylaspartic acid. Methylaspartic acid is normally found as an intermediate of the mesaconate pathway for (*S*)-glutamate fermentation in *Clostridium* spp.[75] and in members of the family *Enterobacteriaceae*[76]. Disruption of the glutamate mutase genes, *glmA* and *glmB* as part of the biosynthetic gene cluster of friulimicins in *Actinoplanes friuliensis* remarkably decreased the friulimicins productivity[77].

HMR 1043, discovered by the Aventis group, exhibits good antimicrobial activity against susceptible and resistant gram-positive bacteria. The activity of HMR 1043 in vitro is less influenced by the presence of calcium ions than that of daptomycin[78].

The structures and antimicrobial activity of the friulimicins and HMR 1043 are related to those of the amophomycins.

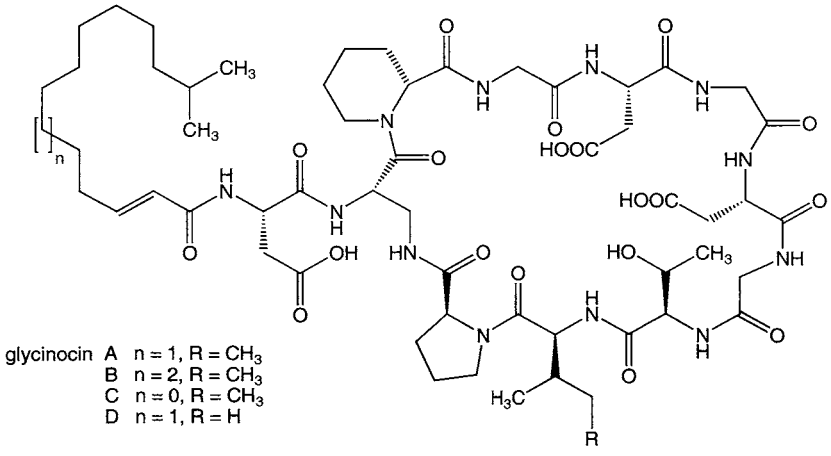


friulimicin B X = NH_2, R_1 = R_2 = H
amphomycin A-1437B X = OH, R_1= CH_3, R_2 = H
HMR 1043 X = NH_2, R_1 = H, R_2 = CH_3

Fig. 19 Structure of friulimicin B and amphomycin A-1437B and HMR1043

Glycinocins

Glycinocins A, B, C and D were discovered by F. Kong and G. T. Carter in 2003, and were isolated from the fermentation broth of an unidentified terrestrial *actinomycete* species. The absolute configurations of the amino acid residues were determined by Marfey's methodology[79].



glycinocin A n = 1, R = CH_3
B n = 2, R = CH_3
C n = 0, R = CH_3
D n = 1, R = H

Fig. 20 Structure of glycinocins

1.6 Guanidine-containing cyclic lipopeptides

Three types of guanidine-containing cyclic lipopeptides have been discovered from microbial metabolites: the first group comprises plusbacins[80,81], empedopeptin[82,83] and tripropeptins[84,85,86]; the second involves fusaricidins[87,88] and heptadepsin[89]; and the third group contains syringomycins[94,95,96].

Structural similarity among the first group is that these compounds have arginine, proline, serine, hydroxyproline and two hydroxyaspartic acids as the common amino acids in their peptide nucleus. The most prominent character of these is the presence of arginine and two hydroxyaspartic acids. Plusbacins, empedopeptin and tripropeptins were discovered as potent antimicrobial compounds especially against gram-positive bacteria, including resistant strains, and show therapeutic effects in models of staphylococcal septicemia in mice.

Plusbacins

Plusbacins were discovered by Shoji *et al.* in 1992 from the gram-negative soil bacterium *Pseudomonas* sp. Plusbacins A_1-A_4 and B_1-B_4 show inhibitory activity against gram-positive bacteria including MRSA (Table 6), and show no inhibitory activity against gram-negative bacteria such as *E. coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis* and *Serratia marcescens* even at 100μg/ml. The therapeutic effects (ED_{50}) of plusbacins A_2 and A_3 in mice infected with *S. aureus* Smith are 2.04 and 0.47 mg/kg, respectively, when plusbacins are given subcutaneously 5 hours after i.p. bacterial challenge[80,81]. Both *in vitro* and *in vivo,* the plusbacin A series antibiotics are more potent than the plusbacin B series. The former contain two hydroxyproline residues in their peptide nucleus, whereas the latter contain one proline and one hydroxyproline. The mode of action of plusbacin A_3 involves blocking transglycosylation and its foregoing steps of cell wall peptidoglycan synthesis, which is distinct from that of vancomycin[90].

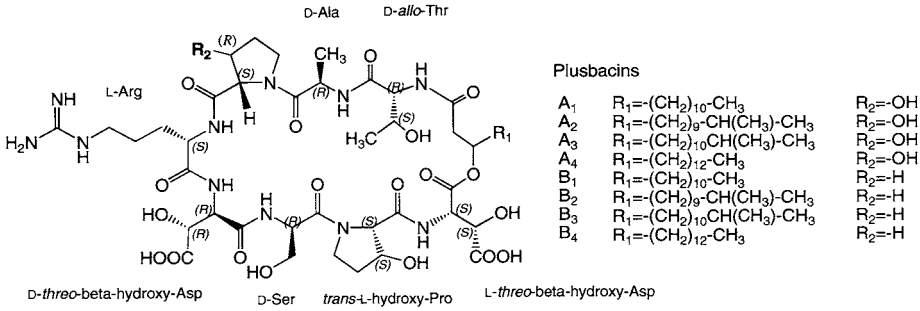


Fig .21 Structure of plusbacins

Table 6. Antimicrobial activities of plusbacins

	plusbacins MIC (μg/ml)							
test organism	A_1	A_2	A_3	A_4	B_1	B_2	B_3	B_4
Staphylococcus aureus 209P JC-1	0.05	0.1	0.1	0.1	0.1	0.1	0.2	0.4
S. aureus Smith	1.6	0.8	0.4	0.4	1.6	0.8	1.6	1.6
S. aureus SR5597(MRSA)	1.6	0.8	0.4	0.4	1.6	0.8	0.8	0.8
S. aureus SR5598(MRSA)	1.6	0.8	0.8	0.4	1.6	0.8	1.6	0.8
S. aureus SR5580(MRSA)	1.6	0.8	0.8	0.4	1.6	0.8	1.6	1.6
S. epidermidis A 14990	0.8	0.2	0.2	0.4	0.8	0.4	0.4	0.8
Enterococcus faecalis SR1001	6.3	3.1	1.6	1.6	6.3	1.6	1.6	3.1
E. faecium SR4512	6.3	3.1	1.6	1.6	12.5	3.1	3.1	6.3

Empedopeptin

Empedopeptin (Fig.22) was discovered by Konishi *et al.* in 1984 from the gram-negative soil bacterium *Empedobacter* sp. Antimicrobial activity of empedopeptin *in vivo* is similar to that of amphomycin and vancomycin in antibacterial spectra, inhibiting predominantly gram-positive aerobic and anaerobic bacteria (Table 7). *In vivo* efficacy of empedopeptin was assessed in experimental infections of mice with sensitive and resistant staphylococci, *Streptococcus pyogenes, Streptococcus pneumoniae* and *Clostridium perfringens*. Enpedopeptins showed the good therapeutic efficacy in these experiments, and they were more potent than amphomycin, but somewhat less potent than vancomycin[82,83].

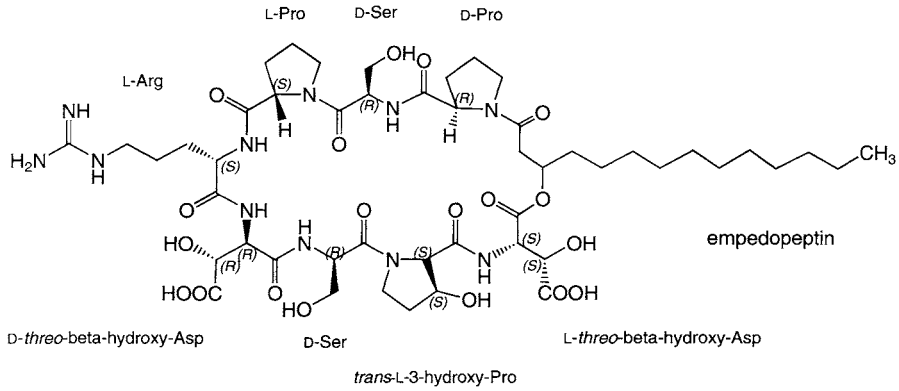


Fig. 22 Structure of empedopeptin

Tripropeptins

Tripropeptins A, B, C, D, E and Z were discovered by Hashizume *et al.* in 2001 from *Lysobacter* sp. isolated from a soil sample in Okinawa prefecture, Japan (Fig. 23). Tripropeptins are active against gram-positive bacteria including MRSA, penicillin-resistant *Streptococcus pneumoniae* (PRSP) and VRE, but are not active against gram-negative bacteria. Antimicrobial activity of tripropeptins seems to be dependent on the length of fatty acyl side chains[84,85,86]. Tripropeptins show good therapeutic efficacy in mice septicemia model. Tripropeptin C shows no subacute toxicity in mice even at 100 mg/kg for 14 days by i.v. injection[91]. The absolute structure of the prolylproline residues was determined by Marfey's amino acid analysis[92] of the compound resulting from Birch reduction[93] of tripropeptin C. The absolute structures of D- and L-hydroxyaspartic acid were also determined by the same method of analysis of the compound obtained by $LiBH_4$ reduction of tripropeptin C, which selectively reduces the hydroxyaspartic acid-forming lactone linkage.

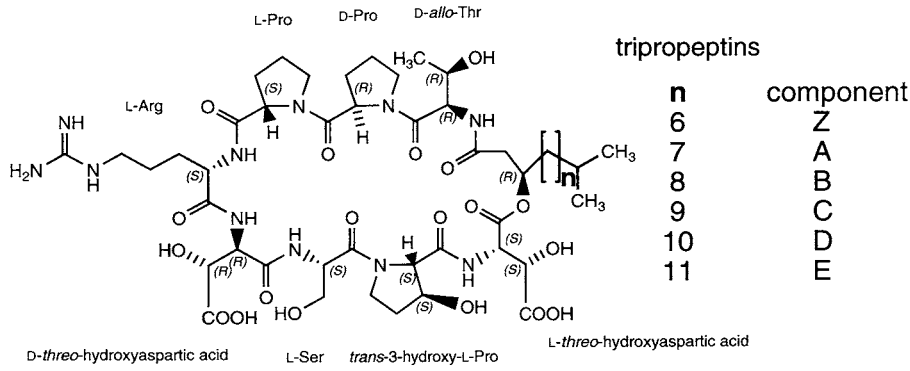


Fig. 23 Structure of tripropeptins

Table 8. Antimicrobial activities of tripropeptins and related compounds against MSSA and MRSA

test sample	MIC (μg/ml)			MIC (μg/ml)		
(Number of strains)	MSSA (10)			MRSA (10)		
	range	MIC_{50}	MIC_{90}	range	MIC_{50}	MIC_{90}
tripropeptin A	3.13~12.5	6.25	6.25	0.78~12.5	6.25	12.5
tripropeptin B	0.78~3.13	1.56	3.13	0.39~3.13	3.13	3.13
tripropeptin C	0.39~0.78	0.78	0.78	0.39~1.56	0.78	1.56
tripropeptin D	0.39~0.78	0.78	0.78	0.39~0.78	0.78	0.78
tripropeptin E	0.39~0.78	0.78	0.78	0.39~1.56	0.78	1.56
tripropeptin Z	12.5~50	50	50	3.13~100	50	100
vancomycin	0.78	0.78	0.78	0.39~1.56	1.56	1.56
teicoplanin	0.39~0.78	0.39	0.78	0.20~1.56	0.78	1.56
meropenem	0.05~0.10	0.10	0.10	6.25~50	12.5	50
levofloxacin	0.05~0.39	0.10	0.20	3.13~>100	25	>100
ofloxacin	0.20~0.78	0.39	0.39	6.25~>100	100	>100
ampicillin	0.10~3.13	0.78	3.13	6.25~50	25	50
arbekacin	0.20~0.78	0.39	0.78	0.20~0.39	0.39	0.39
erythromycin	0.10~>100	0.20	12.5	>100	>100	>100
tetracycline	0.20	0.20	0.20	1.56~50	50	50
fosfomycin	3.13~50	6.25	25	>100	>100	>100

Mueller Hinton agar (Difco), 37℃, 18hours

Fusaricidins and heptadepsin

Fusaricidins A, B, C and D were isolated by Kajimura and Kaneda in 1996 from *Bacillus polymyxa* KT-8 obtained from the rhizosphere of garlic suffering from basal rot caused by *Fusarium oxysporum*[87,88]. Fusaricidins are active against gram-positive bacteria and fungi, and their MICs were as follows: methicillin-sensitive- and -resistant-*S. aureus,* and *Micrococcus luteus* (<0.78-1.56μg/ml), *Fusarium* spp. (1.56μg/ml) and *Aspergillus* spp. (3.12μg/ml). Fusaricidin A shows medium activity against yeasts such as *Candida* spp., whereas fusaricidins C and D show no activities even at 100μg/ml against these yeasts[88].

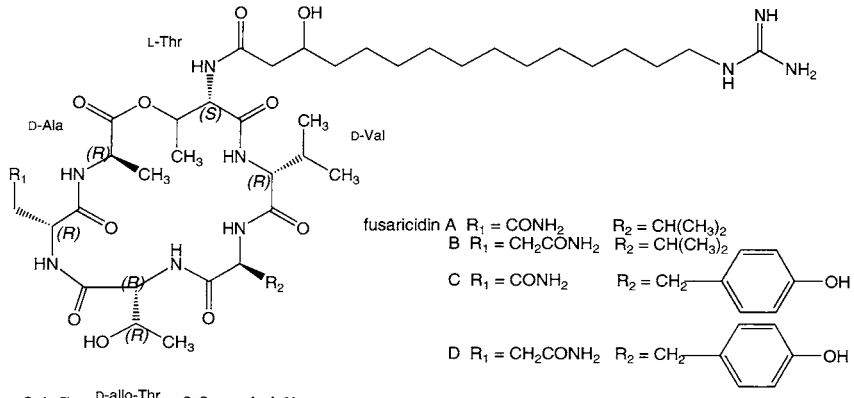


Fig. 24 Structure of fusaricidins

Heptadepsin

Heptadepsin (Fig.25) was discovered by Ohno *et al.* in 2004 from *Paenibacillus* sp. during screening for inhibition of lipopolysaccharide (LPS)-stimulated adhesioin between human umbilical vein endothelial cells and human myelocytic HL-60 cells. Heptadepsin inhibits cell adhesion induced by either LPS or lipid A by direct interaction with LPS, but does not inhibit tumor necrosis factor-α or IL-1β-induced cell adhesion[89].

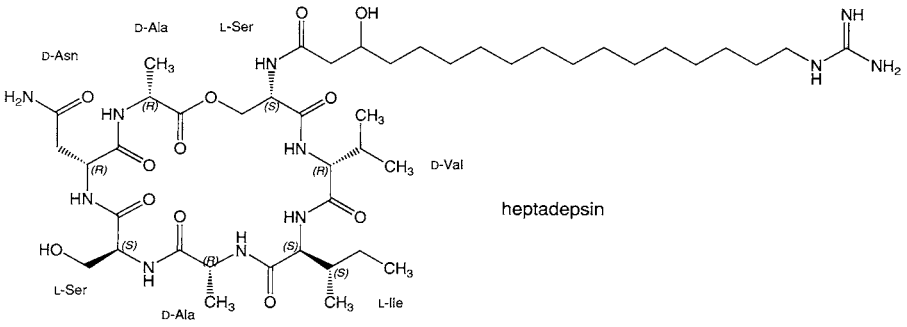


Fig. 25 Structure of heptadepsin

Syringomycins

Syringomycins are produced by *Pseudomonas syringae* pv. *syringae* as well as syringotoxins or syringostatins (Fig. 26). These *Ps. syringae*-cyclic lipodepsinonapeptides are considered putative virulence factors against plants and show antifungal activity. A study using the non-toxigenic, Tn 5-containing mutant of *Ps. syringae* indicates that

syringomycin is not essential for pathogenicity, but contributes significantly to virulence[94]. Syringomycin E and syringostatin A show similar antifungal activities (Table 9) and erythrocyte toxicities, whereas, sringotoxin B is generally less potent against fungi and less toxic to erythrocytes than syringomycin E and syringostatin A[95]. The order of binding of syringomycin E to the fungal wall constituents was as follows: beta-1,3-glucan > chitin > mannan > ergosterol = cholesterol[96].

Ps. syringae pv. *syringae* also produces syringopeptins (Fig. 27). Syringopeptins 22A and PhvA exhibit similar phytotoxic activities, measured as severity of necrotic lesions formed on tobacco leaves. The growth of *Bacillus megaterium* is inhibited by 6.2μM syringopeptin 22A and 25mM syringopeptin 22PhvA. The latter compound does not affect the growth of *Rhodotorula pilimanae* and *Geotrichum candidum,* even at 50μM, whereas the former compound inhibits the growth of these fungi at 12.5 and 25μM, respectively[97].

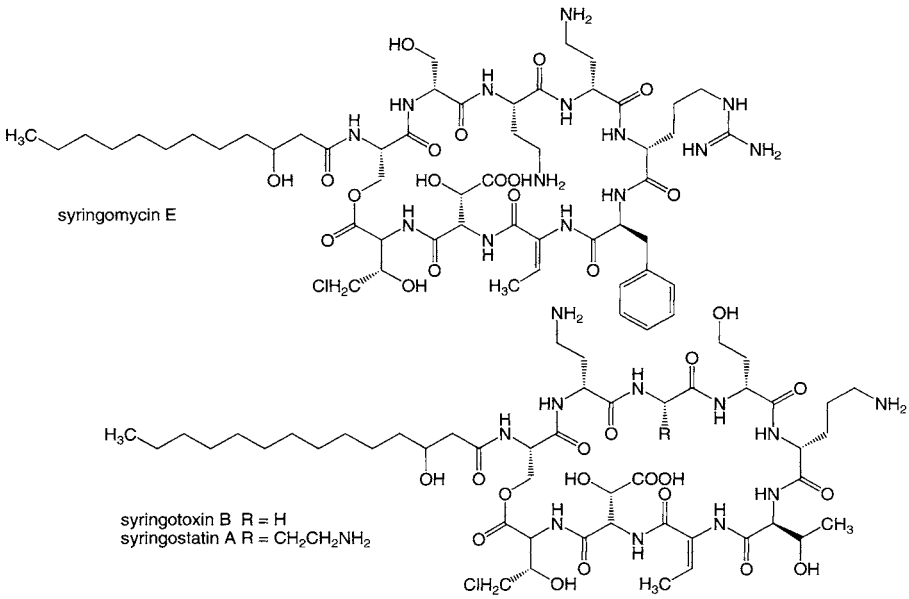
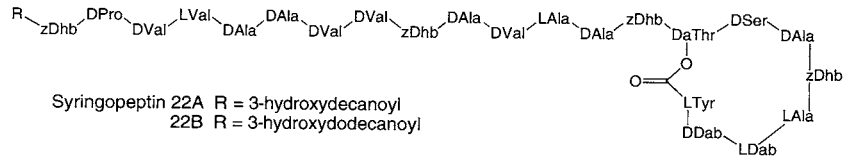


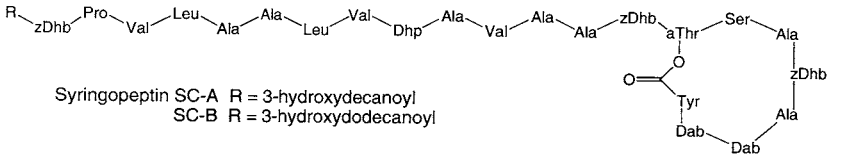
Fig. 26 Structures of syringomycin E, syringotoxin B and syringostatin A

Table 9. Antifungal activities of syringomycin E, syringotoxin B and syringostatin A

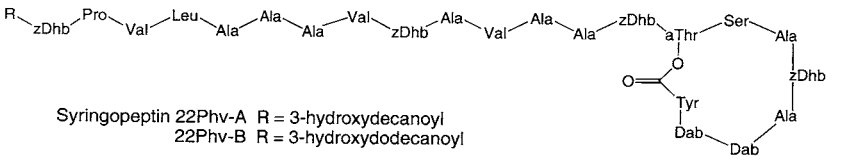
	MIC (μg/ml) range				
test organism (no. of isolates)	syringomycin E	syringotoxin B	syringostatin A	amphotericin B	ketoconazole
Candida albicans (20)	2.5-5	3.2-12.5	2.5-5	<0.04-0.3	<0.02-10
C. krusei (2)	10	12.5-25	10	0.3-0.6	0.15
C. parapsilosis (2)	2.5	6.25-12.5	2.5-5	0.6	<0.02
C. tropicalis (2)	2.5-5	3.2	2.5-5	0.3-1.25	0.08-0.6
Cryptococcus neoformans (14)	2.5-10	0.8-6.25	25.-10	0.08-1.25	0.04-0.6
Aspergillus fumigatus (16)	10-20	6.25-25	5-40	0.15-1.25	0.3->10
Mucor spp. (5)	10->40	6.25-100	10->40	<0.02-0.15	0.6->10
Trichophyton spp. (3)	3.1-6.25	25-200	2.5-5	0.3-0.6	<0.4-3.1



Syringopeptin 22A R = 3-hydroxydecanoyl
22B R = 3-hydroxydodecanoyl



Syringopeptin SC-A R = 3-hydroxydecanoyl
SC-B R = 3-hydroxydodecanoyl



Syringopeptin 22Phv-A R = 3-hydroxydecanoyl
22Phv-B R = 3-hydroxydodecanoyl

DAb, 2,4-diaminobutyric acid; Dhb, dehydrobutirine; aThr, *allo*-threonine;

Fig. 27 Structures of syringopeptins

1.7 Macrocyclic lipopeptides

Ramoplanins

Ramoplanins A_1, A_2 and A_3 (A-16686) were discovered by Cavalleri *et al.* in 1984 (Fig.28) from *Actinoplanes* sp. ATCC 33076[98]. Ramoplanins contain unusual amino acids, hydroxyphenylglycine and *N*-lipo-asparagine residues. Ramoplanins exhibit potent antimicrobial activity against gram-positive bacteria, while no activity against gram-negative bacteria, *Candida albicans*, *Trichophyton mentagrophytes*, *Mycobacterium tuberculosis* H37Rv and *Mycoplasma gallisepticum*[99]. Ramoplanin inhibits bacterial translocases during a late step of peptidoglycan biosynthesis by binding as a dimer to lipid II, the substrate for these enzymes[100,101]. Antimicrobial activity of ramoplanin together with other typical antibiotics against gram-positive cocci and intestinal anaerobic bacteria are shown in Tables 10[102,103] and 11[104], respectively. Ramoplanin is a promising clinical candidate for treatment of infectious diseases caused by gram-positive bacterial, and is now under clinical study.

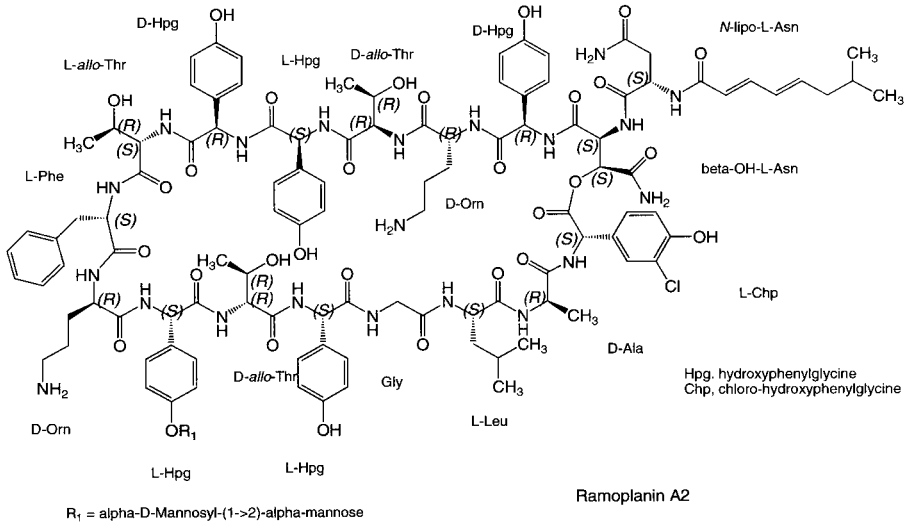


Fig. 28 Structure of ramoplanin A2

Table 11. In vitro activity of ramoplanin and typical antibiotics against gram-positive cocci

test organism (No. of strains)	MIC_{50} (μg/ml) Ramoplanin	Teicoplanin	Vancomycin	Oxacillin	Gentamicin	Ciprofloxacin
Staphylococcus aureus						
Oxacillin susceptible (92)	<0.25	0.3	0.74	0.31	9.0	0.22
Oxacillin resistant (21)	<0.25	0.46	0.97	7.4	>64	0.22
Staphylococcus epidermidis						
Oxacillin susceptible (42)	<0.25	0.82	1.4	1.4	12.8	0.12
Oxacillin resistant (40)	<0.25	0.93	1.87	>8.0	>64	2.0
Streptococcus pneumoniae						
Oxacillin susceptible (19)	<0.25	<0.25	0.43	<0.25	14.8	0.91
Enterococcus spp.						
Oxacillin resistant (21)	<0.25	<0.25	1.69	>8.0	>64	1.0
Enterococcus faecium						
vancomycin susceptible (12)	0.5	1.0	2.0	not tested	not tested	not tested
vancomycin resistant (31)	0.5	>512	128	not tested	not tested	not tested
Enterococcus faecalis						
vancomycin susceptible (12)	0.5	0.5	2.0	not tested	not tested	not tested
vancomycin resistant (15)	0.5	1	512	not tested	not tested	not tested
Leuconostoc spp. (14)	0.125	128	1024	not tested	not tested	not tested
Lactobacillus spp. (23)	0.125	128	1024	not tested	not tested	not tested

Enduracidins

Enduracidins (Fig.29) were isolated from *Streptomyces fungicidicus* No. B5477 by Higashide *et al.* in 1968[105]. Enduracidins are closely related to ramoplanins, both in structure and biological function[101].

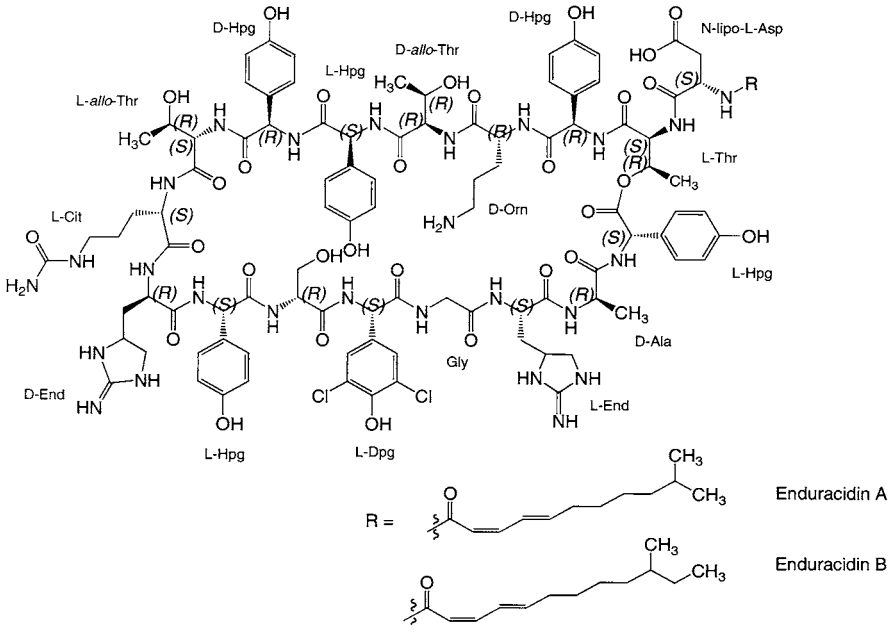
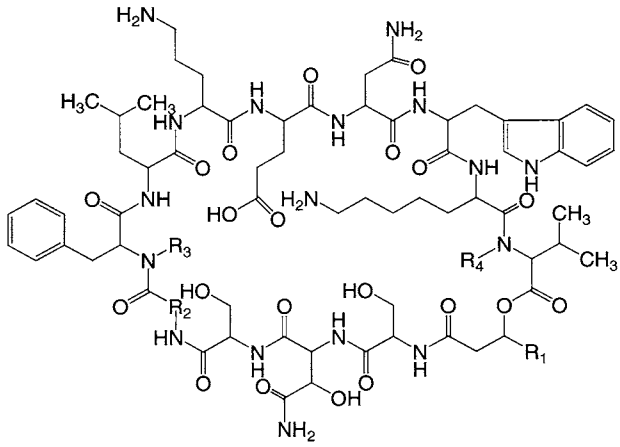


Fig. 29 Structure of enduracidins

WAP-8294A

WAP8294As were isolated from *Lysobacter* sp. by Kato et al. in 1997[106,107]. WAP-8294As exhibit potent antimicrobial activity against gram-positive bacteria, while no activity against gram-negative bacteria and fungi (Table 12)[107]. WAP-8294A2 is also a promising clinical candidate, as well as ramoplanin, for the treatment of gram-positive bacterial infections, and is now under phase I study.



	R_1	R_2	R_3	R_4
WAP-8294A1	$(CH_2)_4CH_3$	CH_2	CH_3	CH_3
WAP-8294A2	$(CH_2)_3CH(CH_3)_2$	CH_2	CH_3	CH_3
WAP-8294A4	$(CH_2)_4CH(CH_3)_2$	CH_2	CH_3	CH_3
WAP-8294Ax8	$(CH_2)_3CH(CH_3)_2$	CH_2	CH_3	H
WAP-8294Ax9	$(CH_2)_3CH(CH_3)_2$	CH_2	H	CH_3
WAP-8294Ax13	$(CH_2)_3CH(CH_3)_2$	$(CH_2)_2$	CH_3	CH_3

Fig. 30 Structure of WAP-8294As

Table 12. Antimicrobial activities of WAP8294As

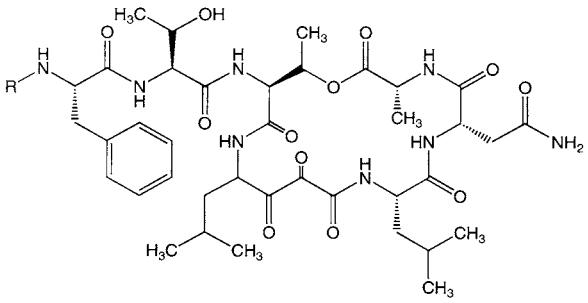
	MIC (μg/ml)					
test organism	A1	A2	A4	Ax8	Ax9	Ax13
Staphylococcus aureus JCM8702 (MRSA)	0.39	0.78	0.78	n.t.	n.t.	n.t.
S. aureus No. 1 (MRSA)	0.39	0.78	0.78	3.13	3.13	3.13
S. aureus No.11 (MRSA)	0.39	0.78	0.78	n.t.	n.t.	n.t.
S. aureus ATCC 25923	0.39	0.78	0.78	3.13	3.13	3.13
S. epidermidis ATCC 12228	0.39	0.78	0.78	1.56	1.56	1.56
Bacillus subtilis ATCC6633	0.78	0.78	0.78	1.56	3.13	3.13
Enterococcus faecium CIP103510 (VRE)	n.t.	6.25	n.t.	n.t.	n.t.	n.t.
Streptococcus pyogenes ATCC19615	6.25	6.25	6.25	>100	>100	>100
Escherichia coli ATCC25922	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa ATCC9027	>100	>100	>100	>100	>100	>100
Candida albicans TIMM 0239	>100	>100	>100	>100	>100	>100
Aspergillus fumigatus IAM2004	>100	>100	>100	>100	>100	>100

1.8 Other lipopeptides

In this section, cyclic lipopeptides not belonging to any of the previous sections will be described.

YM-47141 and YM-47142

YM-47141 and YM-47142 (Fig. 31) were discovered by Orita *et al.* in 1995, as elastase inhibitors from a gram-negative bacterium *Flexibacter* sp. Q17897. YM-47141 and YM-47142 exhibit inhibition of human leukocyte elastase (HLE), and their IC_{50} values are $1.5x10^{-7}$ and $3.0x10^{-7}$ M, respectively. YM-47141 also affects human leukocyte cathepsin G and bovine pancreatic α-chymotrypsin at somewhat higher doses than those active against HLE. On the other hand, YM-47141 does not inhibit the plasmin, bovine thrombin, bovine trypsin and human kallikrein (IC_{50} >50 M)[108]. YM-47141 and YM-47142 are the first bacterial metabolites known to contain 2,3-dioxo-4-amino-6-methyl-heptanoic acid[109].



	R
YM-47141	$COCH_2Ph$
YM-47142	$COCH_2CH(CH_3)_2$

Fig. 31 Structure of YM-47141 and YM-47142

Enopeptins

Enopeptins A and B (Fig. 32), with anti-bacteriophage activity were isolated from *Streptomyces* sp. RK-1051 by Osada *et al.* in 1991. This producing organism was isolated from a soil sample collected in Tsuruoka City, Yamagata Prefecture, Japan. Enopeptin A inhibits plaque formation of bacteriophage B at a concentration of 5μg/disk, and shows antibacterial activity against *Staphylococcus* spp. and *Enterococcus faecalis* at 12.5-25μg/ml (MIC), but not against gram-negative bacteria and fungi even at 200μg/ml[110].

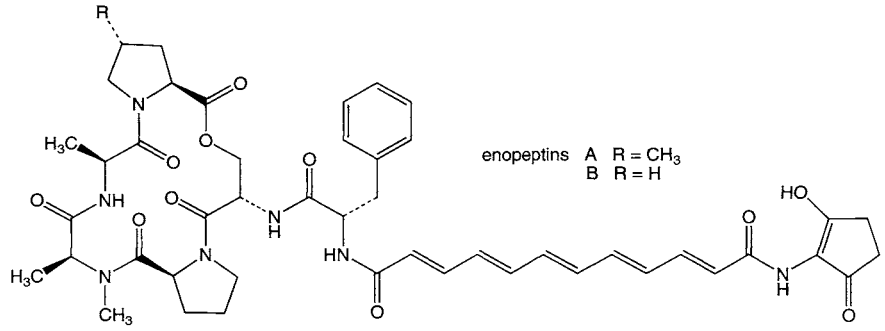


Fig. 32 Structure of enopeptins

Trapoxins and enamidonin

Trapoxins were discovered by Itazaki *et al.* in 1990, from the fungas *Helicoma ambiens* RF-1023. These compounds exhibit detransformation activity against *v-sis* oncogene-transformed NIH3T3 cells. Trapoxin A has no effect on human red blood cells, even at a concentration of 250μg/ml and incubation at 37 °C for 2 days[111]. Trapoxins are known to inhibit histone deacetylase (HDAC1) activity[112] and trapoxin B has no effect on the stability of HDAC6-PP1 complexes or on tubulin acetylation[113].

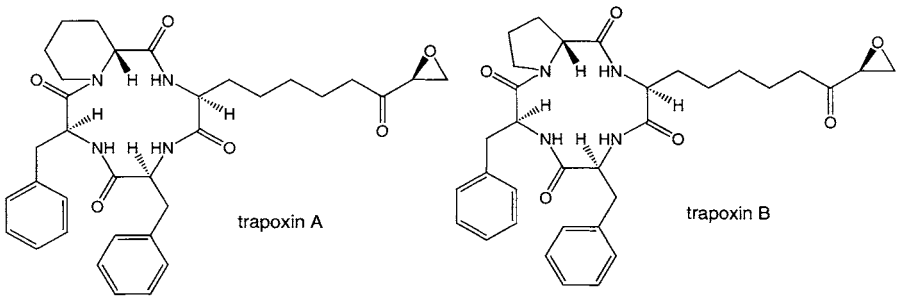


Fig. 33 Structures of trapoxins A and B

Enamidonin

Enamidonin was discovered by Koshino *et al.* in 1995, from *Streptomyces* sp. 91-75 during screening for inhibitors of the mitogenic activity of epidermal growth factor. Enamidonin inhibits epidermal growth factor-dependent uptake of ^{3}H-thymidine into Balb/MK cells with an IC_{50} of 10μg/ml. This compound reverses the transformed morphology of src-ts-NRK to the normal flat morphology with an ED_{50} of 10μg/ml.

Enamidonin shows neither antifungal nor antimicrobial activities in a conventional paper disk-agar method at a concentration of 4μg/disk[114].

Antibacterial or antifungal activities of trapoxins and enamidonin was not reported.

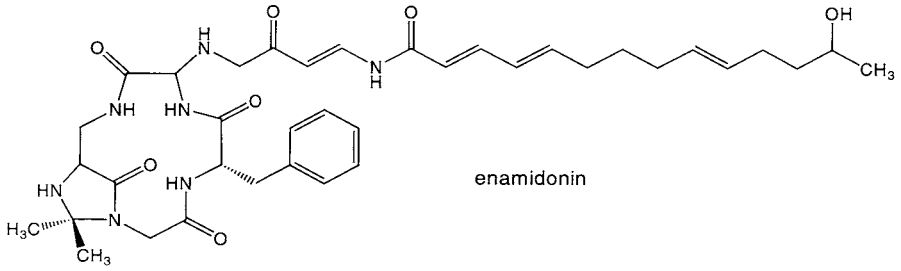


Fig. 34 Structure of enamidonin

Thioviridamide

Thioviridamide was discovered by Hayakawa *et al.* in 2006, from *Streptomyces olivoviridis* during screening for antitumor antibiotics using 3Y1 rat fibroblasts transformed with adenovirus oncogenes. Thioviridamide exhibits selective and potent growth inhibitory activity on Ad12-3Y1 (IC_{50}, 3.9ng/ml) and E1A-3Y1 (IC_{50}, 32ng/ml) cells, both of which contain the adenovirus E1A oncogene[115]. Thioviridamide has five thioamide bonds and two novel amino acids, β-hydroxy-N^1,N^3-dimethylhistidinium and *S*-(2-aminovinyl)cysteine[116].

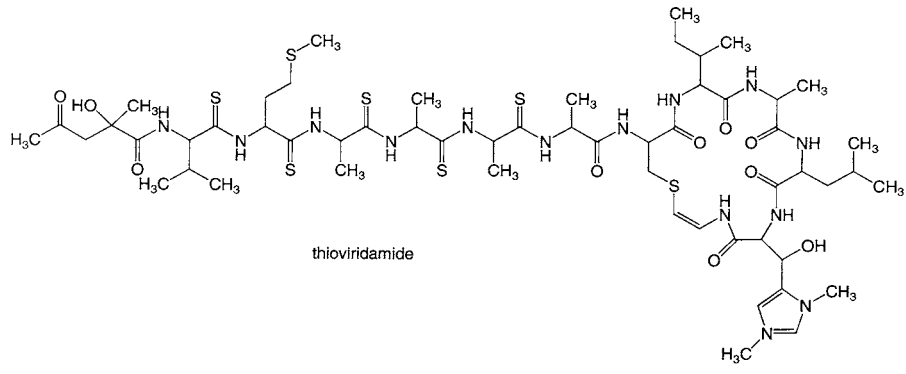


Fig. 35 Structure of thioviridamide

2. CYCLIC LIPOPEPTIDES FROM FUNGAL METABOLITES

2.1 Echinocandin lipopeptides

Echinocandin-type natural products are well known as potent antifungal agents, and include echinocandins, FR901379 and WF11899A, B and C. At present, semi-synthetic echinocandins, such as caspofungin and micafungin are clinically available for the treatment of opportunistic fungal infections, and anidulafungin is also now undergoing phase III study. The antibiotics belonging to this group have common structural motifs such as (2*S*, 3*S*, 4*S*)-4-methyl-3-hydroxy-L-proline, 4-hydroxy-L-proline and 1-(*p*-hydroxyphenyl)-3-amino-propane-2-ene, and inhibit the 1,3-β-D-glucan biosynthetic enzyme in *C. albicans* and *Aspergillus fumigatus* in a non-competitive manner. Therefore, they have potent antifungal activity against *Candida* and *Aspergillus* spp.

Fungal cell walls are composed of three major components, 1,3-β-D-glucan, chitin and mannan, and recent research has revealed that 1,3-β-D-glucan and chitin are essential for fungal survival. Glucan exists in fungal cell walls, but not in mammalian cells, therefore, the echinocandin class of antibiotics are selective antifungal agents.

Echinocandins

Echinocandins were discovered by Benz *et al.* in 1974, from *Aspergillus nidulans var. echinonulatus* and show highly specific anti-yeast antivity[117]. Echinocandins are hardly soluble to water. Echinocandin B (Fig. 36), the major component, inhibits 1,3-β-D-glucan synthase[118].

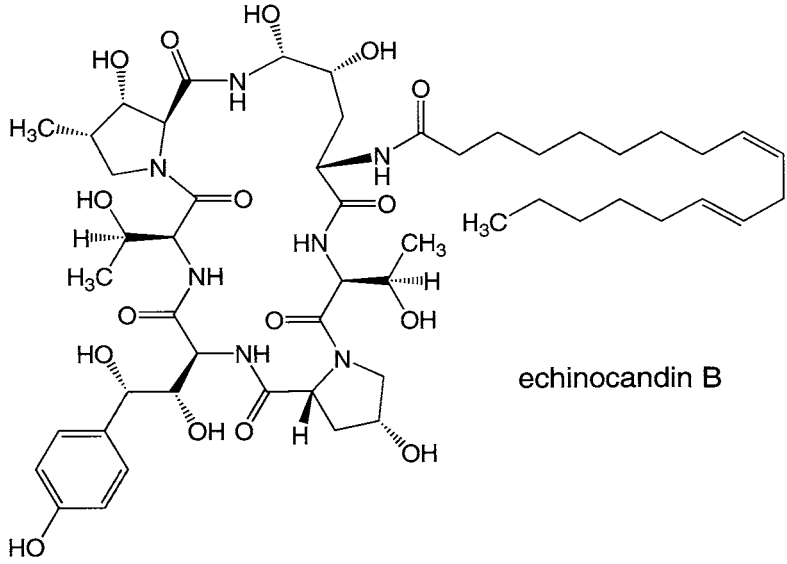


Fig. 36 Structure of echinocandin B

WF11899A, B and C

WF11899A, B and C (Fig.37) were discovered by Iwamoto *et al.* in 1994 as water-soluble antifungal compounds from *Coleophoma empetri* F-11899. These compounds contain a sulfate residue in their structure, while other natural-echinocandins do not. Replacement of the sulfate residue in WF11899A by an arylsulfate greatly diminishes its water solubility, indicating that its excellent water solubility is attributed to the sulfate residue[119].

No lethal toxicity was observed at a dose of 500mg/kg when WF11899A was injected intraperitoneally to 4-week-old ICR mice[120]. In a model of murine systemic infection with *C. albicans*, these compounds significantly prolong the survival of infected mice, and their protective effects are superior to that of echinocandin B, and they are equally as active as fluconazole. WF11899A, B and C have eight fold less hemolytic activity than amphotericin B, but almost the same as that of echinocandin B[121]. However, cilofungin (LY121019, N-*p*-octyloxybenzoylechinocandin B) [122] is not hemolytic[121].

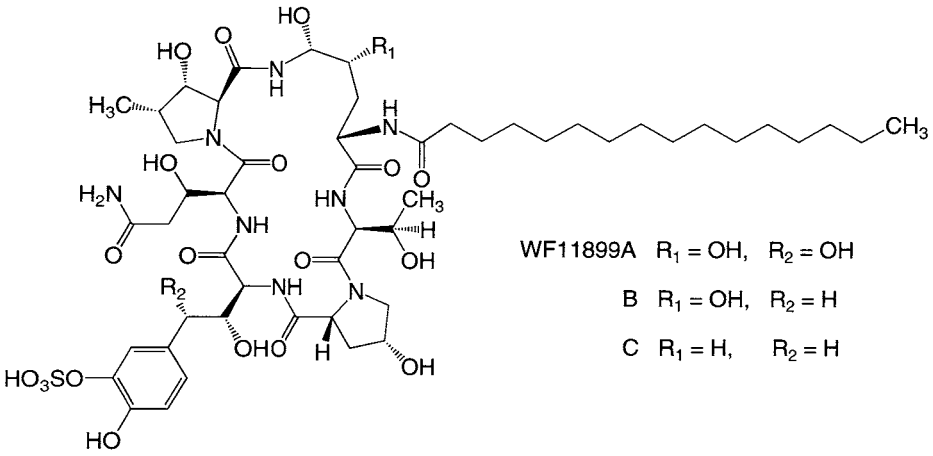


Fig. 37 Structure of WF11899A, B and C

Anidulafungin (LY-303366)

Anidulafungin is a semi synthetic echinocandin class of antibiotics and possesses potent antifungal activity. This compound shows more potent antifungal activity than amphotericin B and itraconazole against *Aspergillus spp. in vitro*[123]. Anidulafungin and itraconazole or voriconazole show a synergistic antifungal activity, while anidulafungin and amphotericin B show low *in vitro* activity against *Aspergillus spp.* and *Fusarium spp.*[124]. Anidulafungin penetrates the esophagus and salivary

glands, and exhibits dose-dependent antifungal efficacy against experimental fluconazole-resistant oropharyngeal and esophageal candidiasis in immunocompromised rabbits[125].

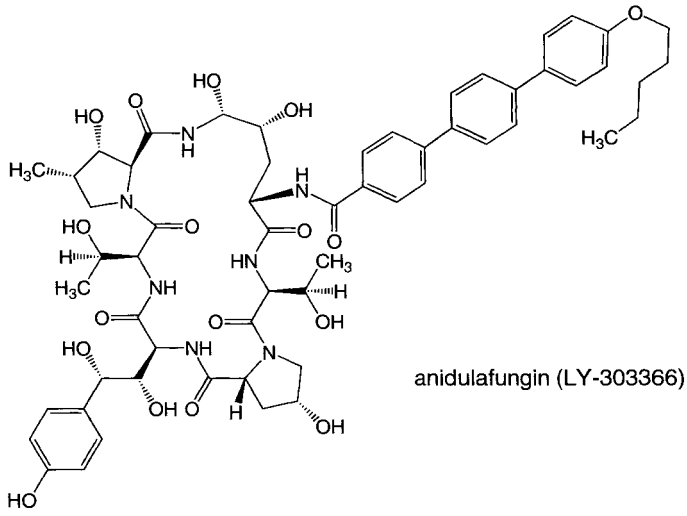


Fig. 38 Structure of anidulafungin (LY-303366)

Micafungin (FK463)

Micafungin (Fig. 39) was obtained from WF11899A by replacement of the side chain with a lipophilic one[119]. The key intermediate of the acyl side chain, methyl 4-(5-(4-pentyl-oxyphenyl)isoxazol-3-yl)benzoate was prepared by regioselective 1,3-dipolar cycloaddition of 4-methylcarbonylbenzhydroxamic acid chloride to 4-pentyloxyphenylacetylene[126]. Micafungin is water-soluble and less hemolytic than the semisynthetic echinocandin class of antibiotics, and was launched as antifungal agent in 2004. Micafungin shows excellent antifungal activity both *in vitro* and *in vivo* against *Aspergillus* spp. and *Candida* spp. but not against *Cryptococcus neoformans* (Table 13).

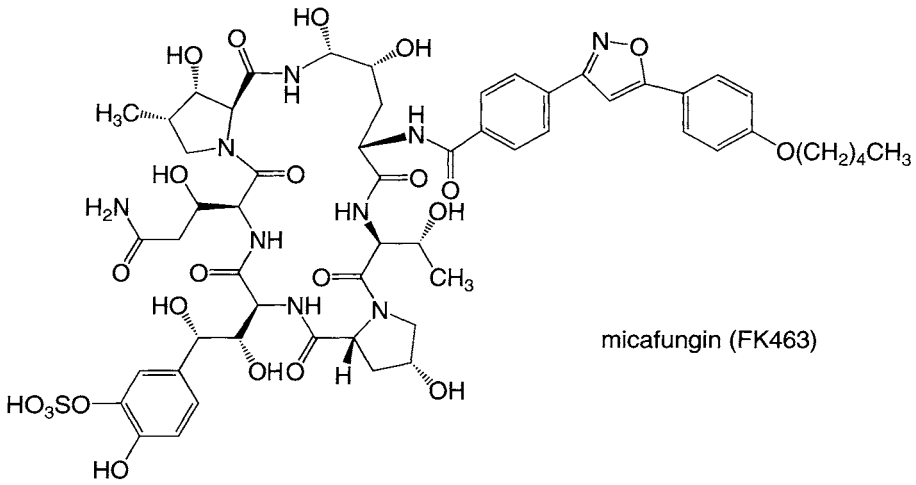


Fig. 39 Structure of micafungin (FK463)

Table 13. MIC_{90} (μg/ml)of micafungin against clinical isolates of fungi

test organism (No. of strains)	micafungin	fluconazole	itaconazole	amphotericin B
Candida albicans (37)	0.0156	0.5	0.0313	0.5
C. albicans flucobnazole-R	0.0313	>64	>8	0.5
C. tropicalis (20)	0.0313	8	0.5	0.125
C. glabrata (20)	0.0156	64	8	1
C. krusei (11)	0.125	64	1	1
C. parasilosis (17)	1	1	0.5	0.5
Cryptococcus neoformans (5)	>64	8	0.5	0.5
Aspergillus fumigatus (29)	0.0156	>64	0.5	1
A. niger (15)	0.0078	>64	1	0.5

FR209602, FR209603 and FR209604

FR209602, FR209603 and FR209604 (Fig. 40) were discovered by Kanasaki *et al.* in 2006 from a fungal strain *Coleophoma crateriformis* No. 738. These compounds are closely related to FR901379 and WF11899A, and contain a sulfate residue in the cyclic peptide portion[127]. These compounds exhibit excellent antifungal activity against *C. albicans* and *A. fumigatus* in the range of 0.02-0.04μg/ml, which is attributed to inhibition of beta-1,3-glucan synthase activity. These antifungal activity and β-1,3-D-glucan synthase inhibitory activities are more potent than those of FR901379. FR209602 and FR209603 show good therapeutic efficacy against *C. albicans* in a model of murine systemic infection, with ED_{50} values of 2.0 and 1.9mg/kg, respectively, which are somewhat less potent than that of FR901379 (ED_{50}, 0.9-1.2mg/kg)[128].

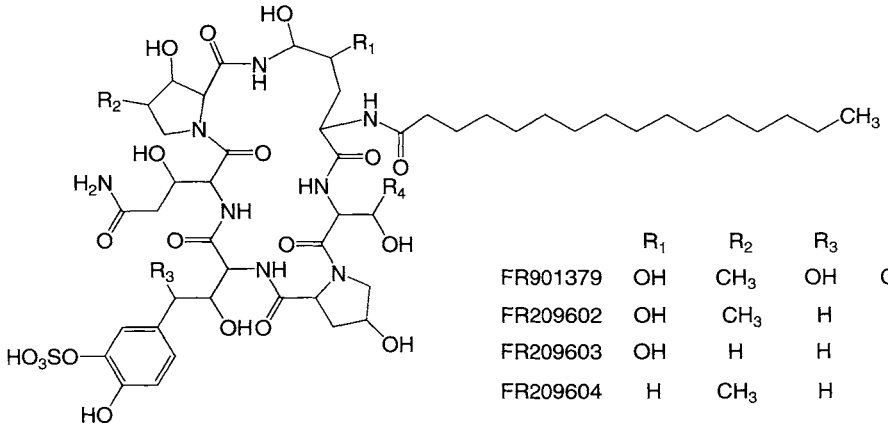


Fig. 40 Structure of FR901379, FR209602, FR902603 and FR902604

FR220897 and FR220899

FR220897 and FR220899 (Fig. 41) were discovered by Kanasaki *et al.* in 2006, from *Coleophoma empetri* No. 14573. In FR220897 and FR220899, the sulfate residue presents at a different position in the aryl ring from that in FR901379 and FR209602-04, mentioned above. FR220897 and FR220899 exhibit 1,3-β-D-glucan synthase inhibitory activity and therapeutic efficacy in a model of murine infection with *C. albicans*, and these effects are less potent than those of FR901379[129].

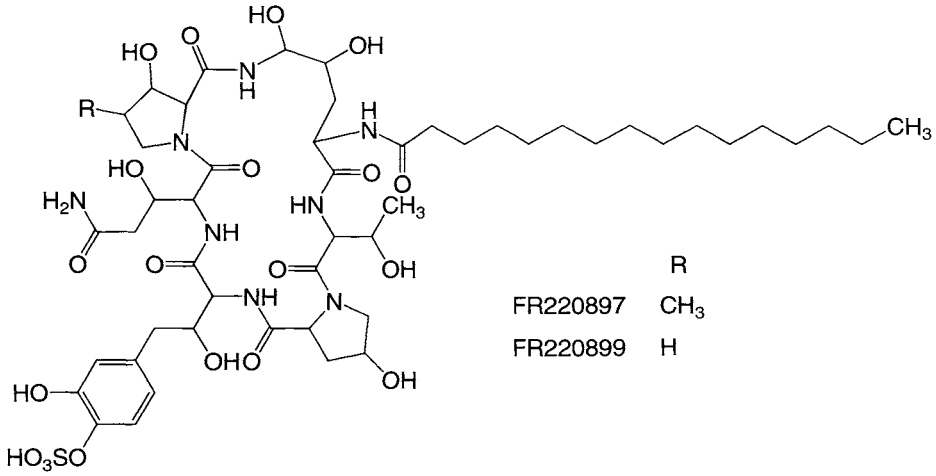


Fig. 41 Structure of FR220897 and FR220899

FR227673 and FR190293

FR227673 and FR190293 (Fig. 42) were discovered by Kanasaki *et al.* in 2006, from a fungal strains *Chalara* sp. No. 22210 and *Tolypocladium parasiticum* No. 16616, respectively. These compounds have the same peptide structure as FR901379, but different side chains. FR227673 and FR190293 show almost equivalent antifungal and 1,3-β-D-glucan synthase inhibitory activities to FR901379[130].

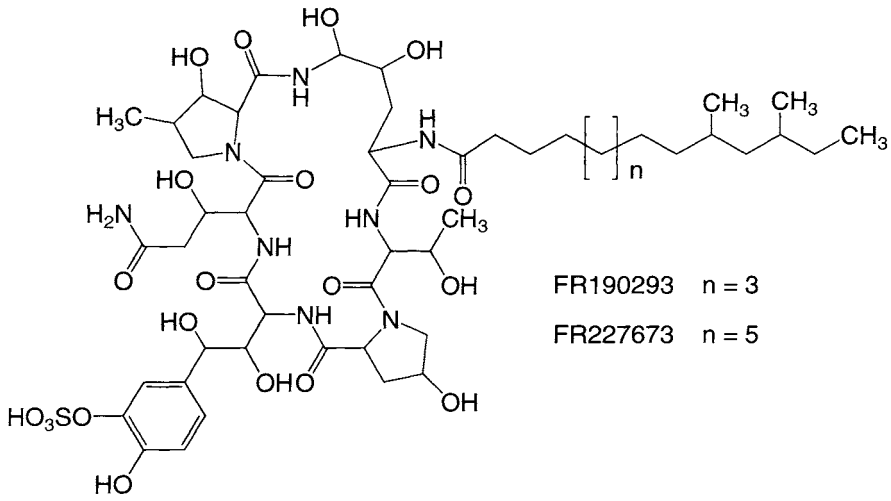


Fig. 42 Structure of FR227673 and FR190293

2.2 Others

FR901469

FR901469 was discovered by Fujie *et al.* in 2000, as a water-soluble antifungal antibiotic from an unidentified fungus No. 11243. This compound inhibits the 1,3-β-D-glucan synthase in *C. albicans* with an IC_{50} of 0.05μg/ml, and displays greater inhibitory activity than other 1,3-β-D-glucan synthase inhibitors such as WF11899A, echinocandin B, aculeacin A and papulacandin B[131]. The core structure of this compound is different from that of echinocandin-type antibiotics.

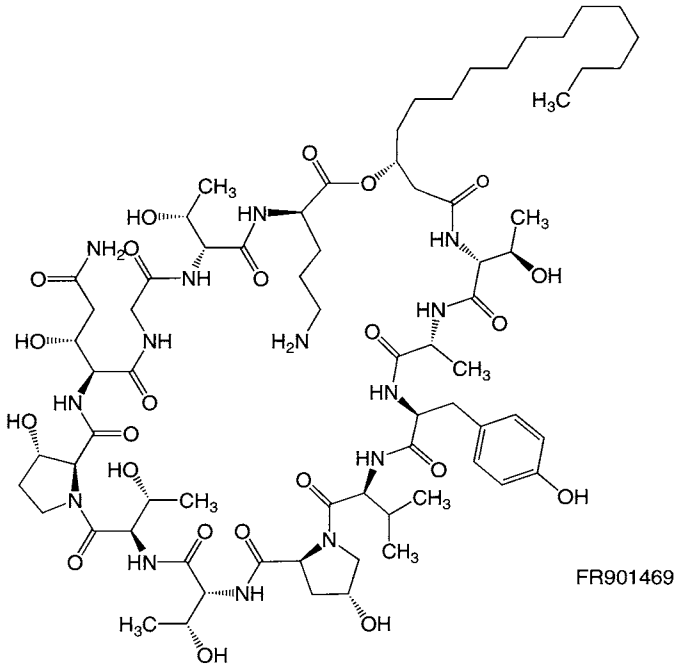


Fig. 43 Structure of FR901469

Enniatins

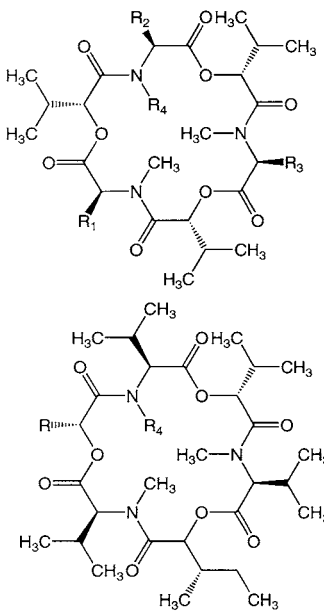
Enniatins (Fig. 44) are produced by *Fusarium* species. Enniatin B exhibits binding to alkali ions, as well as valinomycin. While valinomycin shows preferential binding to K^+, Rb^+, and Cs^+ over Na^+, enniatin B is considerably less specific[132]. Enniatins D, E and F were isolated from *Fusarium* sp. FO-1305 as inhibitors of acyl-CoA : cholesterol acyltransferase (ACAT), by Tomoda *et al.* in 1992 . The IC_{50} values of enniatins D, E and F against ACAT in an enzyme assay using rat liver microsomes are 87, 57 and 40 μM, respectively[133].

Enniatins A, A_1, B_1, E and F show no potent antimicrobial activity against *Staphylococcus, Bacillus, E. coli, Pseudomonas, Candida, Trichphyton, Mucor* and *Aspergillus* spp[133].

Enniatins J_2 and J_3 were isolated by Pohanka *et al.* in 2004 from *Fusarium* sp. strain F31 by bioassay-guided isolation directed against *Botrytis cinerea*[134].

Enniatins inhibit Pdr5p, one of the major multidrug efflux pumps whose

overexpression confers multidrug resistance in *Saccharomyces cerevisiae*. Enniatins B, B_1 and D inhibit strongly Pdr5p-mediated efflux of cycloheximide or cerulenin in Pdr5p-overexpressing cells at a non-toxic concentration of 5μg/ml. Enniatins accumulate the fluorescent dye rhodamine 123, a substrate of Pdr5p, in yeast cells. On the other hand, enniatins do not inhibit the function of Snq2p, a homologue of Pdr5p[135].



enniatin A $R_1 = R_2 = R_3 = CH(CH_3)CH_2CH_3$ $R_4 = CH_3$

enniatin B $R_1 = R_2 = R_3 = CH(CH_3)_2$ $R_4 = CH_3$

enniatin B_1 $R_1 = R_2 = CH(CH_3)_2$ $R_3 = CH(CH_3)CH_2CH_3$ $R_4 = CH_3$

enniatin B_2 $R_1 = R_2 = R_3 = CH(CH_3)_2$ $R_4 = H$

enniatin C $R_1 = R_2 = R_3 = CH_2CH(CH_3)_2$ $R_4 = CH_3$

enniatin D $R_1 = R_2 = CH(CH_3)_2$ $R_3 = CH_2CH(CH_3)_2$ $R_4 = CH_3$

enniatin E $R_1 = CH(CH_3)_2$ $R_2 = CH_2CH(CH_3)_2$ $R_3 = CH(CH_3)CH_2CH_3$ $R_4 = CH_3$

enniatin F $R_1 = CH_2CH(CH_3)_2$ $R_2 = R_3 = CH(CH_3)CH_2CH_3$ $R_4 = CH_3$

enniatin G $R_1 = R_3 = CH_2CH(CH_3)_2$ $R_2 = CH(CH_3)_2$ $R_4 = CH_3$

enniatin J_1 $R_1 = R_2 = CH(CH_3)_2$ $R_3 = CH_3$ $R_4 = CH_3$

enniatin J_2 $R_1 = CH(CH_3)_2$ $R_2 = CH(CH_3)CH_2CH_3$ $R_3 = CH_3$ $R_4 = CH_3$

enniatin J_3 $R_1 = CH(CH_3)_2$ $R_2 = CH_3$ $R_3 = CH(CH_3)CH_2CH_3$ $R_4 = CH_3$

enniatin H $R = CH(CH_3)_2$

enniatin I $R = CH(CH_3)CH_2CH_3$

Fig. 44 Structures of enniatins

Stevastelins

Stevastelins A, A_3, B, B_3, C_3, D_3 and E_3 (Fig. 45) were discovered by Morino *et al.* in 1996, as immunosuppressants that inhibit dual-specificity protein phosphatases from *Penicillium* sp. NK374186[136,137,138,139]. Stevastelin B inhibits IL-2- and IL-6-dependent gene expression but does not inhibit the phosphatase of calcineurin. Structure-activity relationship studies of stevastelins and their analogs indicate that the acidic functional group on the threonine residue and the stearic acid moiety are important for inhibition of the dephosphorylation of VH1-related human protein *in vitro*[139].

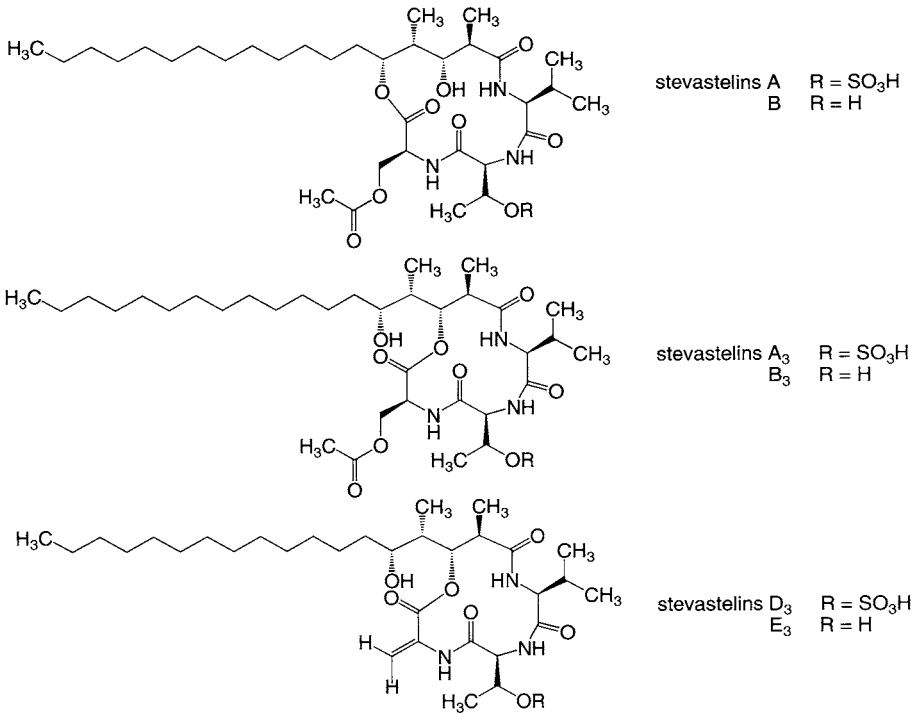
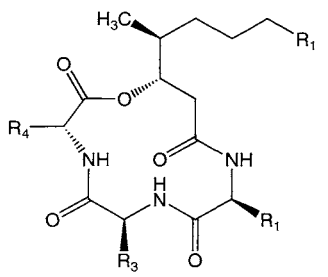


Fig. 45 Structures of stevastelins

Beauveriolides

Beauveriolides (Fig. 46) were discovered by Namatane *et al.* in1999, and D. Matsuda *et al.* in 2004, as inhibitors of lipid droplet formation in mouse peritoneal macrophages from *Beauveria* sp. FO-6979. Beauveriolides I and III exhibit the reduction in the number and size of cytosolic lipid droplets in macrophages at a concentration of 10μM without any cytotoxic effect on macrophages[140,141]. The production of beauveriolides I and III is increased 5-10-fold by fermentation in culture media containing tryptone. Addition of L-leu or L-Ile, but not D-Leu or D-Ile, to the culture medium yields high and selective production of beauveriolide I or III, respectively[142]. Beauveriolides IV, V, VI, VII, VII and IX were isolated from the acetone extract of *Beauveria* sp. FO-6979 mycelia fermented in amino acid-supplemented media. Beauveriolide VII inhibits lipid droplet formation and cholesteryl ester synthesis in macrophages, but beauveriolides IV, V, VI, VIII and IX show slight or almost no effect on lipid droplet formation[143].



Beauveriolides	R_1	R_2	R_3	R_4
I	$-CH_3$	a	b	c
III	$-CH_3$	a	b	d
IV	$-CH_3$	e	b	e
V	$-CH_3$	e	b	d
VI	$-CH_3$	e	b	c
VII	$-CH_3$	a	b	e
VIII	$-(CH_2)_2CH_3$	e	b	d
IX	$-CH_3$	a	a	d

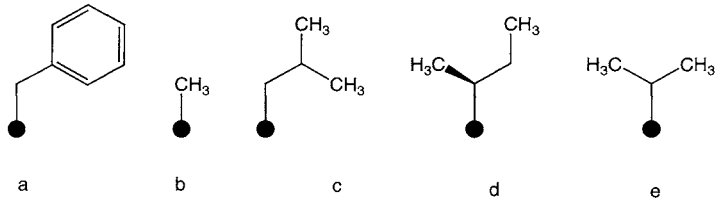


Fig. 46 Structure of beauveriolides

3. LIPOPEPTIDES FROM MARINE-ASSOCIATED ORGANISMS

A lot of interesting lipopeptides, both terms of biological activities and structures, have been discovered from the metabolites of marine-associated organisms. In particular, cyanobacteria produce many kinds of bioactive metabolites including toxins and enzyme inhibitors. Some cyclic lipopeptides from marine-associated organisms have unique structures, which are much different from those of their soil-associated counterparts. For example, the α-diketo structure is in cyclotheonamides: 3-amino-6-hydroxy-2-piperidone is in micropeptins, scyptolins and planktopeptins; and (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid is in the microcystin group. On the other hand, 3-amino-6-hydroxy-2-piperidone-containing cyclic depsipeptides are widely distributed in cyanobacteria such as *Microcystis, Planktothrix, Scytonema* and *Nostoc*. The metabolites produced by marine-associated organisms have different characteristics from those of soil-associated organisms. As a result, marine metabolites have proven to be promising sources for seeking drug candidates.

Micropeptins

Micropeptins EI992 and EI964 (Fig. 47) were isolated as protease inhibitors from the extracts of two samples of non-toxic cyanobacterium

Microcystis aeruginosa strains IL-217 and IL231, collected from the Einan Resevior in the Hula Valley, Israel, by Ploutno et al. in 2002[144]. Micropeptins EI992 and EI964 (Fig. 47) inhibit trypsin with IC_{50} values of 3.8 and 4.2 μg/ml, respectively, but not chymotrypsin at a concentration 45.5 μg/ml. Micropeptins 88-N and 88-Y were isolated as chymotrypsin inhibitors from *Microcystis aeruginosa* NIES-88 by Yamaki *et al.* in 2005, and their chymortrypsin inhibitory activities (IC_{50}) are 15 and 1.3 nM, respectively[145]. Micropeptins containing the unusual amino acid, 3-amino-6-hydroxy-2-piperidone are produced by reduction of the δ carboxyl group of glutamic acid to its corresponding aldehyde, which, in turn, reacts with the amide nitrogen of a neighbouring amino acid to yield a piperidone moiety[154].

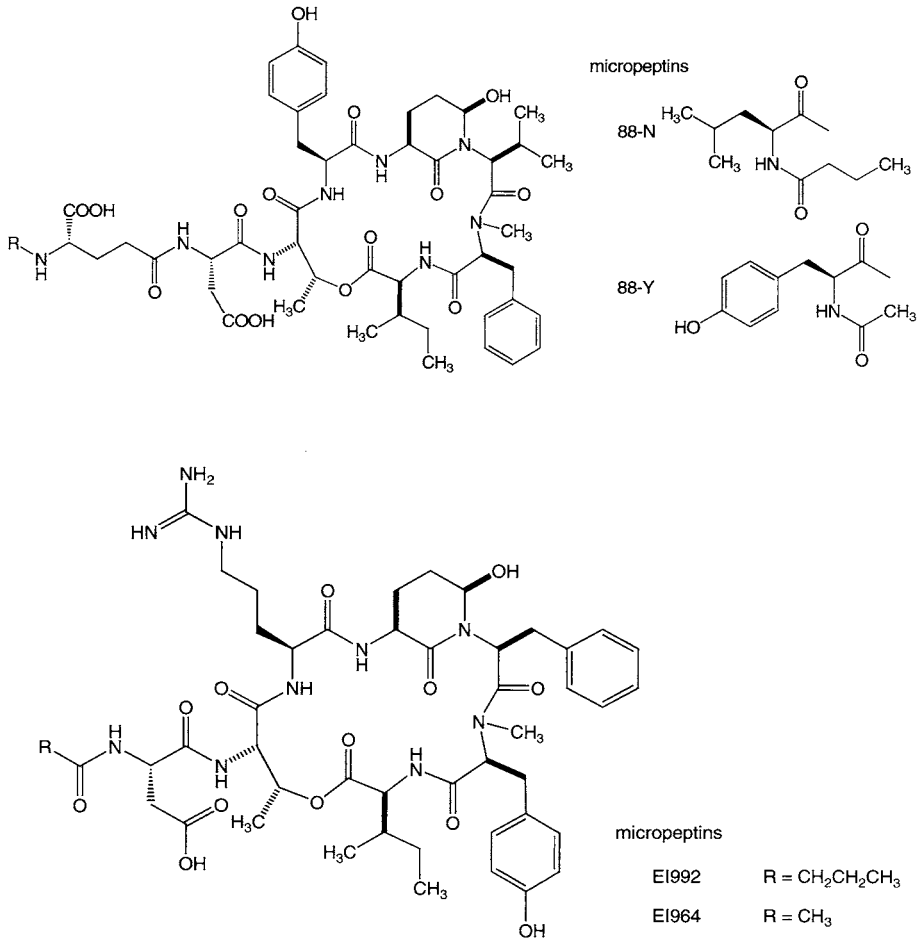


Fig. 47 Structures of micropeptins EI992, EI964, 88-N and 88-Y

Scyptolins

Scyptolins A and B (Fig. 48) were isolated from axenic cultures of the terrestrial cyanobacterium *Scytonema hofmanni* PCC7110 by Matern *et al.* in 2001.They have the uncommon amino acid residue 3'-chloro-*N*-methyl tyrosine and unique side chains. Both scyrtolin A and B exhibit selective inhibition of porcine pancreatic elastase *in vitro* with an IC_{50} of 3.1μg/ml[146].

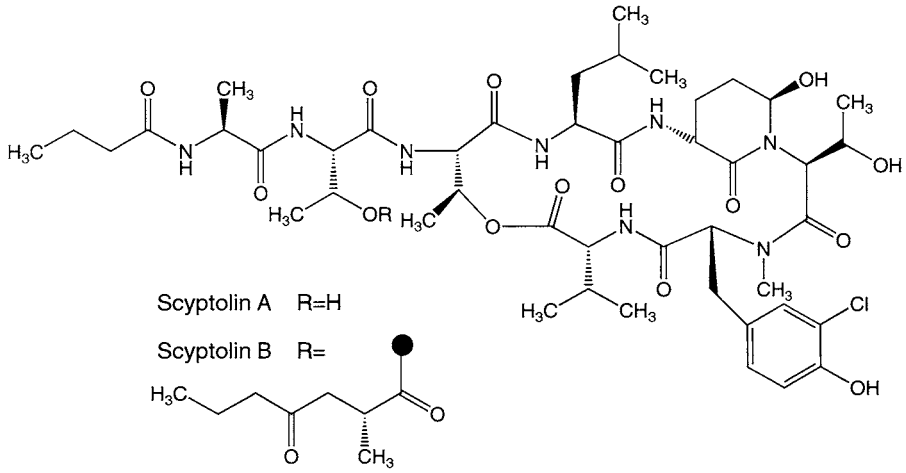


Fig. 48 Structure of scyptolins

Planktopeptins

Planktopeptins BL1125, BL843 and BL1061 (Fig. 49) were isolated from the hydrophobic extract of *Planktothrix rubescens* by Grach-Pogrebinsky *et al.* in 2003. The producing organism, a cyanobacterium, was collected from the Bled Lake in Slovenia. Planktopeptins BL1125, BL843 and BL1061 show inhibitory activity against serine protease elastase *in vitro* with IC_{50} values of 96nM, 1.7μM and 40nM, respectively, and against chymotrypsin *in vitro* with IC_{50} values of 0.8, 14.0 and 2.1μM, respectively, but not against trypsin even at 50 μg/ml[147]. The absolute configuration of glyceric acid was determined by using a chiral HPLC column, and compared with authentic L- and D-glyceric acid.

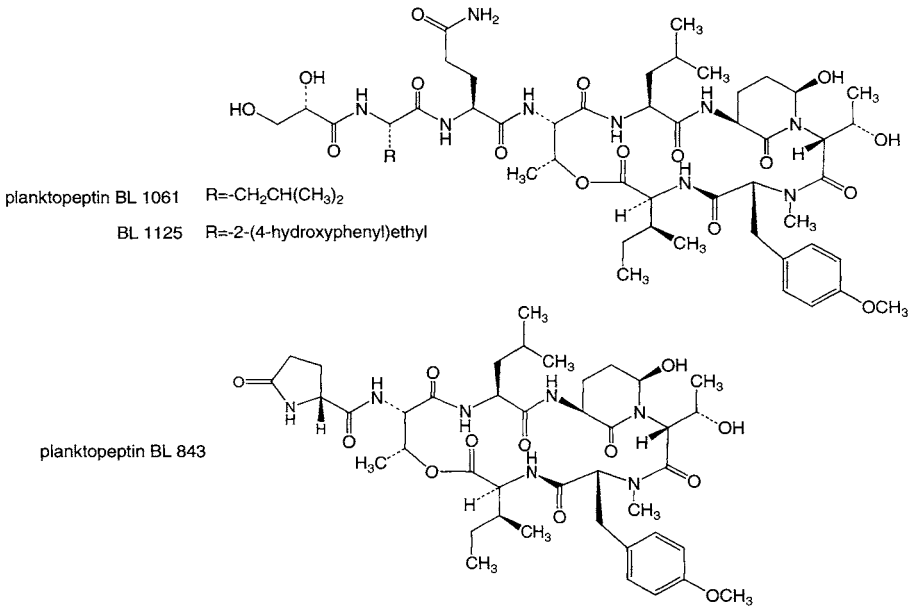


Fig. 49 Structures of planktopeptins BL1125, BL843 and BL1061

Antillatoxin

Antillatoxin (Fig.50), discovered by Orjala *et al.* in 1995, is produced by the pantropical marine cyanobacterium *Lyngbya majuscula*. Blooms of *L. majuscula* have been associated with adverse effects on human health, including respiratory irritation, eye inflammation, and severe contact dermatitis in exposed individuals. Antillatoxin has been reported to be among the most ichthyotoxic metabolites isolated to date from marine microalgae and is exceeded in potency only by the brevetoxins[148].

Natural antillatoxin (*4R, 5R*-isomar) is 25-fold more potent than any of the other stereoisomers [(*4S, 5R*)-, (*4S, 5S*)- and (*4R, 5S*)-,] in the following five assay systems: ichthyotoxicity to goldfish, microphysiometry using cerebellar granule cells (CGCs), lactose dehydrogenase efflux from CGCs and monitoring of intracellular Ca^{++} concentrations in CGCs and cytotoxicity to Neuro 2a cells. The decreased potency of the non-natural antillatoxin stereoisomers is certainly caused by a result of their overall molecular topologies being altered dramatically[149].

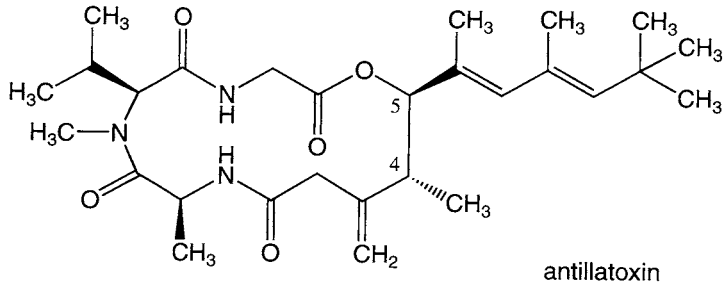


Fig. 50 Structure of antillatoxin

Cryptophycins

Cryptophycins (Figs. 52 and 53) were found in the lipophilic extract of *Nostoc* sp. GSV224 in the course of antitumor activity screening of blue-green algae (cyanobacteria) by Trimurtulu *et al.* in 1994. Lipophilic extracts of cryptophycin-producing organisms exhibit potent fungicidal acitivity against strains of *Cryptococcus,* and cytotoxicity with an IC_{50} of 0.24ng/ml against the KB human nasopharyngeal carcinoma cell line, and 6ng/ml against the LoVo human colorectal adenocarcinoma cell line[150,151].

Structure-activity relationship studies indicate that the intact macrolide ring, the epoxide group, the chloro and *O*-methyl groups in unit B, and the methyl group in unit C are essential for the *in vivo* activity of cryptophycin 1 (Fig. 51)[151].

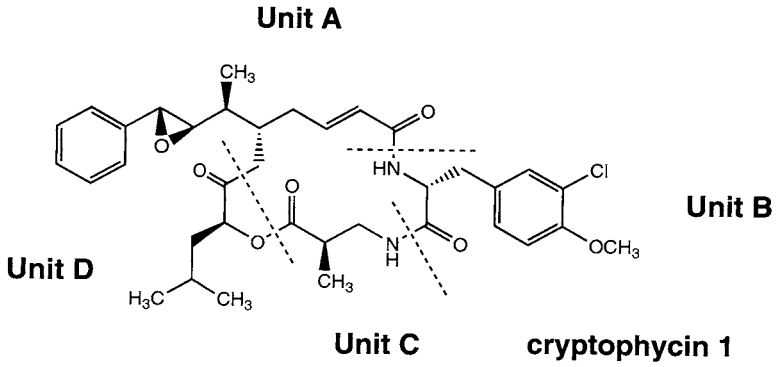
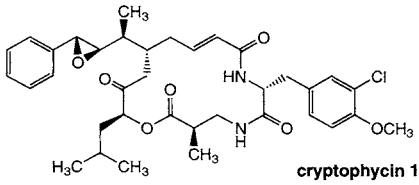
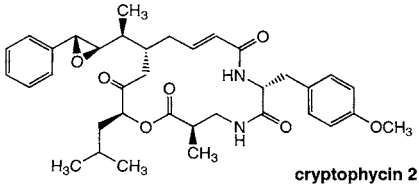


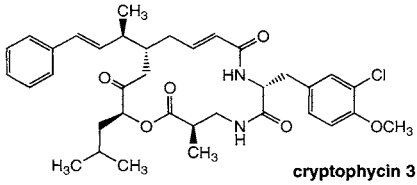
Fig. 51 Structure of cryptophycin 1



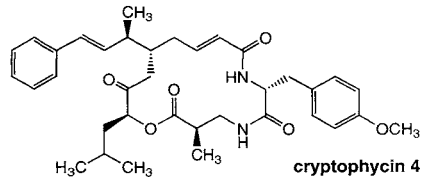
cryptophycin 1



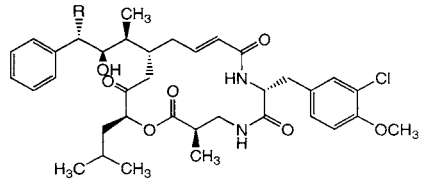
cryptophycin 2



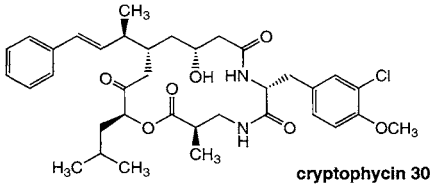
cryptophycin 3



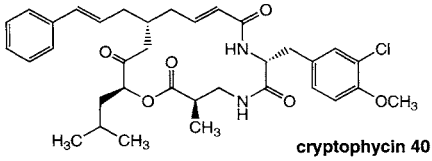
cryptophycin 4



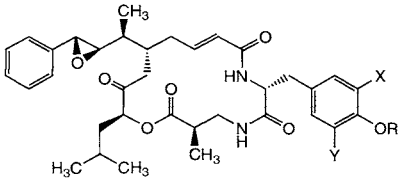
cryptophycin 8 R= Cl
9 R= OCH_3



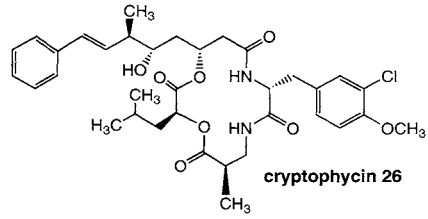
cryptophycin 30



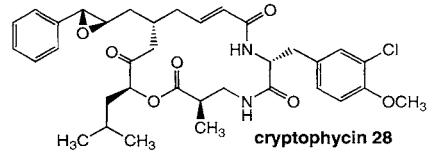
cryptophycin 40



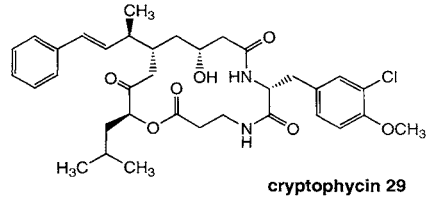
cryptophycin 16 R = H X = Cl Y = H
cryptophycin 23 R = H X = Cl Y = Cl
cryptophycin 31 R = CH_3 X = Cl Y = Cl



cryptophycin 26



cryptophycin 28



cryptophycin 29

Fig. 52 Structures of cryptophycins

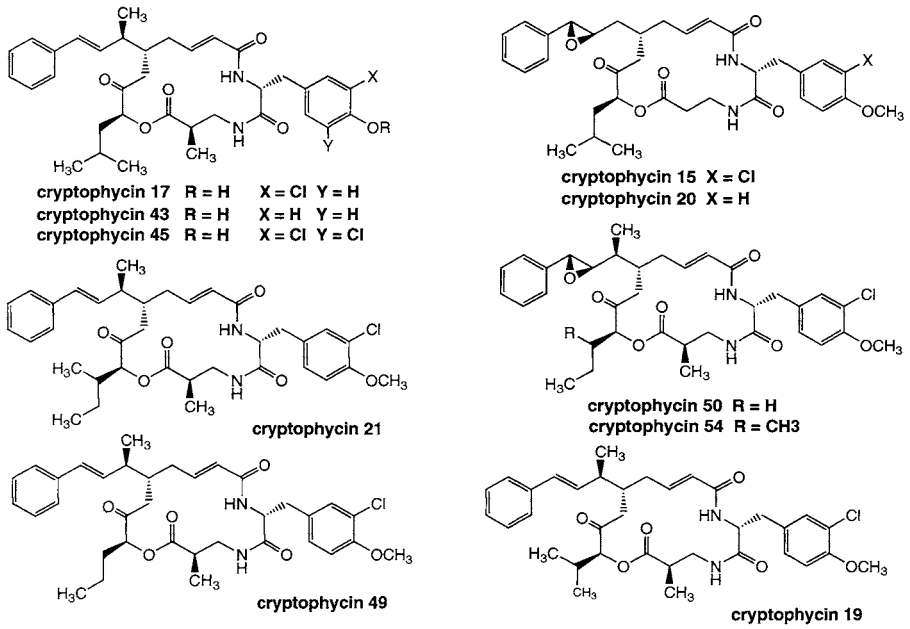


Fig.53 Structures of cryptophycins-2

Cyclotheonamides

Cyclotheonamides A, B, C and D were isolated as slow-binding inhibitors of thrombin and other trypsin-like serine proteinases from the marine sponge *Theonella swinhoei*[152153] by Fusetani *et al.* in 1990 and Nakao *et al.* in 1995. Cyclotheonamide E was isolated from a morphologically different *T. swinhoei*[151] by Y. Nakao *et al.* in 1995. Cyclotheonamides E2 and E3 were isolated by Y. Nakao et al. in 1998 as potent protease inhibitors from ethanol extracts of a *Theonella* species collected from Tanegashima Island, Japan. Cyclotheonamides E4 and E5 were isolated as tryptase inhibitors from a marine sponge of the genus *Irchinia* by Murakami *et al.* in 2002[154]. Tryptase is a protease released from mast cells and is involved in inflammation in allergic diseases. Cyclotheonamides E, E4 and E5 exhibit inhibition of human tryptase *in vitro* with IC_{50} values of 6.9, 5.1 and 84.7nM, respectively, and they also show non-selective inhibition against human thrombin, bovine trypsin and mouse tryptase[152]. They contain two unusual amino acids, α-ketoarginine and vinylogous tyrosine, which are involved in binding to the thrombin molecule[155].

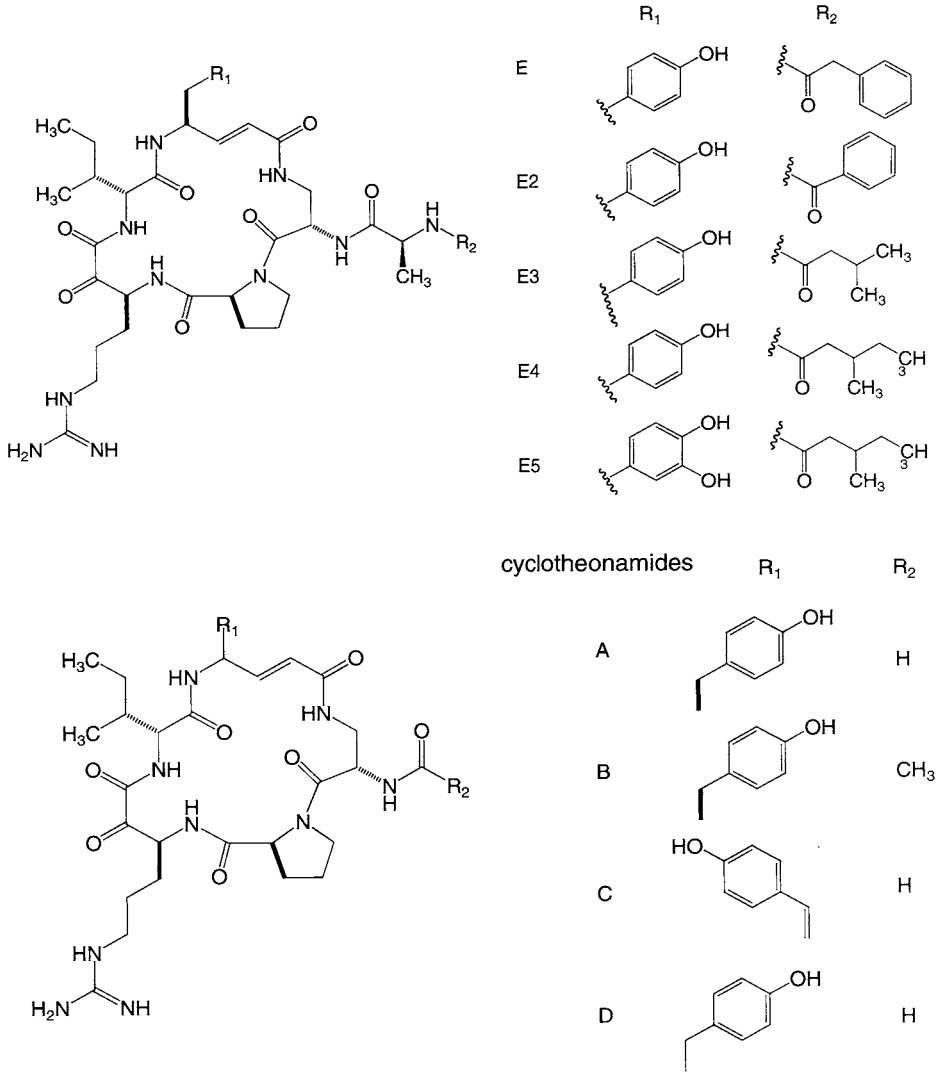


Fig.54 Structures of cyclotheonamides

Keenamide A

Keenamide A (Fig. 55), containing a dehydroxylated leucyl-cystein and isoprene unit, was isolated from the notaspidean mollusk *Pleurobranchus forskalii* by Wesson *et al.* in 1996. Keenamide A exhibits significant cytotoxicity against several tumor cell lines, with IC_{50} values of 2.5μg/ml against P-388, A-549 and MEL-20 cells, but is inactive against the D6 and W2 *Plasmodium falciparum* clones[156].

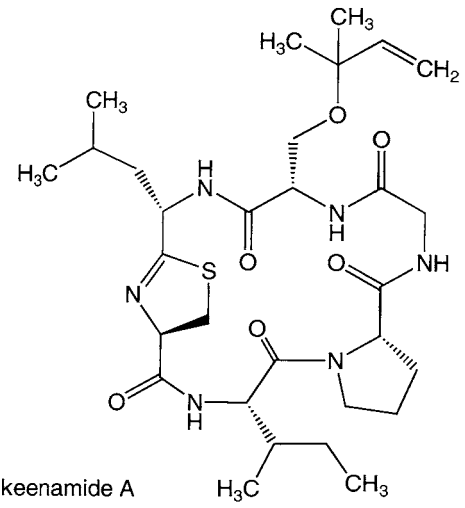


Fig. 55 Structure of keenamide A

Microcystins group [microcystins, hepatotoxins and nodularin (=motuporin)]

Microcystins[157,158159], hepatotoxins[160] and nodularin[161] (Fig. 56) have a unique C_{20} amino acid (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) as a remarkable structural feature. Adda seems to be important for their hepatotoxicity, since hydrogenation or ozonolysis of the diene system gives an inactive compound and stereoisomers at the 6-double bond are inactive[162].

Microcystins were isolated as toxins from *Microcystis aeruginosa* bloom by Elleman *et al.* in 1978 and Kusumi *et al.* in 1987.

Hepatotoxins were isolated as new hepatotoxic compounds from the cyanobacterium *Nostoc* sp. strain 152 and assigned structures based on their high resolution FAB (fast atom bombardment) MS/MS, ^{1}H and ^{13}C NMR spectra, amino acid analysis and gas chromatography on a chiral capillary column by Namikoshi *et al.*[160].

Nodularin was isolated as a potent protein phosphatase (PP1) inhibitor from the Papua New Guinea sponge *Theonella swinhoei* gray[162].

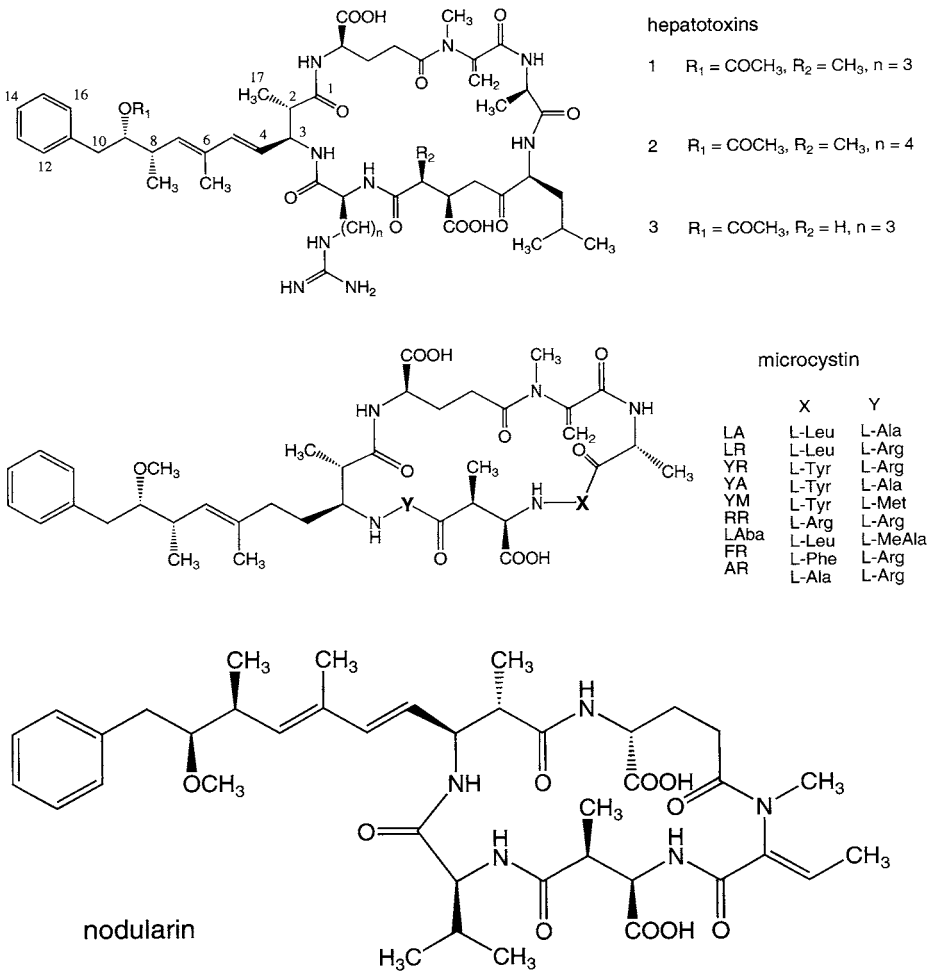


Fig.56 Structures of microcystins group chemicals

Mirixins

Mirixins A, B, and C (Fig. 57) were isolated from an ethyl acetate extract of marine bacterium *Bacillus* sp., obtained from sea mud near the Arctic pole by Zhang *et al.* in 2004. Mirixins contain three asparagine residues and the β-amino fatty acyl side chains. Mirixins A, B and C exhibit growth inhibition with IC_{50} values of 0.68, 1.6 and 1.3μg/ml, respectively against HCT-116 human colon tumor cells[163].

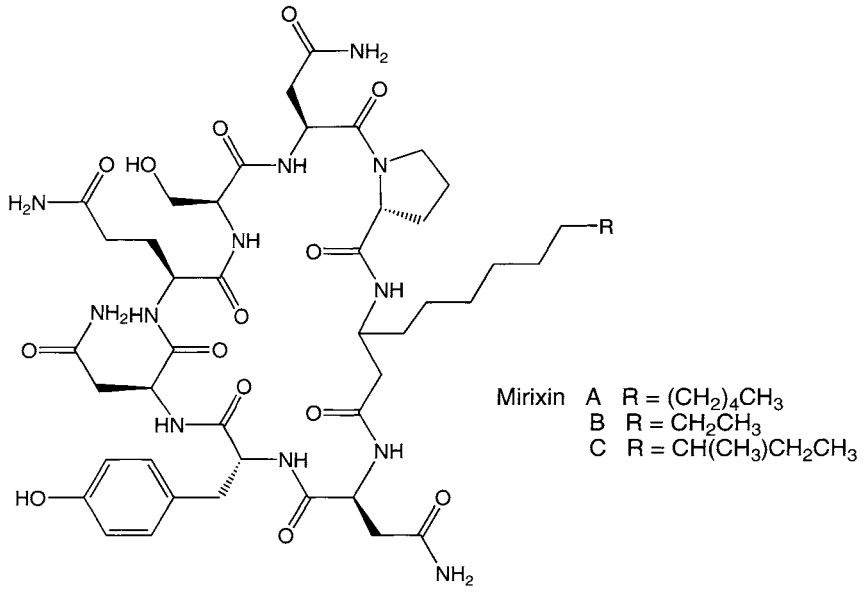


Fig. 57 Structure of mirixins

4. CONCLUSION

Cyclic lipopeptide antibiotics contain various kinds of amino acids including unusual amino acids. Many kinds of cyclic lipopeptides were discovered from microorganisms and they often exhibit a wide variety of biological activities such as antimicrobial, antifungal, antitumor, and enzyme inhibitory activities. It is also of considerable interest that the structural similarities do not always reflect any similarity of biological activities in the cyclic lipopeptides.

Recently, as high through-put screening systems and combinatorial chemistry have progressed rapidly, novel clinically important drugs have been developed efficiently using this new methodology, such as Glivec for the treatment of chronic myelogenous leukemia. However, the new drugs approved in the past were mainly derived from natural products. They include various types of chemicals such as peptides, β-lactams, aminoglycosides, tetracyclines, macrolides, and polyene antibiotics. Cyclic lipopeptides with structurally complex and large molecules may be difficult to obtain even using new synthetic methodology such as combinatorial chemistry. On the other hand, rational drug design based on the cyclic lipopeptide biosynthetic (NRPS) system is showing remarkable progress and leading to effective structural optimization of lead compounds for medical applications.

The structural and biological diversity of the cyclic lipopeptide antibiotics is extremely attractive to chemical, pharmacological and medical sciences. Future rational drug design, chemical modification and discovery of novel cyclic lipopeptides from natural sources will give rise to useful biological tools for research and development in the medicinal sciences.

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BIOACTIVE DITERPENOIDS OF *Salvia* SPECIES

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ABSTRACT: More than 550 diterpenoids have been isolated from *Salvia* species widely distributed in Mexico, Turkey, Spain and China. 23 species grow in Algeria, five of them are endemic. We've recently isolated five abietane diterpenoids from each of the endemic species *S. jaminiana* and *S. verbenaca* subsp. c*landestina* and seven diterpenoids, from which three are new, from the endemic species *S. barrelieri*. We've recently reported the antibacterial activity of the roots of *S. jaminiana* while the antioxidant activity have been established for the seven diterpenoids isolated from *S. barrelieri*. *Salvia* diterpenoids could be divided into 2 categories. The first category includes one monocyclic and bicyclic diterpenoids containing labdanes, clerodanes, neo-clerodanes, seco-clerodanes and other rearranged clerodanes. The second category concerns tricyclic and tetracyclic diterpenes represented by pimaranes and abietanes including quinone abietanes, nor-abietanes, rearranged nor-abietanes, dinor-abietanes, seco-abietanes, tanshinones and other particular rearranged abietanes. A great number of the described *Salvia* diterpenoids exhibited interesting biological activities *e.g* anti-tuberculous, antitumour, antimicrobial, antibacterial, antileishmanial and antispasmolytic.

INTRODUCTION

The *Salvia* genus has been the subject of numerous studies. It's an important genus consisting of 900 species widely used in folk

medicine. Over 275 species grow in America, 90 are distributed in Turkey. 23 Species grow in Algeria [1], 5 of them are endemic such us *S. jaminiana*, *S. verbenaca* subsp. *clandestina* and *S. barreilieri* which have been the subject of recent studie as a part of our works on Lamiaceae [2-4]. A large number of the isolated compounds from *Salvia* species are diterpenoids possessing interesting biological activities.

BIOLOGICAL INTERESTS OF *Salvia* SPECIES

Diterpenoids are the most important compounds responsible of the biological activities found in numerous *Salvia* species. Antituberculous effects have been established for *S. blepharochlyn*a [5], *S. multicaulis* also reported for it's antifungic effect [6] and *S. prionitis* which possess antiphlogistic and antibacterial properties [7,8] beside its antituberculous activity.
An antibiotic activity has been detemined for the spanish species, *S. canariensis* [9]. Many species namely *S. albocaerula* [10], *S. forskhalei* [11], *S. lanigera* [12] and *S. officinalis* [13] have shown an antimicrobial activity. Recently, the latter exhibited antiviral [14] and antioxidant effects [15]. We've also established the antioxidant activity of thr diterpenoids isolated from the endemic species *S. barrelieri* [3]. Antibacterial activities have been established for *S. bracteata* [16], *S. caespitosae* [17], *S. hypargeai [18], S. jaminiana* [2], *S. leriafolia* [19], *S. palaestina* [20], *S. viridis* [21] *and S. syriaca* [22], also reported for its cardioactive effect [23]. *S. glutinosa* and *S. microstegia* possess antitumour properties [24], while cardioactive effects were determined for the *Salvia* species namely *S. amplexicaulis* [25], *S. eriophora* [26], *S. staminea* [27] and the chineese species *S. milthiorrhiza* [28-32] "Danshen" which is the most studied species during a span time covering 50 years, also reported for its use in the treatment of angina pectoris, myocardial infarction, haematological abnormalities and for its cytotoxic effect against human tumour cells. The other chineese species, *S. przewalskii* [33] is used in North Western China (Tibet) as a substitut of *S mil*thiorrhiza. *S. aegyptiaca* [34], *S. cinnabarina* [35], *S. parryi* [36] and *S. polystachya* [37] are used in the treatment

of stomach disorders. Recentely, it has been established that the turkish species *S. cilicic*a [38] possess an antileishamnial activity.

BIOLOGICAL ACTIVITIES OF *Salvia* SPECIES FROM ALGERIA

Antibacterial activity of the roots of S. jaminiana and S. verbenaca subsp. jaminiana

As shown in table 1, the acetone extracts of the roots of the algerian species *S. jaminiana* (extract 1) and *S. verbenaca* subsp. *jaminiana* (extract 2) inhibited remarkably the growth of *B. subtilis*, *S. aureus ATCC 25923* and *S. α-hemolitic* while a very weak antibacterial activity was observed with *E. coli*, *K. pneumoniae* and *P. mirabilis*. To our knowledge, it's the first time that a *Salvia* species gave a so significant inhibition of the growth of *B. subtilis*.

Table 1 Antibacterial activity of the *S. jaminiana* (1) and *Salvia verbenaca* subsp. *jaminiana* (2) roots acetone extracts

Microorganism	Inhibition zone (mm)		MIC (µg/ml)	
	Ampicillin (10µg/ml)	Extract (128µg/ml)	*Salvia* extracts 1, 2 (128 µg/ml)	Ampicillin (10 µg/ml)
Bacillus subtilis	14	28	4	10
Escherichia coli ATCC 25922	18	14	128	10
Klebsiella pneumonia	14	12	>128	32
Proteus mirabilis	16	8	>128	64
Staphyloccocus aureus	30	26	16	5
Streptoccocus -hemolitic	18	22	6	8

Antioxidant activity of the diterpenoids of the roots of Salvia barrelieri

Very recently, we've established the antioxidant activity of six abietane diterpenoids isolated from the roots of the endemic species *Salvia barrelieri* namely, 12-methoxy-7-acetoxyroyleanone (or 7-acetoxybarrilierone) **A1,** 12-methoxy-7-oxoroyleanone (or 7-oxobarrilierone) **A2**, 14-hydroxycryptojaponol **A3**, 7-α-acetylhorminone **A4,** horminone **A5**, royleanone **A6.** The abietanes **A1-2** are new while the abietane **A3** is reported for the first time from the *Salvia* genus [3]. Free radical and superoxide scavenging and β-carotene bleaching methods have been used.

The reducing capability of DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Table 2 illustrates a significant ($P<0.05$) increase the concentration of DPPH radical due to the DPPH scavenging affect ability of each concentration of abietanes and BHT and α-tocopherol. **A3** showed a stronger activity than known standarts BHT and α-tocopherol in all concentrations. The DPPH scavenging effect of abietanes and standarts on the DPPH radical decreased in the order of A3>α-tocopherol>BHT>A2>A1>A6>A5>A4 at all concentrations (10μg, and 25μg).

It appears that 14-hydroxycryptojaponol (inuroyleanol) **A3** showed the highest DPPH scavenging activity while 7-acetyl horminone **A4** showed the highest activity. Interestingly, royleanone **A6** showed higher antioxidany activity than both horminone **A5** and 7-acetyl horminone **A4,** indicating an oxygenated substituent at C-7 would be decreased activity. The new compound **A2** which is a methyl derivative of 7-acetylhorminone at C-12 showed higher activity than the latter, probably because of the higher scavenging power of methoxy group at C-12. Except substituent at C-7, all other substituents were the same in the new compounds **A1** and **A2.** Since, better activity was observed for compound **A1**, we can

conclude that in abietanes with *p*-quinones moiety, the presence of a keto group instead of acetyl at C-7 increased antioxidant activit

Table 2. Free radical scavenging activity of abietanes (A1-7), BHT, α-Tocopherol by 1,1-diphenyl-2-picrylhydrazyl radicals[a].

Sample	2,5 µg	5 µg	10 µg	25 µg
A1	NT	NT	2,9896±1,40	8,3381±0,59
A2	NT	NT	6,3302±0,10	14,4285±0,43
A3	17,2316±0,06	33,4592±0,56	69,3483±0,11	96,6777±0,15
A4	NT	NT	0,6441±0,22	2,6609±0,24
A5	NT	NT	1,6792±0,40	2,9896±0,44
A6	NT	NT	2,5499±0,06	5,8949±0,16
A7	NT	NT	0,7302±0,05	2,5649±0,22
α-Tocopherol[b]	19,9460±0,60	38,2210±0,71	61,1967±0,56	92,6954±0,07
BHT[b]	NT	NT	37,8081±0,68	68,5532±0,09

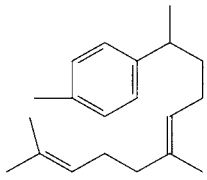
[a] Values expressed are means±S.D. of three parallel measurements. ($P<0.05$).
[b] Reference compounds. NT: not tested, BHT: butylated hydroxytoluene
A1: 12-methoxy-7-acetoxyroyleanone (or 7-acetoxybarrilierone)
A2: 12-methoxy-7-oxoroyleanone (or 7-oxobarrilierone)
A3: 14-hydroxycryptojaponol
A4: 7-α-acetylhorminone
A5: horminone
A6: royleanone

CLASSIFICATION OF THE DITERPENOIDS ISOLATED FROM *Salvia* SPECIES

We've classified more than 550 diterpenoids (listed in Table 3 whith corresponding sources) from all the reported *Salvia* species which we've divided into 2 categories. The first one contains the monocyclic and bicyclic diterpenoids including labdanes and clerodanes and related diterpenes. The second category concerns tricyclic and tetracyclic diterpenes including pimaranes and abietanes and related diterpenes. The clerodane type diterpenes are mainly represented by american species while abietane type are found in european and asian species.

Monocyclic diterpenoids

In our knowledge, 2,6-dimethyl-10-(p-tolyl)undeca-2,6-(E)-diene **(1) is** the alone monocyclic diterpene, it was reported from *S. dorisiana* [39].



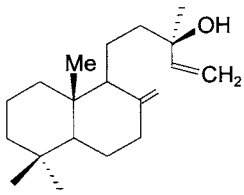
1

Bicyclic diterpenoids

Four bicyclic type diterpenoids have been reported from *Salvia* species: labdanes, neo-clerodanes and clerodanes, seco-clerodanes, rearranged clerodanes.

Labdanes

As listed in Table 3, the major labdane diterpenoids **(2-18)** were isolated from turkish species.



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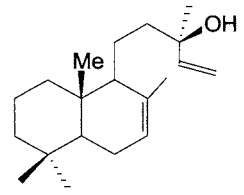

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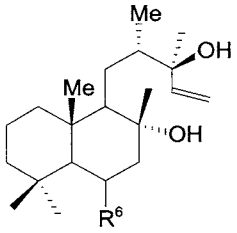
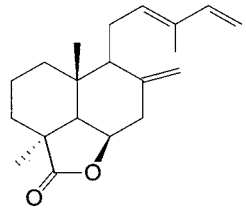


4 R^6=H
5 R^6=OH
6 R^6=H; R^{13}= αOH



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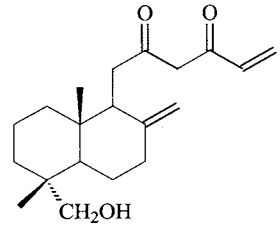

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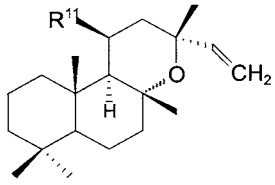
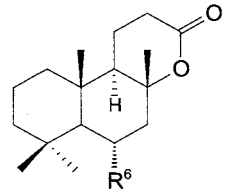
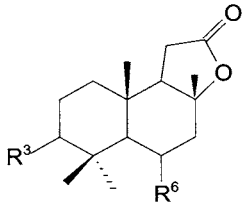


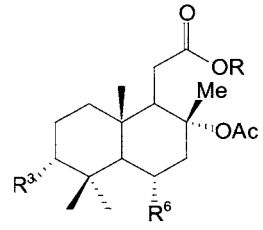
9 R^{11}=H
10 R^{11}=OH
11 R^{11}=H; αMe-17; αMe-16



12 R^6=H
13 R^6=OH



14 R^3= R^6= H
15 R^3= OH ; R^6= H
16 R^3= H ; R^6= OH



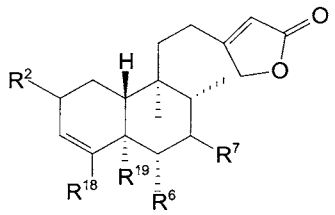
17 R = R^3= H; R^6= OH
18 R= Me; R^3= OH; R^6 = H

Neo-clerodanes and clerodanes

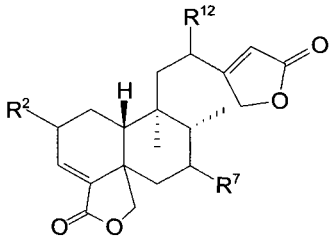
The neoclerodane type diterpenoids (listed in Table 3 with corresponding *Salvia* species and references) may be divided into 4 categories.

The first categoriy is represented by a decalin skeleton containing a C3-C4 double bond, it includes 10 groups:

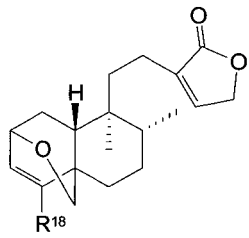
1. Neoclerodan-15,16-olides **(19-22).**
2. Neoclerodan-15,16,18,19-diolides **(23-26)**.
3. 2:19-epoxy-neoclerodan -16,15-olides **(27-28)**
4. Neoclerodan-16,15,18,19-diolides **(29-37).**
5. Neoclerodan-16,15-olides **(38-40)** .
6. Neoclerodan-16,15,20,19-diolides **(41-43)** which have been isolated from *S. madrensis* [69].
7. Neoclerodanes with a furan group on C-12 **(44-48)**.
8. Neoclerodan-18,19-olides where Me-17 is in β-configuration **(49).**
9. Neoclerodan-18,19-olides with a furan group on C-12 and Me-17 in α-configuration **(50-52).**
10. Neoclerodan-18,19-olide with a furan ring on C-12 **(53).**



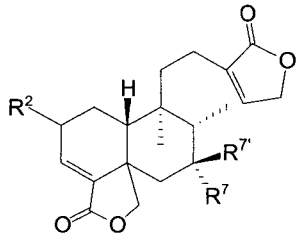
19 R^2= O; R^6= H; R^7=α OAc; R^{18}= R^{19}= Me
20 R^2= R^7= H; R^6= OH; R^{18}= R^{19}= CH_2OH
21 R^2= H; R^6= R^7= OH; R^{18}= R^{19}= CH_2OH
22 R^2= R^7= H; R^6= OH; R^{18}= Me; R^{19}= COOH



23 R^2= R^{12}= OH; R^7= H
24 R^2= R^{12}= H; R^7 =OH
25 R^2= R^{12}= H; R^7= O
26 R^2=H; R^7= OAc; R^{12}=OH

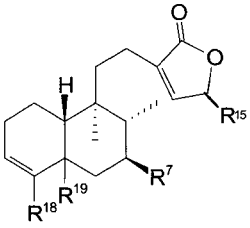


27 R^{18}= CH_2OH
28 R^{18}= CH_2OAc

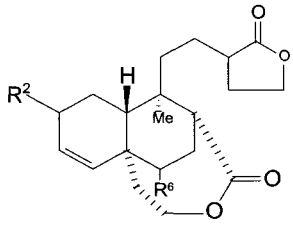


29 R^2= OH; R^7= αOAc
30 R^2= R^7= H; $R^{7'}$= βOH
31 R^2= R^7= H; R^7= αOH
32 R^2= H; R^7= αOAc
33 R^2= R^7= OH

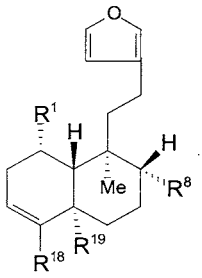
34 R^2= βOAc; R^7= αOH
35 R^2= H; $R^{7'}$, R^7= O, R^7= H
36 R^2= OH; R^7= $R^{7'}$= H
37 R^2= OH; $R^{7'}$, R^7= O



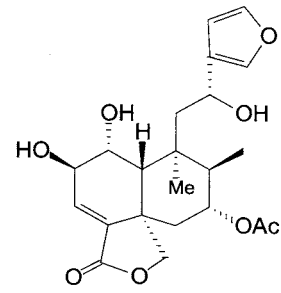
38 R^7= OH; R^{15}= H; R^{18}= R^{19}= CH_2OH
39 R^7= R^{15}= H; R^{18}= CH_2OH; R^{19}= Me
40 R^{15}= OAc; R^{18}= CH_2OAc; R^{19}= CO_2H;



41 R^2= OH
42 R^2= O
43 R^6= OH



44 R^1= H; R^8= R^{18}= Me; R^{19}= CO_2H
45 R^1= H; R^8= R^{19}= Me; R^{18}= CO_2H
46 R^1= OH; R^8= R^{19}= Me; R^{18}= CO_2H
47 R^1= OH; R^8= CH_2OH; R^{18}= CO_2Me;R^{19}= Me
48 R^1= H; R^8= CH_2OAc; R^{18}= CO_2H; R^{19}= Me



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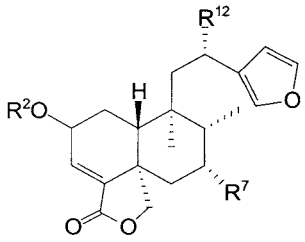
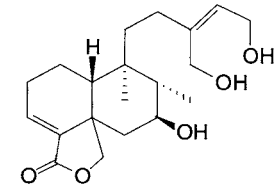


50 R^2= β-D-glucosyl ; R^7= R^{12}= H
51 R^2= Ac ; R^7= OAc ; R^{12}= OH
52 R^2= H ; R^7= OAc ; R^{12}= O



53

The second category is represented by a decalin skeleton with a C-12 lactonisation of the side chain and a furan group derivative (dihydro or tetrahydro) on C-12. It's divided into 6 groups.

1.Neoclerodanes presenting a saturated decalin skeleton **(54-57).**

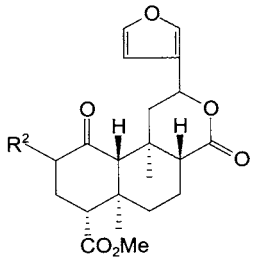
2. Neoclerodanes with a C_3-C_4 double bond **(58-61).**

3.Neoclerodan-18,19-olides with a double bond or more in the decalin skeleton or one double bond and an epoxyde. The C-10 substituent has a β-configuration **(62-78).**

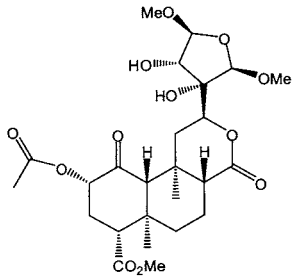
4. Neoclerodan-11,12:16,15:18,19-olide **(79).**

5. Neoclerodan- 18, 10-olide **(80).**

6.Clerodan-18,19-olides of type **3** with a C-10 substituent in α-configuration **(81-90).**



54 R^2= OAc
55 R^2= OH



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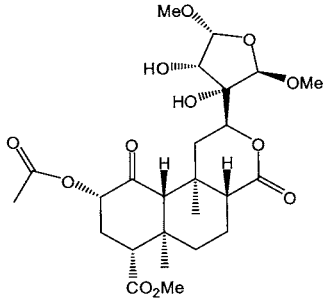

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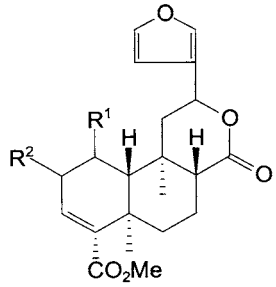
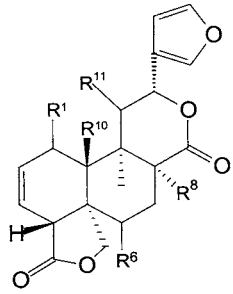
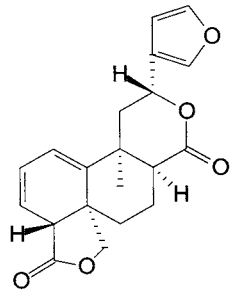


58 R^1= R^2= OAc
59 R^1= OAc; R^2= OH
60 R^1= OH; R^2= OAc
61 R^1= OH; R^2= H

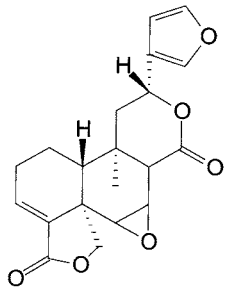


62 R^1= R^8= R^6= R^{10}= R^{11}= H
63 R^1= R^8= R^{10} = R^{11}= H; R^6= OH
64 R^1= R^6= R^{10}= R^{11}= H ; R^8= OH
65 R^1= R^8= R^6= R^{11}= H; R^{10}= OH



71

66 R^1= R^8= R^{10}= R^{11}= H; R^6= OAc
67 R^1= R^6= R^8= R^{11}=H; R^{10}= OAc
68 R^1= R^{11}= OAc; R^8= R^6= R^{10}= H
69 R^1= OH; R^8= R^6= R^{10}= R^{11}= H
70 R^1= R^8= R^6= R^{10}= H; R^{11}= OAc



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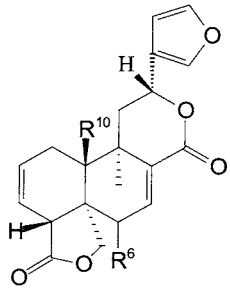
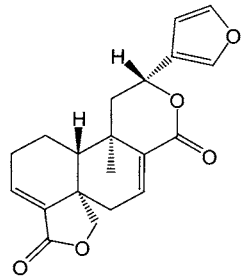


73 R^6= H; R^{10}= OH
74 R^6= OH; R^{10}= H



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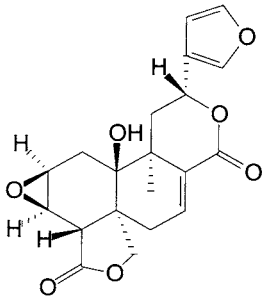

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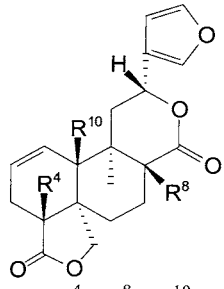
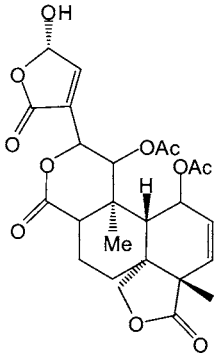


77 R^4= R^8= R^{10}= OH
78 R^4= R^8= R^{10}= H



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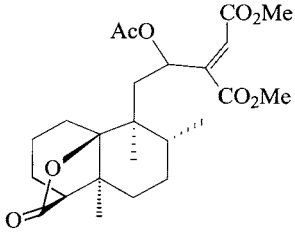

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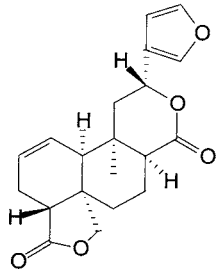

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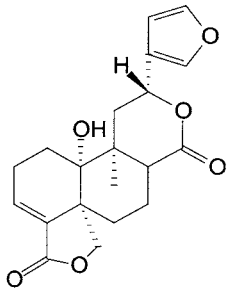

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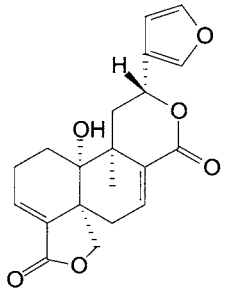

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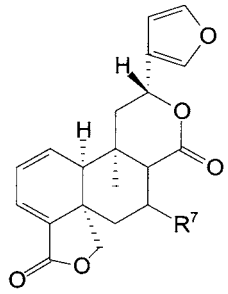
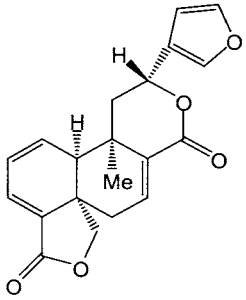


84 R^7= H
85 R^7= OAc



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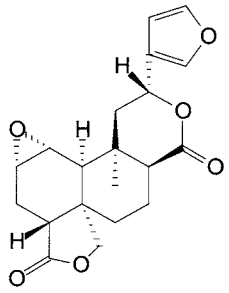

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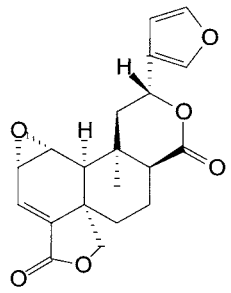

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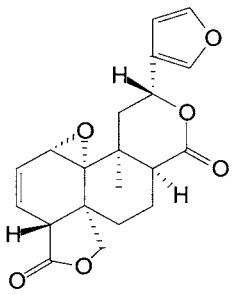

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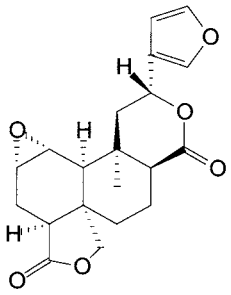
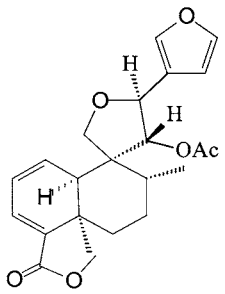


90

The third category is represented with the neoclerodan-18,19-olides, C-9 spiro linked wih a five member etherocycle bearing a furan group on C-12:

1. Neoclerodan-1,3-dien-18,19-olide **(88).**

2. Neoclerodan-12:20-epoxy-18,19-olides **(89-96).**



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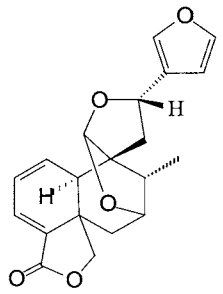

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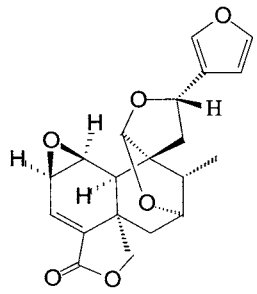

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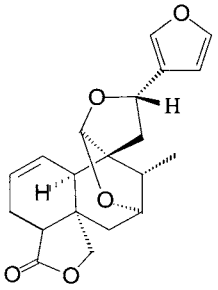

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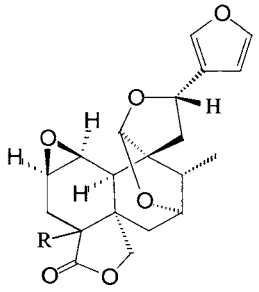
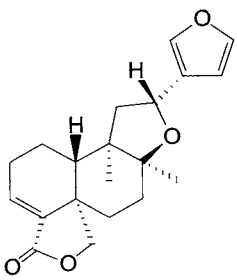


95 R = α-H **96** R = β-H

The fourth category is represented with neoclerodan-3-en-18,19-olides with a C8-C9 fused five member etherocycle with:

1. a furan group on C-12 **(97-98).**

2. a second lactone group on C15-C16 **(99-103).**



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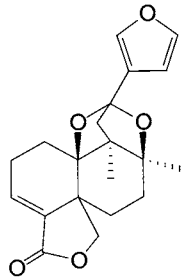

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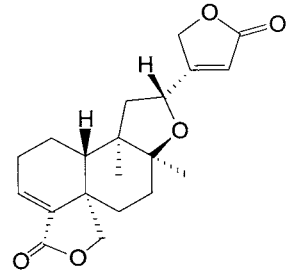

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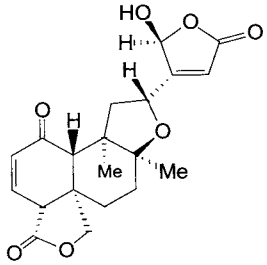
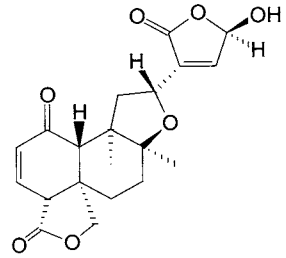


100
101 Epimer at C_{16}

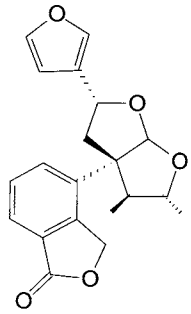


102
103 Epimer at C_{15}

Seco-clerodanes

The following n,m-seco-clerodanes present a diterpenoid skeleton which could derive from the Cm-Cn cleavage bond of a clerodane type.

1. 5,6-seco-clerodanes **(104-108).**
2. 5,10-seco-clerodanes **(109-110).**
3. 9,10- seco-clerodanes **(111-112).**



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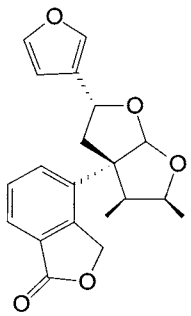

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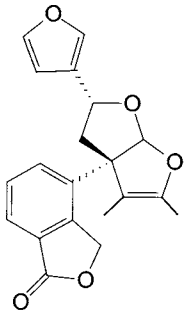

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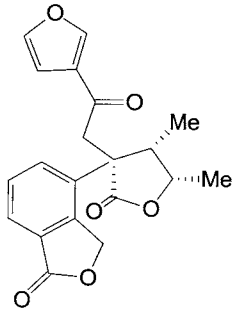

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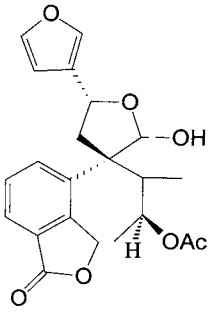

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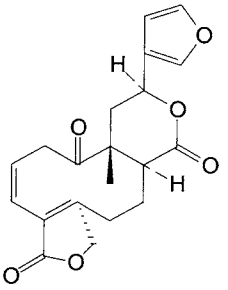
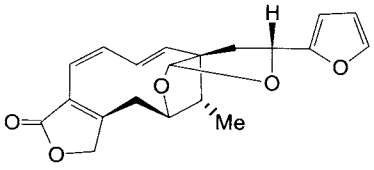
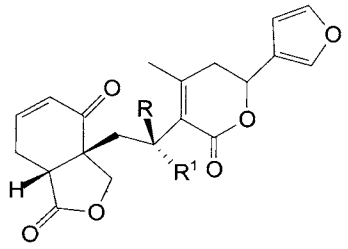


109



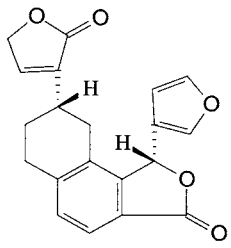
110



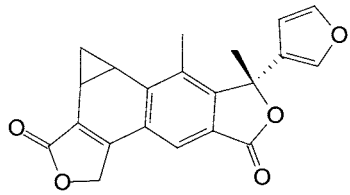
111R = H; R^1= OAc
112 R = OH; R^1= H

Rearranged clerodanes

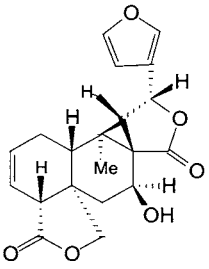
The rearranged clerodane **(113)**, which has an unusual structure, is the first natural product with a substituted tetralin skeleton which could be biogenetically derived from a clerodane precursor.The rearranged clerodane **(114-115)** named salvipuberulin bearing a C3-C6-C6 ring system was isolated from *S.puberula* [106] while the other clerodane blepharolide A **(116)** with a different rearranged C3-C6-C6 was found in *S.blepharophylla* [107].



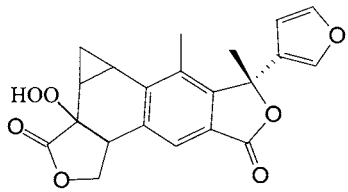
113



114

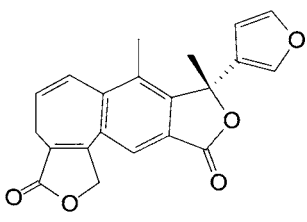


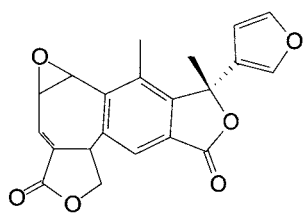
115

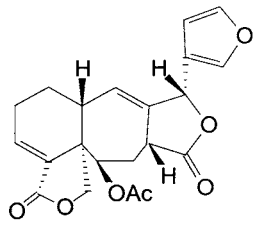


116

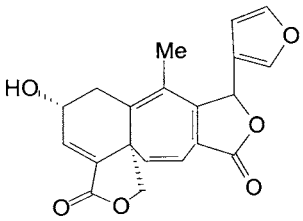
Rearranged neo-clerodanes **(117-118)** found in *S. puberula* [106] and *S. leucantha* [95] respectively, showed a 7/6 A/B ring system while a 6/7 A/B ring system was observed in the three salvigenane diterpenoids **(119-121).**

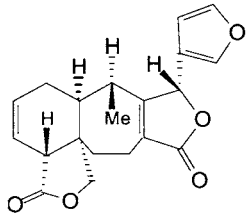






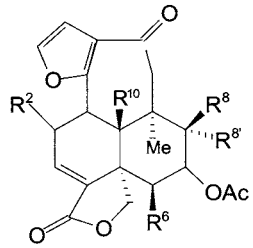
117 **118** **119**



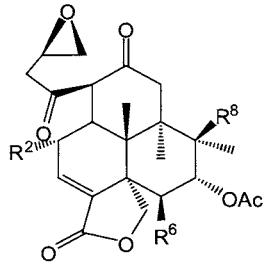


120 **121**

Rearranged clerodane skeleton (**122-126**) named languidulane diterpenes showed a 7 membered ring resulting from the C-1-C-16 linkage in a clerodanic precursor. Other rearranged clerodanes (**127-130**) named salvilanguidulane A-D were found in *S. languidula* [111].



122 R^2= OH; R^6= R^{10}= R^8 =H ; $R^{8'}$= Me
123 R^2= R^6= R^{10}= H ; R^8= $R^{8'}$= CH_2
124 R^2=R^{10}= H; R^6= OH; R^8=H; $R^{8'}$= Me
125 R^2 = R^6= R^8= H; $R^{8'}$= Me; R^{10}= OH
126 R^2 = R^6= R^{10}= H ; R^8 = OH; $R^{8'}$ = Me

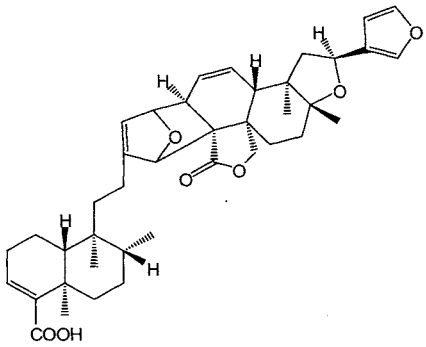


127 R^2= R^6= R^8= H **128** R^2= OH; R^6= R^8= H

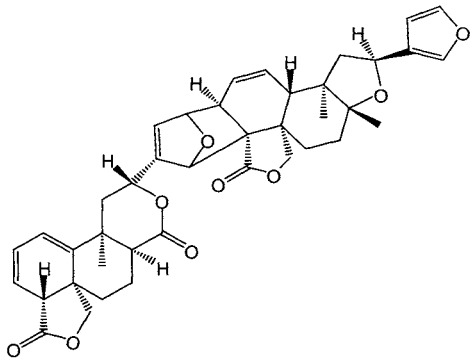
129 R^2= R^8= H; R^6= OH **130** R^2= R^6= H; R^8= OH

Clerodanes diterpene dimers

Recently, 2 clerodane dimers **(131-132)** have been isolated from *S. wagneriana [89].*



131



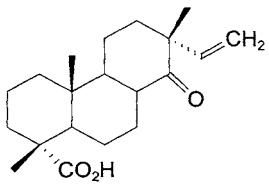
132

Tricyclic diterpenoids

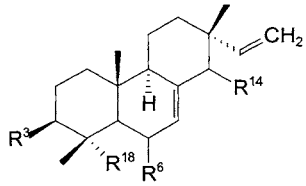
Tricyclic diterpenoids were found in numerous species. They may be divided into 2 categories, pimaranes and abietanes.

Pimaranes

14-oxopimaric acid **(133)** and relatives **(134-155)** have been reported from numerous species listed in Table 3.

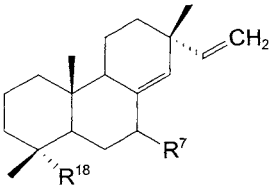


133

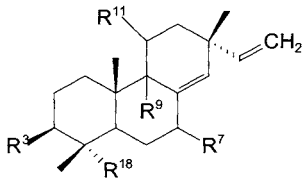


134 R^3= R^6= R^{14}= H; R^{18}= CO_2H
135 R^3= R^6= H; R^{14}= OH; R^{18}= CO_2H
136 R^3= OH; R^6= R^{14}= H; R^{18}= CO_2H
137 R^3= R^{14}= H; R^6= OH; R^{18}= CO_2H
138 R^3= R^6= R^{14}= H; R^{18}= CH_3

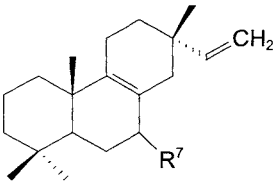
139 R^3= R^6= H; R^{14}= OH; R^{18}= CH_3
140 R^3= R^6= H; R^{14}= OH; R^{18}= CH_2OH
141 R^3= R^6= R^{14}= H; R^{18}= CHO



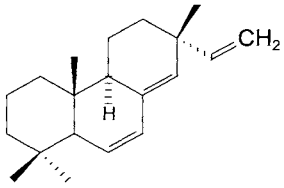
142 R^7= H; R^{18}= CO_2H
143 R^7= OAc; R^{18}= CO_2H
144 R^7= αOH ; R^{18}= CO_2H
145 R^7= βOH ; R^{18}= CO_2H
146 R^7= O ; R^{18}= CO_2Me
147 R^7= OH ; R^{18}= CO_2Me



148 R^3= R^9= R^{11}= H ; R^7= OH; R^{18}= CH_2OH
149 R^3= R^{11}= O; R^7= R^9= OH ; R^{18}= Me
150 R^3= R^7= R^9= R^{11}= H ; R^{18}= CH_3
151 R^7= OH; R^3= R^9= R^{11}= H; R^{18}=Me
152 R^7= O; R^3= R^9= R^{11}= H; R^{18}= Me

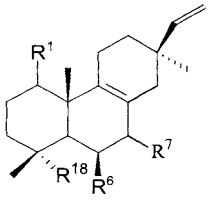


153 R^7= H
154 R^7= O

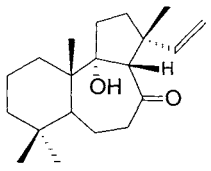


155

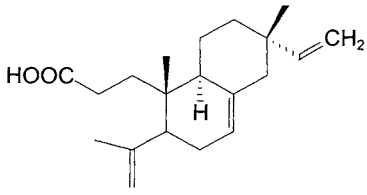
Epipimaranes **(156-157)** were respectively isolated from *S. heldrichiana* [114] and *S. ceratophylla* [119]. A pimarane with a C6-C-7-C-5-ring system **(158)** has been found in *S. parryi* [38] while the seco-pimarane **(159)**, which exhibited *in vitro* non-specific spasmomytic activity, was reported from *S. cinnabarina* [37].



156 R^1= R^6= H; R^7= O ; R^{18}= CO_2H
157 R^1= O; R^6= OH; R^7= H;



158



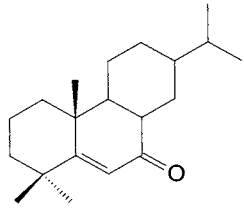
159

R^{18}= Me

Abietanes

They may be divided into 14 categories.

1. Abietanes with one or two carbone-carbone double bonds such as pachystazone **(160)** and relatives **(161-166)** which are abietane type diterpenes possessing one or two double bonds bearing an isopropyl or a propenyl group on C-13. The abietane lactone **(166)** was isolated from *S. heldrichiana* [115].



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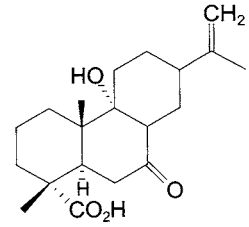
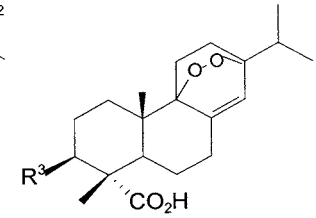
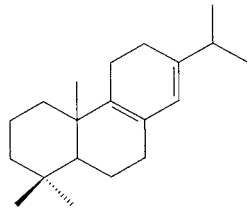


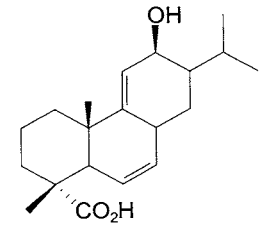
161



162 R^3= OH ; **163** R^3= OAc



164



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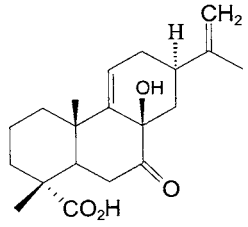
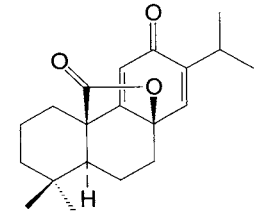


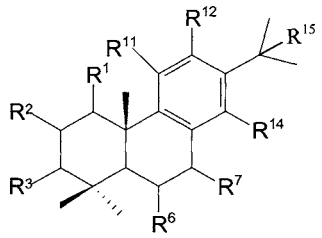
166



167

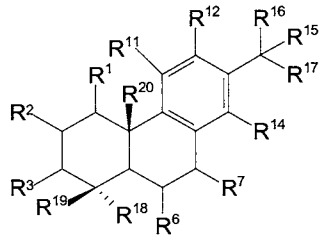
2. Abietanes with an aromatic ring

Among the abietane-type diterpenoids possessing an aromatic ring **(168-198),** inuroyleanol **(198)** was very recently isolated for the first time from the genus and from the Algerian species *S. barrelieri* [3].

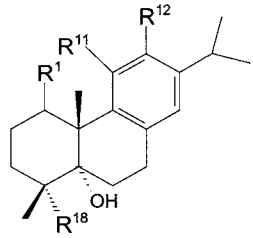


168 R^1= R^2= R^3= R^6= R^7= R^{11}= R^{12}= R^{14}= R^{15}= H
169 R^3 = O
170 R^7 = O
171 R^{11}= OH; R^{12} = OMe
172 R^6 = O; R^{12} = OOH
173 R^3= O; R^{12} = OMe; R^{14} = OH
174 R^3= R^7= O; R^{11}= R^{12}= R^{14}= OH
175 R^7= O; R^{11}= OH; R^{12}= OMe
176 R^7= O; R^{11}= R^{12}= OH
177 R^7= O; R^3= R^{11}= R^{12}= OH
178 R^7= O; R^6= R^{11}= R^{12}= OH
179 R^2= R^{11}= R^{12}= OH
180 R^{12}= OH
181 R^1= O; R^{12}= OH
182 R^6= O; R^{12}= OH
183 R^{12} = R^{14}= OH
184 R^{12}= OMe
185 R^2= R^{12}= OMe
186 R^1= R^7= O; R^6= βOH; R^{12}= OH
187 R^6= R^7= O; R^{12}= OH
188 R^1= O; R^{11}= R^{12}= OH
189 R^7=O;R^{11}=R^{14}=OH;R^{12}= OMe
190 R^2= OH
191 R^2= OH; R^7= O
192 R^2= R^{12}= OH
193 R^6=R^7=R^{11}=OH; R^{12}= OMe
194 R^7=O.;R^{12}=OH
195 R^2= R^{12}= OH ; R^7= O
196 R^6= O; R^7 = R^{12}= OH
197 R^6=R^{11}= OH; R^{12}= OMe
198 R^7= O; R^{11}= R^{12}= OH R^{12}=OMe

In abietatrienes **(199-236)**, including the antibacterials **(204)** [10], **(226)** [11] and **(232)** [5], the Me-15, Me-16, Me-18 and Me-19 have been oxidized. Abietatetraenes **(237-238)** bearing a 5-OH group were isolated from
S. microstegia [154] and *S. candidissima* [40], respectively.

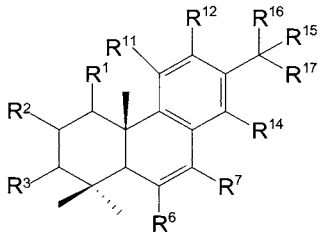


199 R^{18}= CO_2H
200 R^3= OH; R^{18}= CO_2H
201 R^1= O; R^{18}= CO_2H
202 R^7= R^{15}= OH
203 R^7= O; R^{15}= OH
204 R^7= OH; R^{18}= OH
205 R^3= R^{12}=R^{16}=OH
206 R^{11}= R^{12}= OH; R^{20}= CH_2OH
207 R^{11}= R^{12}= R^{16}= OH; R^{20}= COOH
208 R^{11}= R^{12}= OH; R^{20}= CO_2H
209 R^7=O, R^{11}=R^{12}=OH; R^{20}= CO_2H
210 R^{11}= R^{12}= OH; R^{16}= CH_2OH; R^{20}= CO_2H
211 R^{11}= OH; R^{12}= OMe; R^{20}= CO_2H
212 R^{11}= OAc; R^{12}= OH; R^{20}= CO_2H
213 R^{11}= R^{12}= OH; R^{16}= OAc; R^{20}= CO_2H
214 R^3= O ; R^{20}= CO_2H
215 R^6= O; R^7= αOH; R^{11}= R^{12}= R^{20}= CO_2H
216 R^6= O; R^7= βOH; R^{11}= R^{12}= OH; OH; R^{20}= CO_2H
217 R^{11}=R^{12}=OH; R^{20}=CO_2Me
218 R^{11}=R^{12}=R^{15}=OH; R^{20}= CHO
219 R^{12}= OH; R^{18}= CHO
220 R^{11}= R^{15}= OH
221 R^{12}= OH; R^{16}= CH_2OH
222 R^7=O; R^{12}= OH; R^{18}= CHO
223 R^{12}= OH; R^{20}= COMe
224 R^6= R^7= O;R^{11}= R^{14}= OH; R^{12}= OMe; R^{20}= CH_2OH
225 R^7= O; R^{12}=R^{15}= OH
226 R^7= O; R^{11}= R^{14}=OH; R^{12}= OMe; R^{20}= CHO
227 R^2= R^{14}= OH; R^{18}= CO_2H
228 R^{12}= OH ; R^{20}= CHO
229 R^{12}= OH ; R^{20}= CO_2H
230 R^{12}= OMe ; R^{20}= CO_2H
231 R^{12}= OMe; R^{20}= CO_2Me
232 R^{18}= CH_2OH
233 R^{12}= OH; R^{18}= CH_2OAc
234 R^{11}= OH; R^{12}= OMe; R^{18}= CO_2H
235 R^2= R^{15}= OH; R^7= O
236 R^7= O; R^{18}= CH_2OAc

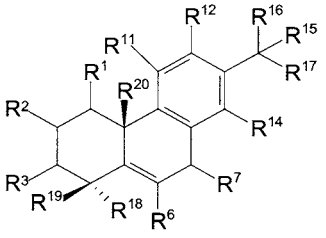


237 R^1= H; R^{11}= R^{12}= OH; R^{18}= Me
238 R^1= O; R^{11}= R^{12}= H; R^{18}= CO_2H

Abietatetraenes bearing a fourth double bond on C6-C7 **(239-247)** or on C5-C6 **(248-257)**, beside the aromatic cycle have been found in numerous species *e.g* cryptanol **(242)** which was isolated from 14 species comprising the algerian species *S. jaminiana* [2], as well as 6-hydroxysalvinolone **(255)**.



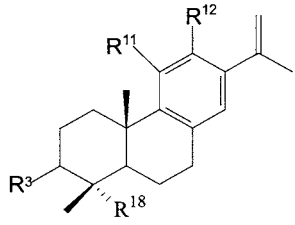
239 R^6= R^{12}= R^{14}= OH
240 R^{11}= R^{12}= OMe; R^{20}= CO_2Me;
241 R^{11}= R^{12}= R^{14}= OH; R^{20}= CO_2H
242 R^{11}= R^{12}= R^{14}= OH
243 R^{12}= OH
244 R^{12}= OH ; R^{16}= CH_2OH
245 R^2= O ; R^{12}= OH
246 R^2= OH
247 R^7= R^{12}= OH



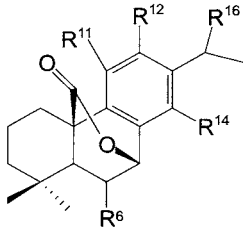
248 R^7= O; R^{11}= R^{12} = R^{14}= OH; R^{20}= CH_2Me
249 R^6= R^{11}= OH; R^{12}= OMe
250 R^{12}= OMe; R^{20}= CO_2H
251 R^6= R^{12}= OH; R^7= O
252 R^6= OH; R^7= O; R^{12}= OMe
253 R^1= R^7= O; R^6= OH
254 R^7= O; R^{11}= R^{12}= OH
255 R^6= R^{11}= R^{12}= OH; R^7= O
256 R^7= O; R^{11}= OH; R^{12}= OMe
257 R^{11}= R^{12}= OH

Antiochic acid **(258)** [169] and tchihatine **(259)** [167] are two abietatrienes bearing a propenyl group on C-14.

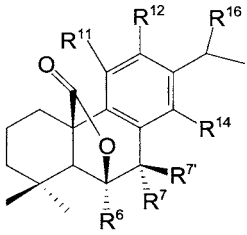
Abietatrien-20,7-olides **(260-269)** and abietatrien-20,6-olides **(270-281)** have been isolated from numerous species (Table 3).



258 R^3= OH; R^{18}= CO_2H
259 R^3= R^{12}= H; R^{11}= OH; R^{18}= Me



260 R^{11}= R^{12}= OH
261 R^{11}= R^{12}= OH; R^{16}= CH_2OH
262 R^{11}= R^{12}= OH; R^{16}= CH_2OAc
263 R^{11}= OH; R^{12}= OMe
264 R^{11}= R^{12}= OMe
265 R^{11}= R^{14}= OH
266 R^{11}= R^{12}= R^{14}= OH
267 R^6= O; R^{11}= R^{12} =OH
268 R^6= R^{11}= R^{12}= OH
269 R^6= R^{11}= R^{12}= OH; R^{16}= CH_2OH



270 $R^{7'}$, R^7= O; R^{11}= R^{12}= OH
271 $R^{7'}$= H; R^7= R^{11}= R^{12}= OH
272 $R^{7'}$= H; R^7= R^{11}= R^{12}= OH;
273 $R^{7'}$= H; R^7= OEt; R^{11}= R^{12}= OH ; R^{16}= CH_2OH
274 $R^{7'}$= H; R^7= OMe; R^{11}=R^{12}= OMe
275 $R^{7'}$=H; R^7= OH; R^{11}=R^{12}= OH

276 $R^{7'}$= H; R^{7}= OMe; R^{11}= R^{12}= OH; R^{16}= CH_2OH

277 R^{7}= H; $R^{7'}$= R^{11}= R^{12}= OH

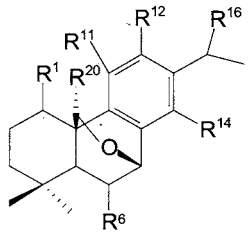
278 R^{7}= H; $R^{7'}$= R^{11}= R^{12}= OH

279 R^{6}= $R^{7'}$= OMe; R^{11}=R^{12}= OH; R^{7}= H; R^{16}= OMe

280 R^{7}= OMe; R^{11}=R^{12}= OH; R^{16}= Me

281 $R^{7'}$= OMe; R^{11}=R^{12}= OH; R^{16}= Me

6:20-epoxy abietatrienes **(282-286)** have been found in numerous species, as shown in Table 3.



282 R^{11}= R^{12}= OH

283 R^{12}= OMe; R^{11}= OH

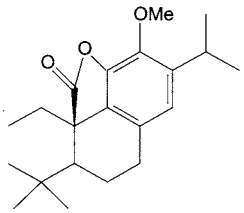
284 R^{11}= R^{12}= OH; R^{16}= CH_2OH

285 R^{6}= R^{11}= R^{12}= R^{20}= OH

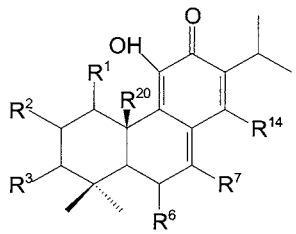
286 R^{1}= O; R^{12}= OMe

3. Methylene quinones abietanes

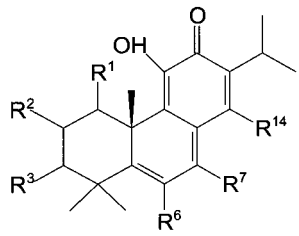
The serie of Methylene quinones abietanes (286-303) comprising taxodone **(288)**, possessing an antitumour activity [142] have been reported from a large number of *Salvi*a species.

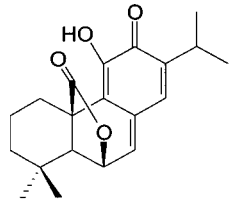


287



288 R^6= OH
289 R^6= O
290 R^2= OH; R^6= O
291 R^2= R^6= O
292 R^7= OH; R^6= O
293 R^2= OH
294 R^2= R^6= OH
295 R^2= R^6= R^7= OH
296 R^6= OH; R^{14}= OMe
297 R^2= R^7= R^{11}= OH



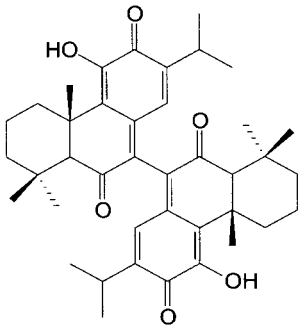


298 R^2= R^{11}= OH
299 R^1 to R^{14}= H
300 R^7= OH
301 R^2= R^7= OH
302 R^6= OMe

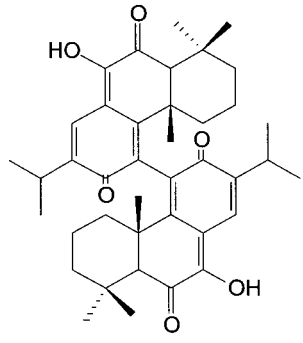
303

4. Abietane dimers

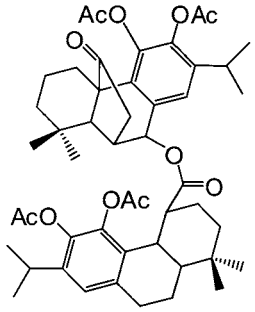
Seven abietane dimers **(304-310)** were found in *S. montbretti* [130], *S. canariensis* [1581] and *S. prionitis* [204, 205].



304



305



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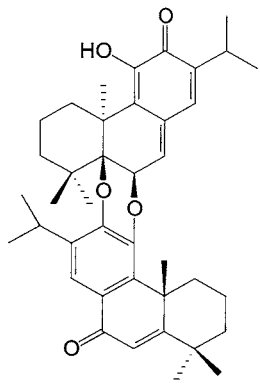
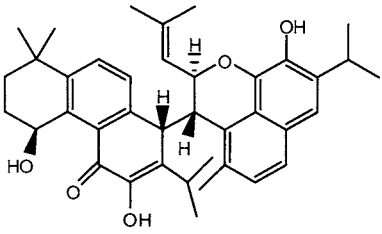


307



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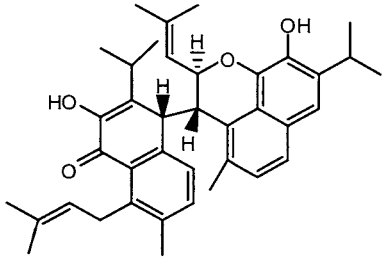
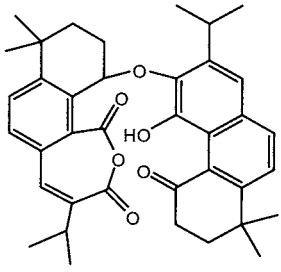


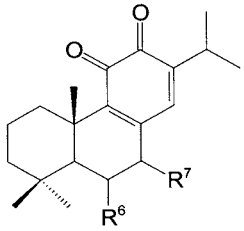
309

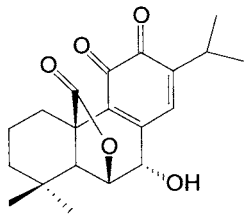


310

5. Orthoquinone abietanes

Orthoquinones abietanes **(311-315)** were reported from 8 *Salvia* species (Table 3).





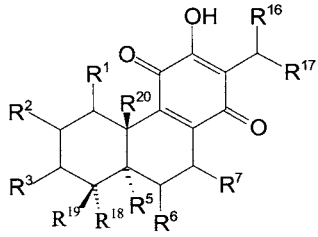
311 R^6= R^7= H
312 R^6= OH; R^7= H
313 αR^6= βR^7= OH
314 R^6= H; R^7= OH

315

6. Paraquinone abietanes

Royleanone **(316),** found in 17 *Salvia* species and relatives **(317-341)** including horminone **(319)** and the new diterpenes 7-oxobarrilierone **(340)** and its acetoxyderivative **(341)** which we have recently isolated from *Salvia barrelieri* [3]. The paraquinone abietanes may be 6,7-unsaturated **(342-345)** or 5,6-unsaturated **(346-347).** The double bond may be external **(348).**

Paraquinones abietanes bearing a lactone group in different positions have been reported from various species **(349-355).** The decalin moiety may be epoxidized at various positions **(356-362).**

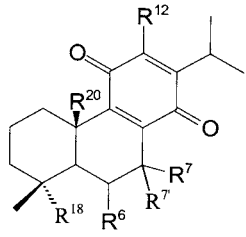


316 R^1 to R^7= H; R^{16} to R^{20}= Me
317 R^2= OH
318 R^6=β OH
319 R^7=α OH
320 R^7=β OH

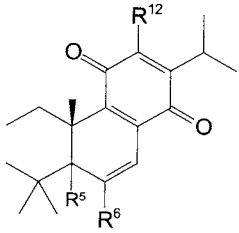
326 R^7= OAc; R^{19}= CH_2OH
327 R^7= OAc; R^{20}= CH_2OH
328 R^6= O; R^{18}= CO_2H
329 R^7= OH; R^{18}= CO_2H
330 R^7= OH; R^{18}= CHO

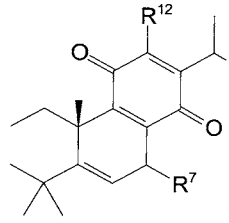
321 R^{16}= CH_2OH
322 R^{19}= CH_2OH
323 R^7= OAc
324 R^7= O
325 R^6= OH; R^7= OAc

331 R^7= OAc; R^{20}= CHO
332 R^7= OH; R^{20}= CHO
333 R^{16}= R^{17}= CH_2OH
334 R^3= R^7= O



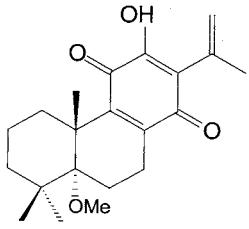
335 R^6= $R^{7'}$= H; R^7= OH; R^{12}= OMe; R^{18}= R^{20}= Me
336 R^6= R^7= $R^{7'}$= R^{12}=H; R^{18}= R^{20}= Me
337 R^6= O; R^{12}= OMe; R^{18}= CO_2H; R^{20}= Me
338 R^6= O; R^7= $R^{7'}$= OMe; R^{12}= H; R^{18}= R^{20}= Me
339 R^6= R^7= $R^{7'}$= H; R^{12}= OMe; R^{18}= Me; R^{20}= CO_2H
340 R^6=H; R^7= $R^{7'}$= O; R^{12}= OMe; R^{18}= R^{20}= Me
341 R^6= R^7=H; = $R^{7'}$= OAc; R^{12}= OMe; R^{18}= R^{20}= Me





342 R^5= R^{12}= OH; R^6= H
343 R^5= R^6= H; R^{12}= OH
344 R^5= R^6= R^{12}=H
345 R^5= R^{12}= H; R^6= OH

346 R^7=OH; R^{12}= OMe
347 R^7= OAc; R^{12}= OMe



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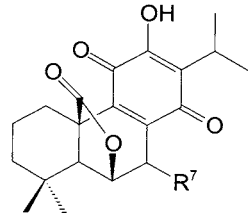
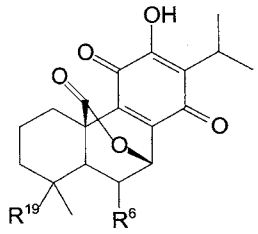
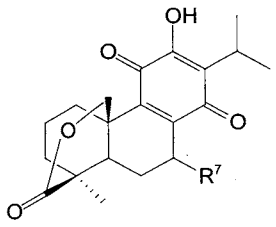


349 R^7= OEt

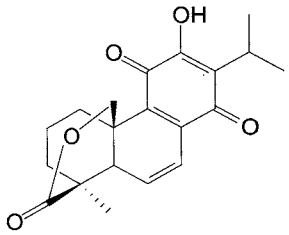
350 R^7= αOH



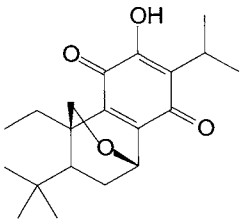


351 R^6= OH, R^{19}= CH_2OH
352 R^6= H; R^{19}= Me

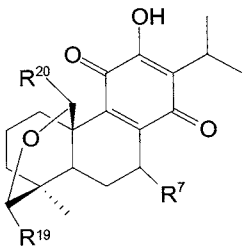
353 R^7= 7α-(3β-hydroxyolean- 12- en-28-oate)
354 R^7= OAc
355 R^7= OH



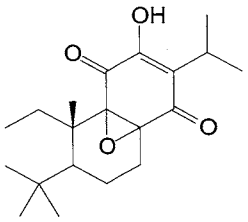
356



357



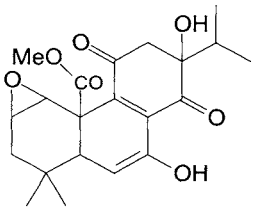
358 R^7= R^{20}= OH; R^{19}= H
359 R^7= OMe; R^{19}= H; R^{20}= OH
360 R^7= OH; R^{20}= H; R^{19}= OH
361 R^7= OH; R^{20}= H; R^{19}= OM



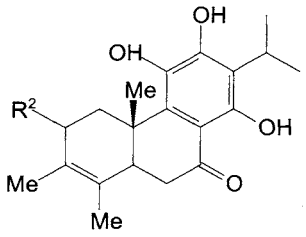
362

7. Rearranged abietanes

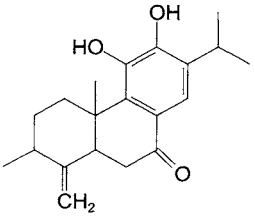
Rearranged abietanes and other rearranged abietanes **(363-367)** were found in numerous species as mentioned in Table 3.



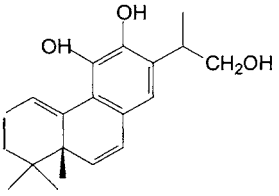
363



364 R^2= H
365 R^2= O



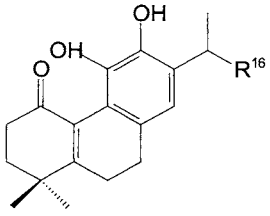
366



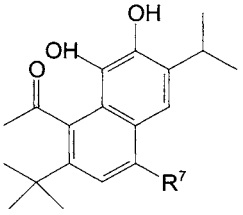
367

8. Nor-abietanes

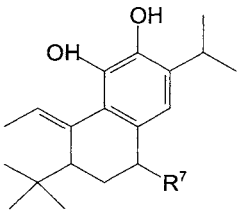
Abietanes **(368-385)** corresponding to the desappearence of Me-20, such us arucadiol **(355)** exhibiting a strong cytotoxicity against P338 leukaemia cells have been described.



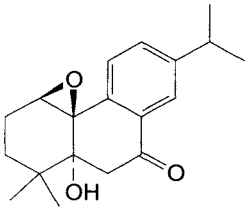
368 R^{16}= Me
369 R^{16}= CH_2OH

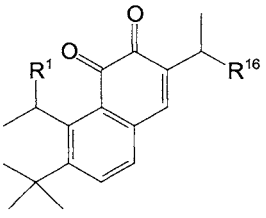


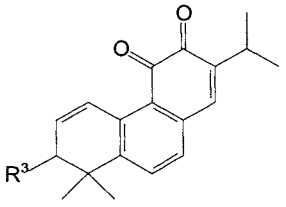
370 R^7= H
371 R^7= OH



372 R^7= O
373 R^7= H



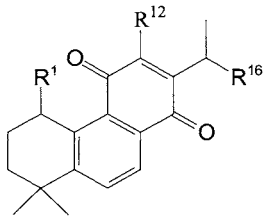




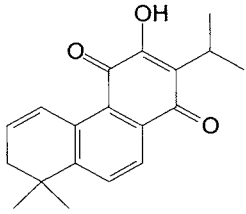
374

375 R^1= H; R^{16}= Me
376 R^1= OH; R^{16}= Me
377 R^1= H; R^{16}= CH_2OH

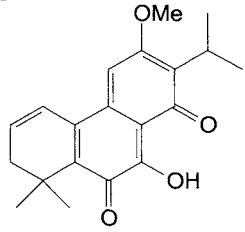
378 R^3= H
379 R^3 = O



380 R^1= H; R^{12}= OH; R^{16}= CH_2OH
381 R^1= H; R^{12}= OH; R^{16}= CH_2O-oleoyl
382 R^1= O; R^{12}= OH; R^{16}= Me
383 R^1= H; R^{12}= H; R^{16}= CH_2OH

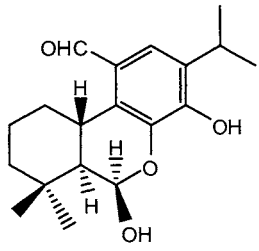


384



38

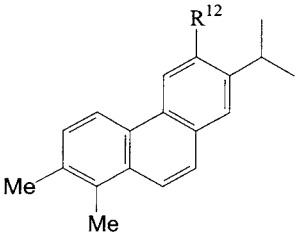
Very recently, an heterocylic nor-abietane **(386)** has been isolated from *S. dorri* [199]



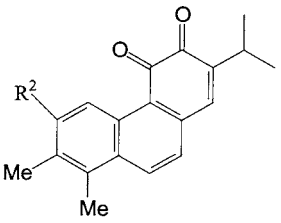
386

9. Rearranged nor-abietanes

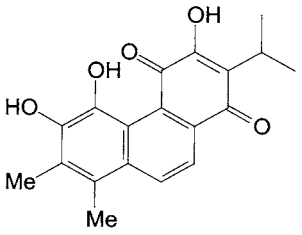
Nor-abietanes **(387-388)** possessing an anti-tuberculous activity against *Mycobacterium* [5, 6] and nor-abietanes **(389-391)** resulted from the migration of Me-18 to C -3.



387 R^{12}= OMe
388 R^{12}= OH



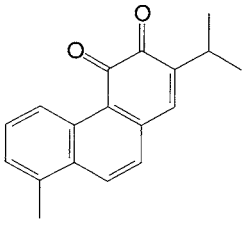
389 R^2= OMe
390 R^2= OH



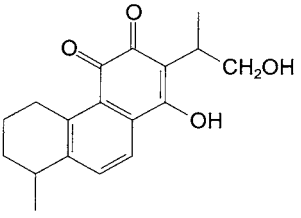
391

10. Dinor-abietanes

Nor-abietanes **(392-402)** corresponding to the desappearance of Me-20 and Me-18 or the substitution of the isoproyl group by the methyl group have been reported from few species



392



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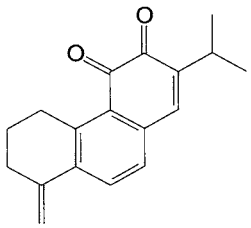
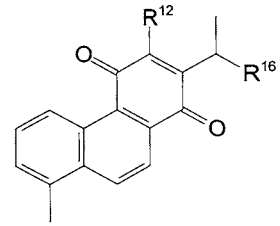
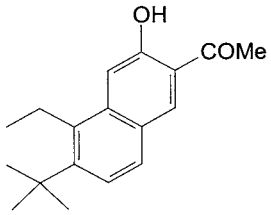


394



395 R^{12}= OH; R^{16}= Me
396 R^{12}= H; R^{16}= Me
397 R^{12}= H; R^{16}= CH_2OH
398 R^{12}= H; R^{16}= CH_2Ooleoyl



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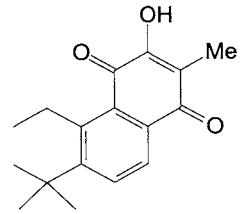

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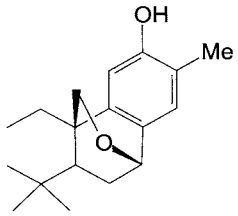

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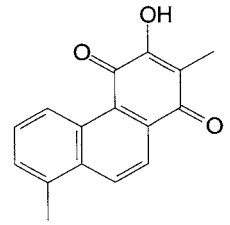
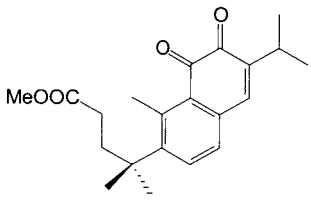


402

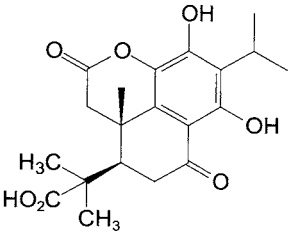
11. Seco-abietanes

Seco-n,m-abietanes resulted from the cleavage of the Cn-Cm bond. The following seco-abietane have been reported from *numerous Salvia* species (Table 3).

. Seco-1,2-abietane **(403)**
. Seco-2,3-abietanes **(404-405)**
. Seco-3,4-abietanes **(410-409)**
. Seco-4,5-abietanes **(408-437)**, including the cardioactives **(417)** and **(421)** and the hypertensive **(424)**.
. Seco-6,7-abietanes **(438-442)**
. Seco-7,8-abietane **(443)**



403



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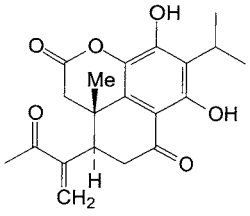
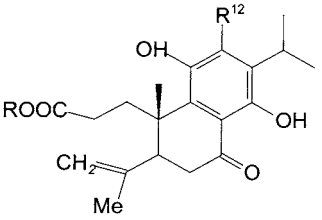
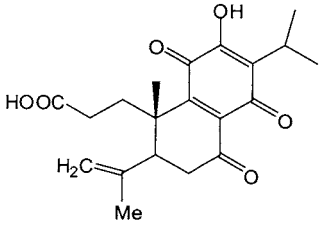


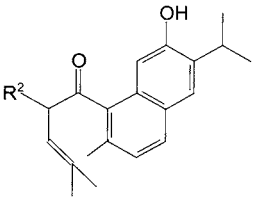
405



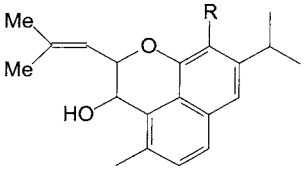
406 R^{12} = OH ; R = H
407 R^{12} = OMe; R = H
408 R^{12} = OH; R = Me



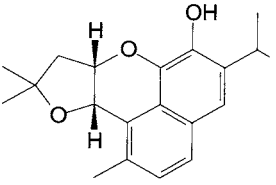
409



410 R^2= OH
411 R^2= OAc



412 R= OEt
413 R= H



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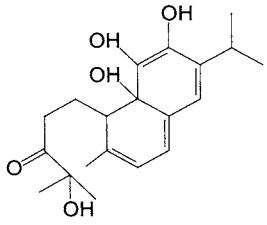

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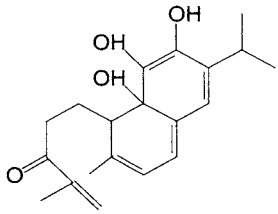

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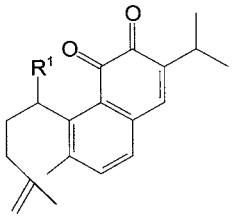
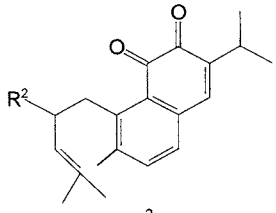
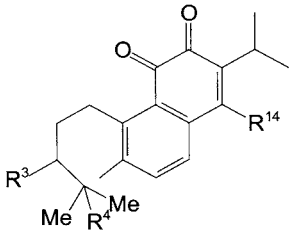


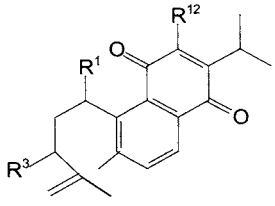
417 R^1= H
418 R^1= O



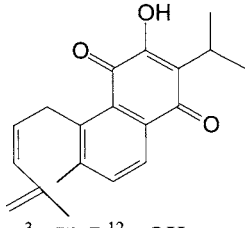
419 R^2= H
420 R^2= OH



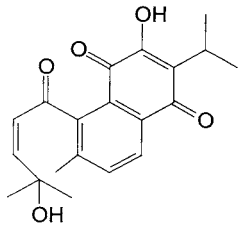
421 R^3= H; R^4= OH R^{14}= H
422 R^3= H; R^4= R^{14}= OH
423 R^3= O; R^4= OH; R^{14}= H
424 R^3= O; R^4= R^{14}= H



425



426 R^1= R^3= H; R^{12}= OH
427 R^1= O; R^3= H; R^{12}= OH
428 R^1= H; R^3= O; R^{12}= OH
429 R^1= R^3= H; R^{12}= OAc
430 R^1= H; R^3= O; R^{12}= OCH_2-CH = $C(Me)_2$



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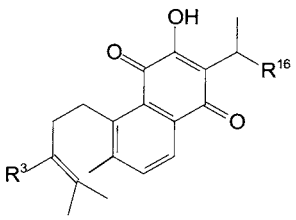
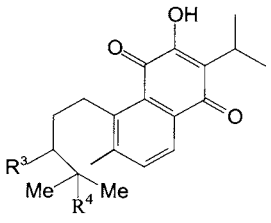
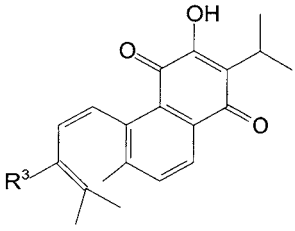


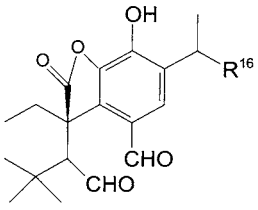
432 R^3= H; R^{16}= Me
433 R^3= H; R^{16}= CHO
434 R^3= OH; R^{16}= Me



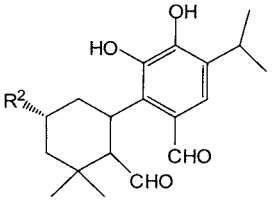
435 R^3= H; R^4= OH
436 R^3= O; R^4= H



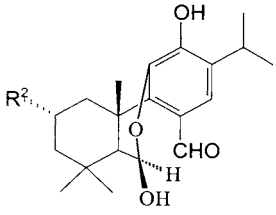
437 R^3= OH



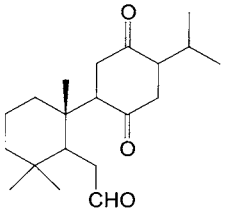
438 R^{16}= Me
439 R^{16}= CH_2OH



440 R^2= OH



441 R^2= H
442 Epimere of **422**



443

12. Tanshinones

As listed in Table 3, the following tanshinones were mainly found in Asian countries:

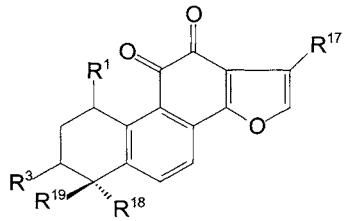
. Tanshinones IIA and derivatives **(444-453)**

. Tanshinone **(454)** bearing a C1-C2 double bond

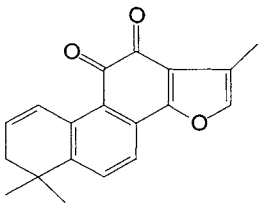
15,16-dihydrotanshinone and relatives **(455-459)**

.13,14-dihydrotanshinone **(460)** reported from *S. milthiorrhiza* [161, 253] as well as miltionone [257] **(461)** and danshenol B [164, 262] **(462)** while lanugon Q [133] **(463)** and 6-deoxo-5,6-dehydrolanugon Q [135] **(464)** were found in *S. apiana* [140, 142]

. Tanshinones I and relatives, possessing an A aromatic ring **(465-470)**
1,2:15,16 tetrahydrotanshinquinone and 1,2 dihydrotanshinquinone **(471-472)**.
. Methylene tanshinquinone **(473)** and its hydroxy derivative **(474)** and nor-tanshinone **(475)** and przewaquinone F **(476)** and their dehydro derivatives
(478-479).
Isotanshinones **(480-485)** presenting a furan or a dihydrofuran ring on C12-C13.



444 R^1= R^3= H; R^{17}= R^{18}= R^{19}= Me
445 R^1= OH; R^3= H; R^{17}= R^{18}= R^{19}= Me
446 R^1= H; R^3= OH; R^{17}= R^{18}= R^{19}= Me
447 R^1= H; R^3= OH; R^{17}= CH_2OH; R^{18}= R^{19}= Me
448 R^1= R^3= H; R^{17}= R^{19}= Me; R^{18}= CH_2OH
449 R^1= R^3= H; R^{18}= R^{19}= Me; R^{17}= CH_2OH
450 R^1= R^3= H; R^{17}= R^{18}= Me; R^{19}= CO_2Me
451 R^1= H; R^3= OH; R^{18}= OH; R^{19}= Me
452 R^1= H; R^3= OH; R^{18}= Me; R^{19}= OH
453 R^1= R^3= H; R^{18}= Me; R^{19}= OH



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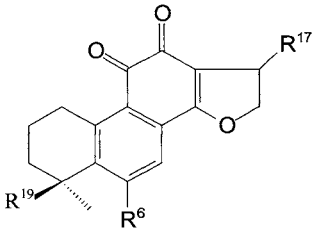
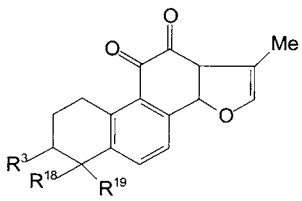
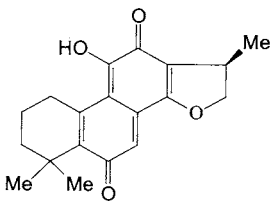


455 R^{17}= R^{18}=Me; R^6= H
456 R^6= R^{17}= R^{18}= Me
457 R^6= H; R^{19}= Me; R^{18}= CO_2Me
458 R^{18}= R^{19}= Me; R^{17}= CH_2OH
459 R^{17}= R^{19}= Me; R^{18}= CHO



460 R^3= H; R^{18}= CH_2OH; R^{19}= OH



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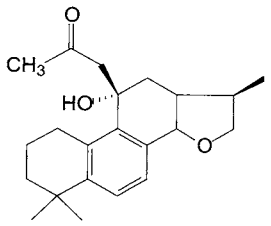

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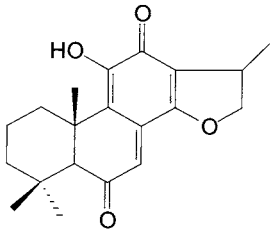

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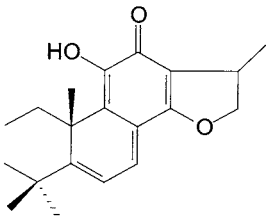

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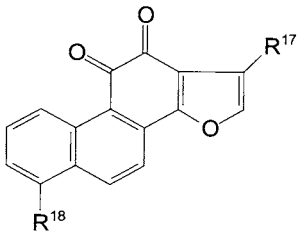
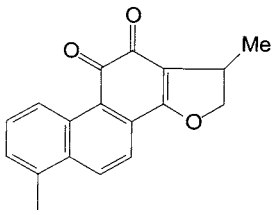


465 R^{17}= R^{18}= CH_3
466 R^{17}= CH_2OH, R^{18}= CH_3
467 R^{18}= CHO, R^{18}= CHO



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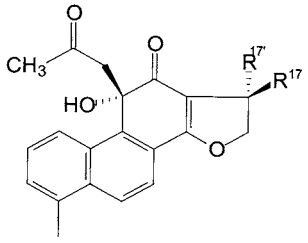
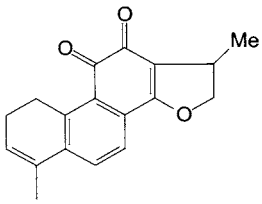


469 R^{17}= Me; $R^{17'}$= H
470 R^{17}= H; $R^{17'}$= Me



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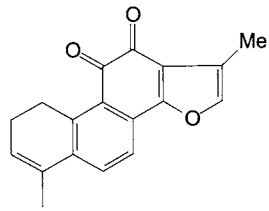

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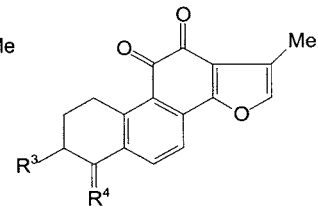
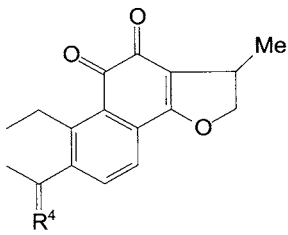
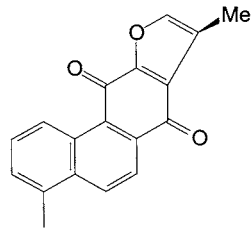


473 R^4= CH_2; R^3= H
474 R^4= CH_2; R^3= OH
475 R^4= O; R^3= H
476 R^4= CH_2OH, H ; R^3= H



477 R^4= O
478 R^4= CH_2



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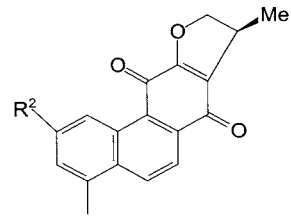
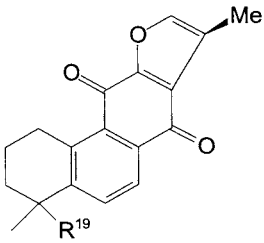
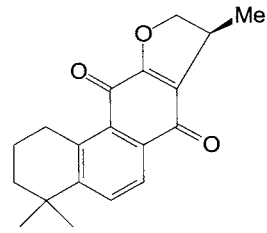


480 R^2=H
481 R^2=OH



482 R^{19}= Me
483 R^{19}= CH_2OH



484

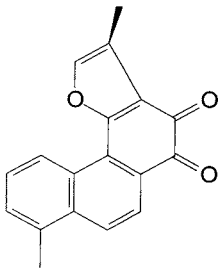
13. Tanshinones derivatives

Isotanshinones **(485-486)** presenting a furan or dihydrofuran on C11-C13.

. Tanshinlactones, dihydrofuranolactonized tanshinones and dihydrofuranoanhydrides as well as the pyranotanshinones **(497-492),** reported from *S. milthiorhiza.*

. Cryptoacetalides **(493-494)** danshenspirolactones **(495-498)** found in *S. aegyptiaca* and *S. milthiorrhiza.*

. Neoprzewaquinone A **(499)** isolated from *S. przewalskii*



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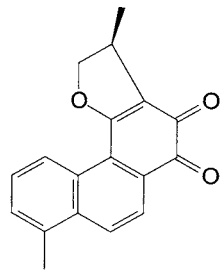

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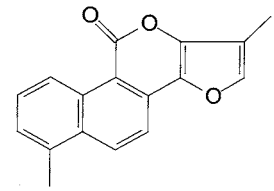

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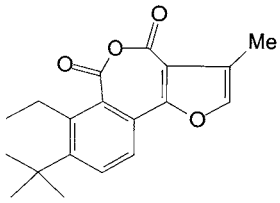

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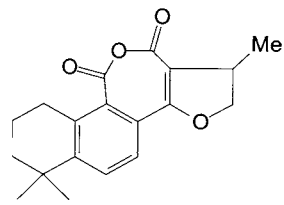

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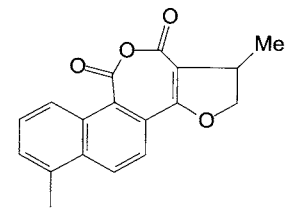

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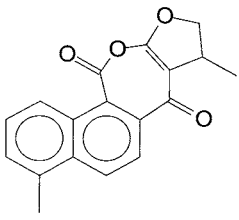

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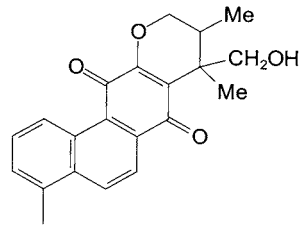

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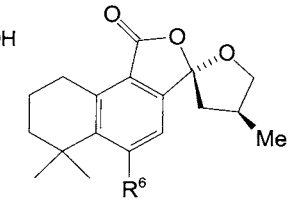
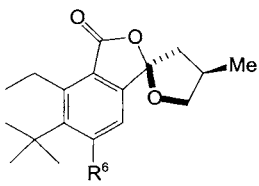
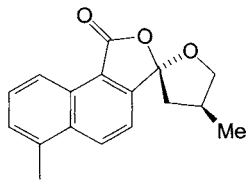


493 R^6= H
494 R^6= Me



495 R^6= H
496 R^6= Me



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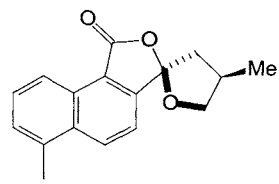

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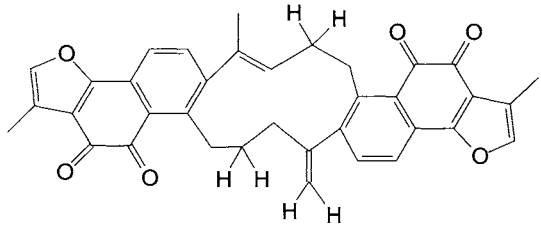
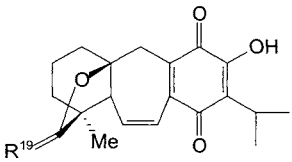


499

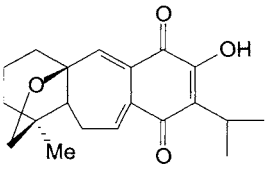
14. Rearranged abietane diterpenoids

As shown in Table 3, the following abietanes resulted from different rearrangements:

. Icetexone **(500)** and relatives **(501-514)**
. Icetexan-18,6-olides **(515-516)**
. Rearranged abietanes with:
6,6,7-ring **(517-518)**
6,7,7-ring **(519)**
7,6,6-ring **(520-524)**
6,6,6-ring **(525- 527)**
6,5,6-ring **(528-530)**
5,7,6-ring **(531)**
6,5-heterocycle, 6-ring **(532-534)**
6,6-lactonic, 6-ring **(535)**
6,7-lactonic, 5-ring **(536)**
. Microstegiol and derivatives **(537-539)**
. Particular rearranged abietanes **(540-547)** isolated from *S. prionitis*.



500 R^{19}= O ; H-5α
501 R^{19}= O; H-5β
502 R^{19}= H, H
503 R^{19}= OAc, H
504 R^{19}= OAc, H



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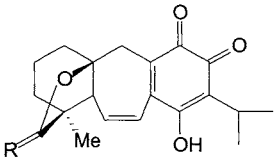

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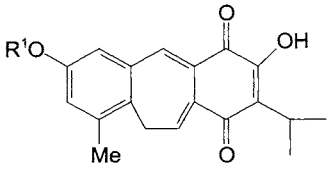
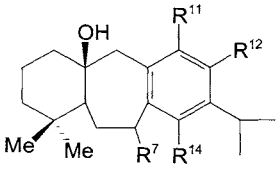
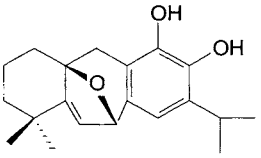


507 R^1= Me
508 R^1= H



509 R^7= R^{14}= H; R^{11}= OH; R^{12}= OMe
510 R^7= R^{14}= H; R^{11}= R^{12}= OH
511 R^7= O; R^{11}= R^{14}= OH; R^{12}= OMe
512 R^7= R^{11}= R^{14}= H; R^{12}= OH



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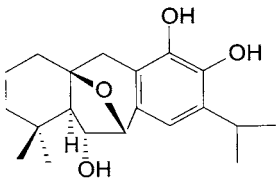

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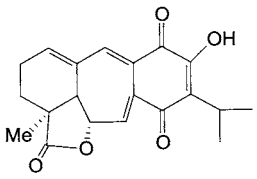

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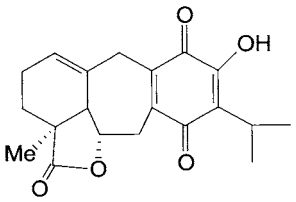

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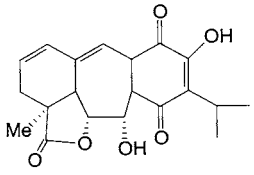

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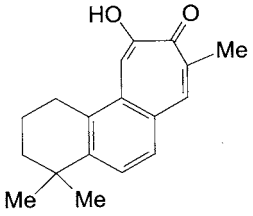

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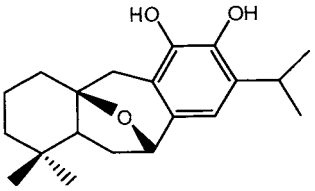

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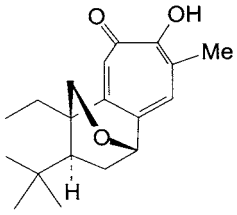

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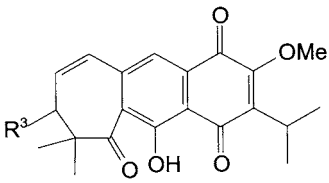
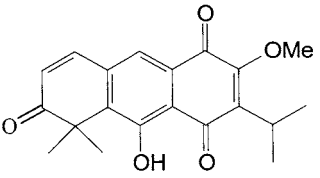


521 R^3= H
522 R^3= OH
523 R^3= O



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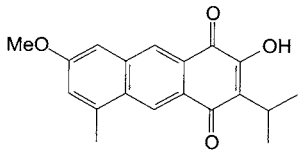

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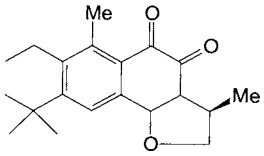

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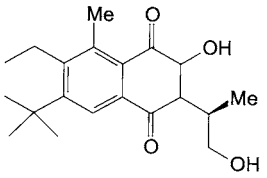

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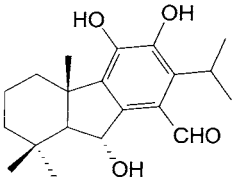

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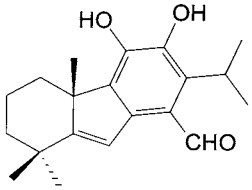

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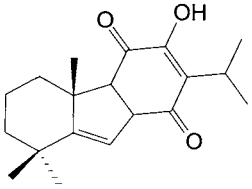

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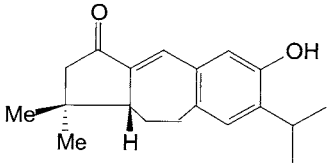

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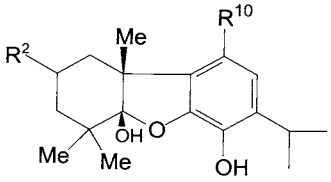
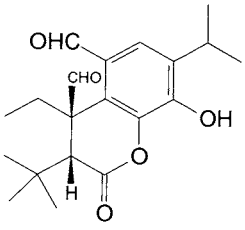


532 R^1= H; R^{10}= CO_2H
533 R^2= H; R^{10}= CHO
534 R^2= OH; R^{10}= CO_2H



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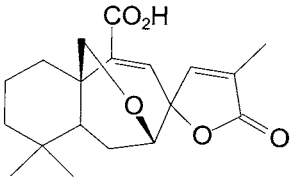

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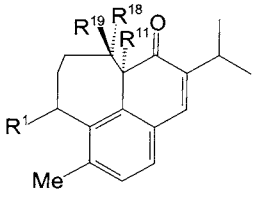
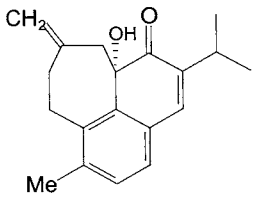


537 R^1= H; R^{11}= OH; R^{18}= R^{19}= Me
538 R^1= H;R^{11}=R^{19}= Me; R^{18}= OH
539 R^1= O; R^{11}= R^{19}= Me; R^{18}= OH



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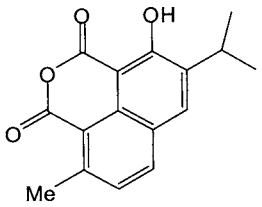

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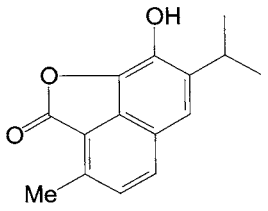

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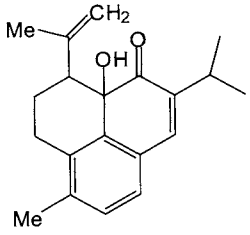

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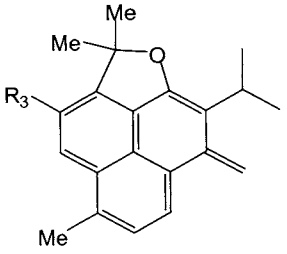
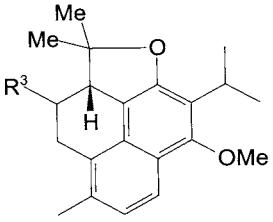


544 $R^3 = H$
545 $R^3 = OH$



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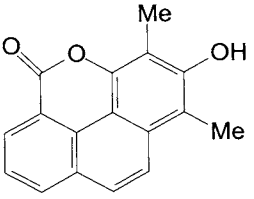
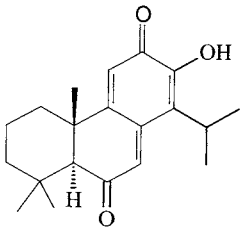


547

Tetracyclic diterpenes

4 cassanes and kauranes type diterpenoids have been isloated from *Savia* species **(548-551).**



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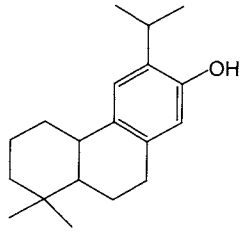

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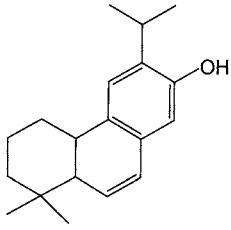

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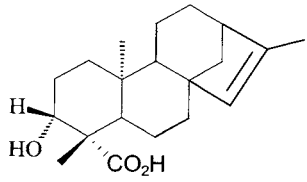

551

Table 3 : Diterpenoids of *Salvia* genus

Compound	Species	Reference
2,6-dimethyl-10-(p-tolyl)undeca-2,6-(E)-diene **(1)**	*S. dorisiana*	*39*
Manool **(2)**	*S. candidissima*	*40*
	S. limbata	*41, 42*
	S. multicaulis	6
	S. officinalis	43, 44
	S. sclarea	45, 46
Δ^7-Manool **(3)**	*S. sclarea*	47
Sclareol **(4)**	*S. limbata*	42
	S. moorcraftiana	48
	S. sclarea	45, 46, 49,50
6β-Hydroxysclareol **(5)**	*S. moorcraftiana*	48
Ent-sclareol **(6)**	*S. candidissima*	40
	S. sclarea	50
Labda-8(17),12E,14-triene-6,19-olide **(7)**	*S. leriafolia*	19
Salvicine **(8)**	*S. verbenaca*	51
Manoyl oxide **(10)**	*S. candidissima*	40, 52, 53, 54
	S. cyanescence	52
	S. staminea	27
11β-Hydroxymanoyl oxide **(11)**	*S. candidissima*	40
	S. cyanescence	53
8,13-Diepimanoyl oxide **(12)**	*S. candidissima*	54, 55
Ambreinolide **(13)**	*S. yosgadensis*	56
6α-Hydroxyambreinolide **(12)**	*S. yosgadensis*	56
Norambreinolide **(14)**	*S. yosgadensis*	56
3α- Hydroxynorambreinolide **(15)**	S. *aethiopis*	57
6α- Hydroxynorambreinolide **(16)**	*S. yosgadensis*	56
6α-Hydroxy-8α-acetoxy-13,14,15,16-tetranorlabdane-12-oic acid **(17)**	*S. yosgadensis*	56
Methyl-3α-hydroxy-8α-acetoxy- 13,14,15,16-tetranorlabdane-12-oate **(18)**	S. *aethiopis*	57
Lasianthin **(19)**	*S. lasiantha*	*58*
Thymonin **(20)**	*S. thymoides*	*59*
7β-Hydroxythymonin **(21)**	*S. thymoides*	*59*
Melisodoric acid **(22)**	*S. melissodora*	*60*
Semiatrin **(23)**	*S. semiatratha*	*61*
7α-Hydroxyneocleroda-3,13-diene-18,19:15,16-diolide **(24)**	*S. fulgens*	*62*
	S. melissodora	*63*
	S. semiatratha	*61*
	S. thymoides	*59*
7-Oxoneocleroda-3,13-diene-18,19:15,16-diolide **(25)**	*S. thymoides*	*59*
Kerlinolide **(26)**	*S. kerlii*	*64*
Brevifloralactone **(27)**	*S. breviflora*	*65*
	S. melissodora	*63*
Brevifloralactone acetate **(28)**	*S. breviflora*	*65*
7α-Acetoxy-2β-hydroxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(29)**	*S. melissodora*	*66*
7β-Hydroxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(30)**	*S. melissodora*	*66*
7α-Hydroxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(31)**	*S. melissodora*	*63, 66*
7α-Acetoxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(32)**	*S. melissodora*	*66*
2β,7α-Dihydroxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(33)**	*S. melissodora*	*66*
2β-Acetoxy-7α-hydroxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(34)**	*S. melissodora*	*66*
7-Oxo-ent-clerodan-3,13-dien-18,19:16,15-diolide **(35)**	*S. melissodora*	*66*
2β-Hydroxy-ent-clerodan-3,13-dien-18,19:16,15-diolide **(36)**	*S. melissodora*	*66*
2β-Hydroxy-7-oxo-ent-clerodan-3,13-dien-18,19:16,15-diolide **(37)**	*S. melissodora*	*66*
7β,18,19-Trihydroxy-ent-cleroda-3,13-dien-16,15-olide **(38)**	*S. melissodora*	*66*
Clerodermic acid **(39**	*S. regla*	*67*
15,18 acetoxy-ent-cleroda-3,13-dien-16,15-olide-19-oic-acid **(40)**	*S. tenorioi*	*68*
Salvimadrensinol **(41)**	*S. madrensis*	*69*

Compound	Species	Reference
Salvimadrensinone (**42**)	*S. madrensis*	*69*
Salvimadrensin (**43**)	*S. madrensis*	*69*
Kerlinic acid (**44**)	*S. kerlii*	*70*
Hardwickiic acid (**45**)	*S. divinorum*	*71*
	S. regla	*67*
Divinatorin A (**46**)	*S. divinorum*	*71*
Divinatorin B (**47**)	*S. divinorum*	*71*
Divinatorin C (**48**)	*S. divinorum*	*71*
Ent-(5R,9R,10S)-7S-acetoxy-15,16-epoxy-1S,2S,12-trihydroxycleroda-3,13(16),14-trien-18,19-olide (**49**)	*S. haenkei*	*72*
Amarisolide (**50**)	S. *amarissima*	*73*
Salvigresin (**51**)	*S. greggi*	*74*
2α-Hydroxy-7α-acetoxy-12-oxo-15:16- epoxy- neoclerodan-3,13(16),14-trien-18:19-olide (**52**)	*S. ramosa*	*68*
	S. urolepis	*75*
Portulide C (**53**)	*S. melissodora*	*63*
Salvinorin A (**54**)	*S. divinorum*	*71*
Salvinorin B (**55**)	*S. divinorum*	*71*
Salvinicin A (**56**)	*S. divinorum*	*76*
Salvinicin B (**57**)	*S. divinorum*	*76*
Salvinorin C (**58**)	*S. divinorum*	*71*
Salvinorin D (**59**)	*S. divinorum*	*71*
Salvinorin E (**60**)	*S. divinorum*	*71*
Salvinorin F (**61**)	*S. divinorum*	*71*
Salviarin (**62**)	*S. carnea*	*68*
	S. gensneraeflora	*77*
	S. greggi	*74*
	S. reflexa	*78*
	S. rhyacophylla	*79*
	S. sousae	*80*
	S. splendens	*81*
6β-Hydroxysalviarin (**63**)	*S. reflexa*	*78*
	S. rhyacophylla	*79*
15,16-Epoxy-8α-hydroxyneocleroda-2,13(16),14-triene-17,12R:18,19-diolide (**64**)	*S. reflexa*	*78*
7,8β-Dihydrosalviacoccin (**65**)	*S. greggi*	*82*
	S. sousae	*80*
6β -Acetoxysalviarin (**66**)	*S. rhyacophylla*	*79*
10β -Acetoxysalviarin (**67**)	*S. rhyacophylla*	*79*
Splendidin (**68**)	*S. splendens*	*83*
Splenolide A (**69**)	*S. splendens*	*84*
Splenolide B (**70**)	*S. splendens*	*84*
1(10) Dehydrosalviarin (**71**)	*S. karwinskii*	*80*
	S. lineata	*85*
Derivative of linearifolin (**72**)	*S. sousae*	*86*
Salviacoccin (**73**)	*S. coccinea*	*87*
	S. plebeia	*88*
6β-Hydroxy-7,8-dehydrobacchatricunaetin (**74**)	*S. rhyacophylla*	*79*
(**75**) New	*S. wagneriana*	*89*
Epoxysalviacoccin (**76**)	*S. plebeia*	*88*
Infuscatin (**77**)	*S. infuscata*	*90*
Genesnerofolin A (**78**)	*S. gensneraeflora*	*91*
Splenolide C (**79**)	*S. splendens*	*84*
80	*S. adenophora*	*68*
Ent-(4S,5R,9S,10R)-15,16- epoxycleroda-1,13(16),14-triene-17,12S:18,19-diolide (**81**)	*S. haenkei*	*72*

Compound	Species	References
Ent-(5R,9R)-15,16-epoxy-10S hydroxycleroda-3,13(16),14-triene-17,12S:18,19-diolide **(82)**	*S. haenkei*	*72*
Ent-(5S,9R)-15,16-epoxy-10S- hydroxycleroda-3,7,13(16),14-tetraene- 17,12S:18,19-diolide **(83)**	*S. lineata*	*85*
Linearolactone **(84)**	*S. polystachya*	*37*
	S. reptans	*91*
7α-Acetoxy-7,8α-dihydrogensnerofolin B **(85)**	*S. gensneraeflora*	*77*
Gensnerofolin B **(86)**	*S. gensneraeflora*	*77, 91*
Polystachyne D **(87)**	*S. polystachya*	*37*
Polystachyne E **(88)**	*S. polystachya*	*37*
1α,10α-Epoxysalviarin **(89)**	*S. lineata*	*85*
1α,2α-Epoxy-3,4-dihydrolinearilactone **(90)**	*S. reptans*	*92*
Salvifolin **(91)**	*S. tiliaefolia*	*93*
Salvifaricin **(92)**	*S. farinacea*	*94*
	S. leucantha	*95*
	S. polystachya	*37*
	S. tonalensis	*96*
Salvifarin **(93)**	*S. farinacea*	*92, 97*
Polystachyne A **(94)**	*S. polystachya*	*37*
Polystachyne B **(95)**	*S. polystachya*	*37*
Polystachyne C **(96)**	*S. polystachya*	*37*
Dehydrokerlin **(97)**	*S. polystachya*	*37*
	S. rhyacophyla	*79*
Salviandulin D **(98)**	*S. blepharophylla*	*98*
	S. lavanduloide	*99*
Kerlin **(99)**	*S. kerlii*	*64*
16-Hydroxy-1-oxo-8 :12(R)-epoxy neocleroda-13(14)diene18,19 ;15,16-diolide **(100)**	*S. urolepis*	*75*
Epimer of 104 at C_{16} **(101)**	*S. urolepis*	*75*
15-Hydroxy-1-oxo-8 :12(R)-epoxy neocleroda-2,13(14)diene18,19 ;16,15-diolide **(102)**	*S. urolepis*	*72*
Epimere of 106 at C_{15} **(103)**	*S. urolepis*	*75*
Rhyacophillin **(104)**	*S. rhyacophylla*	*79*
7-Epirhyacophilline **(105)**	*S. rhyacophylla*	*100*
7,8-Didehydrorhyacophilline **(106)**	*S. reflexa*	*78*
Salvireptalolide **(107)**	*S. reptans*	*92*
Salviandulin C **(108)**	*S. lavanduloide*	*101*
Cardiophyllidin **(109)**	*S. cardiophylla*	*102, 103*
Tonalensin **(110)**	*S. tonalensis*	*96, 104*
Salviandulin A **(111)**	*S. haenkei*	*68*
	S. lavanduloide	*105*
Salviadulin B **(112)**	*S. lavanduloide*	*105*
Tilifodiolide **(113)**	*S. tiliaefolia*	*93*
SalviPuberulin (Puberulin) **(114)**	*S. puberula*	*106*
115	*S. carnea*	*68*
Blepharolide A **(116)**	*S. blepharophylla*	*107*
SalviIsopuberulin (Isopuberulin) **(117)**	*S. puberula*	*106*
	S. tiliaefolia	*93*
Salvileucantholide **(118)**	*S. leucantha*	*95*
Salvigenolide **(119)**	*S. fulgens*	*62, 108*
Salvianduline E **(120)**	*S. leucantha*	*95*
Blepharolide B **(121)**	*S. blepharophylla*	*107*
Salvisousolide **(122)**	*S. sousae*	*86*
	S. tonalensis	*96*
	S. urolepis	*75*
Languduline **(123)**	*S. languidula*	*109*

Compound	Species	Species
Isosalvisousolide **(124)**	*S. sousae*	*75*
Salvimexicanolide **(125)**	*S. mexicana*	*110*
Tonalenin **(126)**	*S. tonalensis*	*96*
Salvialangudilin A **(127)**	*S. languidula*	*111*
Salvialangudilin B **(128)**	*S. languidula*	*111*
Salvialangudilin C **(129)**	*S. languidula*	*111*
Salvialangudilin D **(130)**	*S. languidula*	*111*
Dimer **131**	*S. wagneriana*	*89*
Dimer **132**	*S. wagneriana*	*89*
14-Oxopimaric acid **(133)**	*S. candidissima*	112
	S. wiedmannii	113
Isopimaric acid **(134)**	*S. caespitosa*	17
	S. greggi	74, 82
	S. heldrichiana	114
	S. wiedmannii	113
14α-hydroxyisopimaric acid **(135)**	*S. greggi*	74, 81
	S. microphylla	115
3β-hydroxyisopimaric acid **(136)**	*S. greggi*	82
6β-hydroxyisopimaric acid **(137)**	*S. caespitosa*	17
Isopimaradiene **(138)**	*S. mellifera*	116
14 α-Hydroxyisopimara-7,15-diene **(139)**	*S. parryi*	38
14 α,18-Dihydroxyisopimaradiene **(140)**	*S. greggi*	82
	S. microphylla	115
Isopimaradienal **(141)**	*S. caespitosa*	17
Sandaracopimaric acid **(142)**	*S. caespitosa*	17
	S. fulgens	62
7α-Acetoxysandaracopimaric acid **(143)**	*S. microphylla*	117
7α-Hydroxysandaracopimaric acid **(144)**	*S. microphylla*	117
7β-Hydroxysandaracopimaric acid **(145)**	*S. heldrichiana*	114
	S. wiedmannii	113
Methyl-7-oxosandaracopimarate **(146)**	*S. microphylla*	115
Methyl-7α-hydroxysandaracopimarate **(147)**	*S. microphylla*	115
Sandaracopimara-8(14),15-diene-7α,18-diol **(148)**	*S. microphylla*	117
Salvipimarone **(149)**	*S. multicaulis*	6
Isopimara-8(14), 15-diene **(150)**	*S. multicaulis*	6
	S. parryi	36
7α-Hydroxyisopimara-8(14),15-diene **(151)**	*S. parryi*	36
Isopimara-8(14), 15-dien-7-one **(152)**	*S. parryi*	36
Isopimara-8(9),15-diene **(153)**	*S. argentea*	118
Isopimara-8,15-dien-7-one **(154)**	*S. parryi*	36
Isopimara-6,8(14),15-triene **(155)**	*S. parryi*	36
7-Oxo-13-epi-pimara-8,15-dien-18-oic acid **(156)**	*S. heldrichiana*	114
6β-Hydroxypimara-8,15-dien-1-one **(157)**	*S. ceratophylla*	119
Parryin **(158)**	*S. parryi*	36
3,4-Seco-isopimara-4(18),7,15-triene-3-oic acid **(159)**	*S. cinnabarinna*	37
Pachystazone **(160)**	*S. candidissima*	42, 55
	S. napifolia	120
	S. pachystachys	121
Wiedemannic acid **(161)**	*S. heldrichiana*	114
	S. wiedmannii	113
3β-hydroxy-abiet-8(14)-en-18-oic,9α,13α –endoperoxide acid **(162)**	*S. oxyodon*	122
3β-acetoxy-abiet-8(14)-en-18- oic-9α,13α -endoperoxide acid **(163)**	*S. oxyodon*	122
Abieta-8,13-die	*S. phlomoide*	123

Compounds	Species	References
Longipedin **(165)**	*S. longipedicellata.*	124
Heldrichinic acid **(166)**	*S. heldrichiana*	114
Wiedelactone **(167)**	*S. heldrichiana*	114
	S. multicaulis	125
	S. recognita	126
	S. wiedmannii	127
Abieta-8,11,13-triene **(168)**	*S. limbata*	42
	S. mellifera	128
	S. phlomoide	123
	S. prionitis	129
3-oxo- abieta-8,11,13-triene **(169)**	*S. wiedmannii*	113
7-oxo- abieta-8,11,13-triene **(170)**	S. *amplexicaulis*	25
11-hydroxy-12-methoxy-abieta-8,11,13-triene **(171)**	*S. candidissima*	40, 112
	S. monbretii	130
6-oxo-12-peroxyabieta-8,11,13-triene **(172)**	*S. multicaulis*	125
14-hydroxy-12-methoxy-3-oxo-abieta-8,11,13-triene **(173)**	*S. multicaulis*	125
Candelabrone **(174)**	*S. candelabrum*	131, 132, 133
	S. palaestina	134
Cryptojaponol **(175)**	*S. candidissima*	40
	S. napifolia	120
	S. phlomoide	123
	S. sclarea	45
	S. staminea	25
Demethylcryptojaponol **(176)**	*S. canariensis*	135
	S. monbretii	136
	S. phlomoide	123
3-hydroxydemethylcryptojaponol **(177)**	*S. pubescens*	137
6α-hydroxydemethylcryptojaponol **(178)**	*S. canariensis*	135
Euphraticol **(179)**	*S. euphratica*	138
Ferruginol **(180)**	S. *aethiopis*	139
	S. *amplexicaulis*	25
	S. apiana	140
	S. argentea	118, 141
	S. barrelieri	3
	S. blepharochlyna	5
	S. bracteata	16
	S. caespitosa	17
	S. candidissima	40, 112
	S. ceratophylla	119
	S. cilicica	38
	S. cyanescence	53
	S. eriophora	26
	S. hypargeaie	18, 142
	S. jaminiana	2
	S. lanigera	143
	S. limbata	42
	S. mellifera	128
	S. microstegia	144
	S. milthiorrhiza	145, 146
	S. monbretii	136
	S. multicaulis	125
	S. munzii	147
	S. napifolia	120
	S. potentillifolia	148
	S. prionitis	149
	S. recognita	126

Compound	Species	References
	S. sclarea	45, 46, 47
	S. staminea	27
	S. syriaca	22
	S. tiliaefolia	150
	S. trijuga	151
	S. triloba	152
	S. verbenaca subsp *clandestina*	4
	S. virgata	153
	S. viridis	21
	S. wiedmannii	113
	S. multicaulis	6
1-Oxoferruginol **(181)**	*S. napifolia*	120
	S. viridis	21
	S. multicaulis	125
6-Oxoferruginol **(182)**	*S. napifolia*	120
	S. monbretii	136
14-Hydroxyferruginol **(183)**	*S. bracteata*	14
12-Methoxyferruginol **(184)**	*S. monbretii*	130
	S. pachystachys	121
	S. pommifera	154
2,12-Dimethoxyferruginol **(185)**	*S. pachystachys*	121
Hypargenin A **(186)**	*S. hypargeaie*	18, 142
Hypargenin C **(187)**	*S. hypargeaie*	18
Hypargenin E **(188)**	*S. hypargeaie*	18
Inuroyleanone **(189)**	*S. cilicica*	40
	S. coulteri	155
	S. lavanduliefolia	156
	S. pratensis	157
Pomiferin D **(190)**	*S. pommifera*	154
Pomiferin E **(191)**	*S. pommifera*	154
Salviol (isorol) **(192)**	*S. canariensis*	158
	S. cardiophylla	93
	S. sclarea	159
	S. tomentosa	160
	S. milthiorrhiza	161
	S .texana	159
Salviviridinol **(193)**	*S. canariensis*	158
	S. viridis	21
Sugiol **(194)**	S. *albocaerula*	10
	S. *amplexicaulis*	25
	S. aspera	162
	S. bracteata	16
	S. canariensis	135
	S. candelabrum	131
	S. coulteri	155
	S. lanata	163
	S. milthiorrhiza	161, 164
	S. napifolia	120
	S. pachystachys	121
	S. phlomoide	123
	S. pratensis	157
	S. prionitis	149
	S. recognita	126
	S. tomentosa	160

Compound	Species	References
	S. trijuga	151
	S. triloba	165
	S. viridis	21
2α-hydroxysugiol **(195)**	*S. cardiophylla*	93
	S. texana	166
Trilobinone **(196)**	*S. triloba*	152
	S. broussonetti	167
Iguestol **(197)**	*S. barrelieri*	3
Inuroyleanol (14-hydroxycryptojaponol) **(198)**	*S. tchihatchiffi*	168
Dehydroabietic acid **(197)**	*S. tomentosa*	160, 169
3-Hydroxy-abieta-8,11,13-triene-18-oic acid **(200)**	*S. oxyodon*	122
1-Oxo-abieta-8,11,13-triene-18-oic acid **(201)**	*S. tomentosa*	170
7β,15- Dihydroxy-abieta-8,11,13-triene **(202)**	*S. sapinae*	171
15- Hydroxy-7-oxoabieta-8,11,13-triene **(203)**	S. *albocaerula*	10
18- Hydroxy-7-oxo-abieta-8,11,13-triene **(204)**	*S. pommifera*	154
11,12,16-Trihydroxy-abieta-8,11,13-triene **(205)**	*S. mellifera*	172
11,12,20-Trihydroxy-abieta-8,11,13-triene **(206)**	*S. mellifera*	172
11,12,16,20- Tetrahydroxy-abieta-8,11,13-triene **(207)**	*S. mellifera*	173
Carnosic acid **(208)**	*S. apiana*	174
	S. canariensis	158, 175
	S. columbariae	176
	S. mellifera	172, 173, 177
	S. munzii	178
	S. officinalis	13, 44, 179
	S. willeana	180
	S. canariensis	158
7-Oxocarnosic acid **(209)**	*S. apiana*	175
6-Hydroxycarnosic acid **(210)**	*S. chinopeplica*	181
	S. mellifera	173
	S. munzii	178
	S. lanigera	182
12-Methoxycarnosic acid **(211)**	*S. officinalis*	13, 44
	S. tomentosa	160
	S. canariensis	9
11-Acetoxycarnosic acid **(212)**	*S. columbariae*	183
	S. mellifera	173
16-Acetoxycarnosic acid **(213)**	*S. ceratophylla*	119
3-Oxo-abieta-8,11,13-triene-20-oic acid **(214)**	*S. canariensis*	158
7α-Hydroxy-6-oxocarnosic acid **(215)**	*S. canariensis*	158
7β-Hydroxy-6-oxocarnosic acid **(216)**	*S. lanigera*	182
	S. officinalis	44
Carnosate methyl ester **(217)**	*S. ceratophylla*	119
Euphracal **(218)**	*S. euphratica*	138
	S. multicauli	125
Ferruginal **(219)**	*S. pisidica*	184
15-Hydroxyferruginol **(220)**	*S. pachystachys*	121
16-Hydroxyferruginol **(221)**	*S. chinopeplica*	181
7-Oxoferruginol-18-al **(222)**	*S. sclarea*	*45*
10-Acetylferruginol **(223)**	*S. microstegia*	*144*
Forskalinone **(224)**	*S. forskhalei*	*11*
Hypargenin B **(225)**	*S. hypargeaie*	*118*
20-Oxo-inuroyleanone **(226)**	*S. coulteri*	*155*
	S. lanigera	*185*
Nemorosin **(227)**	*S. nemorosa*	*186*
Pisiferal **(228)**	*S. broussonetii*	*168*
	S. lanigera	*186, 187*

Compound	*Species*	Reference
	S. mellifera	*174*
	S. microstegia	138
	S. multicaulis	6
	S. pisidica	*185*
	S. wiedmannii	*113*
Pisiferic acid **(229)**	*S. blepharochlyna*	5
12-Methoxypisiferic acid **(230)**	*S. blepharochlyna*	5
	S. candelabrum	131
	S. palaestina	134
	S. wiedmannii	113
12-Methoxypisiferic acid methyl ester **(231)**	*S. blepharochlyna*	5
Pomiferin A **(232)**	*S. pomifera*	154
Pomiferin B **(233)**	*S. pomifera*	154
Pomiferin C **(234)**	*S. pomifera*	154
Pomiferin F **(235)**	*S. pomifera*	188
Pomiferin G **(236)**	*S. pomifera*	188
5,11,12-Trihydroxy-abieta-8,11,13-triene **(237)**	*S. microstegia*	144
5-Hydroxy-1-oxo-abieta-8,11,13-trien-18-oic acid **(238)**	*S. candidissima*	40
6,12,14-Trihydroxyabieta-6,8,11,13-tetraene **(239)**	*S. napifolia*	120
11,12-Dimethoxyabieta-6,8,11,13-tetraen-20-oic acid methyl ester **(240)**	*S. canariensis*	158
14-Hydroxy-6,7-dehydrocarnosic acide **(241)**	*S. lanigera*	189
Cryptanol **(242)**	*S. candidissima*	112
	S. cilicica	38
	S. cryptantha	190
	S. euphratica	138
	S. hypargeaie	18
	S. jaminiana	2
	S. multicaulis	125
	S. napifolia	120
	S. pachystachys	121
	S. pisidica	184
	S. potentillifolia	148
	S. triloba	152
	S. verbenaca subsp. *clandestina*	4
	S. virgata	153
	S. wiedmannii	113
6,7-Dehydroferruginol **(243)**	*S. apiana*	140
	S. munzii	*147*
16-Hydroxy-6,7-dehydroferruginol **(244)**	*S. apiana*	*140*
Hypargenin D **(245)**	*S. hypargeaie*	*18, 142*
6,7-Dehydrosalviol **(246)**	*S. texana*	*191*
Trilobiol **(247)**	*S. triloba*	*152*
7-Oxo-11,12,14-trihydroxy-20-methoxy-abieta-5,8,11,13-tetraene **(248)**	*S. candidissima*	*40*
6,11-Dihydroxy-12-methoxy-abieta-5,8,11,13-tetraen-7-one **(249)**	*S. phlomoide*	*192*
Blephaein **(250)**	*S. blepharochlyna*	5
Montbretol **(251)**	*S. milthiorrhiza*	193
	S. monbretii	136
Montbretyl-12-methyl ether **(252)**	*S. candidissima*	112
	S. monbretii	136
Pedicellatin **(253)**	*S. longipedicellata*	124
Salvinolone **(254)**	*S. bracteata*	16
	S. candidissima	40

Compound	Species	References
	S. dichroanta	154
	S. monbretii	130
	S. prionitis	194
	S. sclarea	47
6-Hydroxysalvinolone **(255)**	*S. hypargeaie*	142
	S. jaminiana	2
	S. monbretii	130
	S. phlomoide	123
	S. verbenaca subsp. *clandestina*	4
Salvinolonyl-12-Me-ether **(256)**	*S. candidissima*	40
Salvitchihatine **(257)**	*S. tchihatchiffi*	167
Antiochic acid **(258)**	*S. tomentosa*	169
Tchihatine **(2519)**	*S. tchihatchiffi*	167
Carnosol **(260)**	*S. canariensis*	158, 175
.	*S. chinopeplica*	181
	S. columbariae	176
	S. mellifera	172, 173, 177
	S. munzii	178
	S. officinalis	15, 44, 195
	S. rubescens	196
	S. triloba	197
16-Hydroxycarnosol **(261)**	*S. chinopeplica*	181
	S. mellifera	116
	S. munzii	178
	S. rubescens	196
16-Acetoxycarnosol **(262)**	*S. canariensis*	175
12-Methoxycarnosol **(263)**	*S. broussonetii*	168
	S. officinalis	15
11,12-Dimethoxycarnosol **(264)**	*S. columbariae*	176
	S. rubescens	196
Isocarnosol **(265)**	*S. lanigera*	182, 175, 198
12-Hydroxy-isocarnosol **(266)**	*S. lanigera*	183
Isogaldosol **(267)**	*S. mellifera*	177
Isorosmanol **(268)**	*S. columbariae*	176
	S. mellifera	172, 173
	S. munzii	178
	S. officinalis	15, 44, 179
	S. willeana	180
16-Hydroxy-isorosmanol **(269)**	*S. mellifera*	173
	S. munzii	178
Galdosol **(270)**	*S. canariensis*	158
	S. mellifera	173, 177
	S. officinalis	15
	S. lavanduliefolia	122
Rosmanol **(271)**	*S. canariensis*	158, 9
	S. columbariae	176
	S. mellifera	172, 173
	S. munzii	178
	S. officinalis	15, 44
	S. rubescens	196
	S. tomentosa	160
16-Hydroxyrosmanol **(272)**	*S. mellifera*	173
	S. munzii	178
7-Ethoxyrosmanol **(273)**	*S. canariensis*	9
	S. columbariae	183

Compound	Species	References
	S. officinalis	43
7-Methoxyrosmanol **(274)**	*S. canariensis*	9, 175
	S. chinopeplica	181
	S. columbariae	183
	S. mellifera	173
	S. munzii	178
	S. officinalis	44
11,12-Dimethoxyrosmanol **(275)**	*S. columbariae*	176
16-Hydroxy-7-methoxyrosmanol **(276)**	*S. munzii*	178
	S. rubescens	196
Epirosmanol **(277)**	*S. columbariae*	176
	S. mellifera	173
	S. munzii	178
	S. officinalis	44
16-Hydroxyepirosmanol **(278)**	*S. mellifera*	173
	S. munzii	178
6,7-Dimethoxy-7-epi-rosmanol **(279)**	*S. officinalis*	44
Epimer **(280)**	*S. dorrii*	199
Epimer **(281)**	*S. dorrii*	199
20-Deoxocarnosol **(282)**	*S. candidissima*	52
	S. columbariae	183
12-Methoxy-20-deoxocarnosol **(283)**	*S. canariensis*	135
16-Hydroxy-20-deoxocarnosol **(284)**	*S. chinopeplica*	181
	S. mellifera	173
6-Epidemethylesquirolin D **(285)**	*S. aspera*	162
Salvibractone **(286)**	*S. bracteata*	16
12-Methoxy-abieta-8,11,13-trien-20,11-olide **(287)**	*S. officinalis*	43
Taxodone **(288)**	*S. hypargeaie*	142
	S. munzii	147
	S. phlomoide	123
Taxodione **(289)**	*S. aspera*	162
	S. broussonetii	168
	S. hypargeaie	18
	S. lanigera	*200*
	S. mellifera	*128*
	S. monbretii	*130, 136*
	S. moorcraftiana	*201*
	S. munzii	*147*
	S. nipponica	*202*
	S. pachystachys	*121*
	S. phlomoide	*123*
	S. prionitis	*8*
	S. staminea	*27*
	S. verbenaca	*200*
2α-Hydroxytaxodione **(290)**	*S. texana*	*159*
2-Oxotaxodione **(291)**	*S. texana*	*166*
7-Hydroxytaxodione **(292)**	*S. monbretii*	*130*
6-Deoxo-2α-hydroxytaxodione **(293)**	*S. texana*	*159*
2 α-Hydroxytaxodone **(294)**	*S. texana*	*159*
2α,7-Dihydroxytaxodone **(295)**	*S. texana*	*159*
Viridinol **(296)**	*S. viridis*	*21*
2α,7,11-Trihydroxy-abieta-7,9(11),13-trien-12-one **(297)**	*S. texana*	*166*
2α,11-Dihydroxyabieta-5,7,9(11),13-tetraen-12-one **(298)**	*S. texana*	*166*
15-Deoxyfuerstione **(299)**	*S. mellifera*	*128*
	S. moorcraftiana	*201*
5,6-Dehydro-7-hydroxytaxodone **(300)**	*S. munzii*	*147*

Compound	Species	References
5,6-Dehydro-2α,7dihydroxytaxodone **(301)**	*S. texana*	*203*
Viridone **(302)**	*S. syriaca*	*22*
	S. viridis	*21*
Sagequinone methide A **(303)**	*S. officinalis*	*44*
7,7'-Bistaxodione **(304)**	*S. monbretii*	*130*
11,11'-Didehydroxy-7,7'-hydroxytaxodione **(305)**	*S. monbretii*	*130*
Rosmanoyle carnosate **(306)**	*S. canariensis*	*158*
Hongencaotone **(307)**	*S. prionitis*	*204*
Bisprioterone A **(308)**	*S. prionitis*	*205*
Bisprioterone B **(309)**	*S. prionitis*	*205*
Bisprioterone C **(310)**	*S. prionitis*	*205*
11,12-Dioxo-abieta-8,13-diene **(311)**	*S. candidissima*	*40*
	S. hypargeaie	*142*
	S. napifolia	*120*
6α-Hydroxy-11,12-dioxo-abieta-8,13-diene **(312)**	*S. recognita*	*126*
Salviphlomone **(313)**	*S. phlomoide*	*123*
Deoxysalviphlomone **(314)**	*S. milthiorrhiza*	*161*
	S. nutans	*206*
Rosmaquinone **(315)**	*S. canariensis*	*9*
Royleanone **(316)**	S. *aethiopis*	139, 207
	S. barrerlieri	3
	S. buccharia	208
	S. lanata	209
	S. lanigera	198
	S. lavanduliefolia	156
	S. mellifera	128
	S. moorcraftiana	210
	S. nemorosa	211
	S. nutans	204
	S. officinalis	195
	S. pachystachys	121
	S. phlomoide	123
	S. pratensis	157, 212
	S. prionitis	149
	S. tomentosa	213
	S. triloba	166
2β-Hydroxyroyleanone **(317)**	*S. cryptantha*	90
6β -Hydroxyroyleanone **(318)**	*S. triloba*	152
7α-Hydroxyroyleanone (Horminone) **(319)**	S. *amplexicaulis*	25
	S. austriaca	213
	S. blepharochlyna	5
	S. bracteata	16
	S. candidissima	112
	S. carducea	211?
	S. cryptantha	190
	S. deserta	211?
	S. dracocephaloide	208?
	S. eriophora	26
	S. hypargeaie	18
	S. korolkovii	208?
	S. lanata	214
	S. mellifera	128
	S. moorcraftiana	210
	S. multicaulis	6
	S. munzii	147
	S. napifolia	120

Compound	Species	References
	S. nutans	206
	S. officinalis	195
	S. pachystachys	121
	S. palaestina	134
	S. pisidica	184
	S. potentillifolia	148
	S. pratensis	157, 212
	S. tomentosa	160, 213
	S. triloba	152
	S. verbenaca	200, 213
	S. verticillata	213?
	S. virgata	153
	S. wiedmannii	113
7β-Hydroxyroyleanone (**320**)	*S. lanigera*	*185, 200*
	S. moorcraftiana	*210*
16-Hydroxyroyleanone (**321**)	*S. apiana*	*140*
19-Hydroxyroyleanone (**322**)	*S. chinopeplica*	*181*
7α-Acetoxyroyleanone (**323**)	S. *amplexicaulis*	25
	S. austriaca	*213*
	S. barrelieri	3
	S. blepharochlyna	5
	S. bracteata	16
	S. buccharia	215
	S. candelabrum	131
	S. candidissima	112
	S. carducea	211
	S. cryptantha	190
	S. cyanescence	52
	S. deserta	211
	S. eriophora	26
	S. fruticulosa	216
	S. lanata	214
	S. lavanduliefolia	156
	S. moorcraftiana	201, 210
	S. multicaulis	125
	S. napifolia	120
	S. nutans	206
	S. officinalis	195
	S. phlomoide	123
	S. pisidica	184
	S. potentillifolia	148
	S. pratensis	155, 200
	S. pubescens	137
	S. tomentosa	160, 212
	S. triloba	152, 165
	S. verbenaca	210
	S. verticillata	213
	S. virgata	153
	S. wiedmannii	115
7-Oxoroyleanone (**324**)	*S. lanigera*	217
	S. moorcraftiana	209
	S. pratensis	157
	S. sclarea	45, 46
7α-Acetoxy-6β-hydroxyroyleanone (**325**)	*S. verbenaca*	200
7α-Acetoxy-19-hydroxyroyleanone (**326**)	*S. regla*	218

Compound	Species	References
7α-Acetoxy-20-hydroxyroyleanone **(327)**	*S. lanata*	208
6-Oxoroyleanone-18-oic acid **(328)**	*S. divaricata*	219
Horminone-18-oic acid **(329)**	*S. divaricata*	219
Aldehyde horminone-18-al **(330)**	*S. candicans*	220
Nemorone **(331)**	*S. nemorosa*	221
	S. pubescens	137
Desacylnemorone **(332)**	*S. lanata*	*213*
	S. lanigera	*190, 200*
	S. nemorosa	*211*
	S. pubescens	*137*
Virgatol **(333)**	*S. triloba*	*152*
	S. virgata	*153*
Candelabroquinone **(334)**	*S. candelabrum*	*133*
12-Methoxyhorminone **(335)**	*S. bracteata*	*16*
12-Deoxyroyleanone **(336)**	*S. cilicica*	*38*
6-Oxo-12-methoxyroyleanone-18-oic acid **(337)**	*S. divaricata*	*219*
12-Deoxy-7,7'-dimethoxy-6-oxoroyleanone **(338)**	*S. nutans*	*206*
Trilobic acid **(339)**	*S. triloba*	*222*
7-OxoBarrilierone **(340)**	*S. barrilieri*	*3*
7-Acetoxybarrilierone **(341)**	*S. barrilieri*	*3*
Hypargenin F **(342)**	*S. hypargeaie*	*18*
	S. monbretii	*136*
6,7-Dehydroroyleanone **(343)**	*S. bracteata*	*16*
	S. jaminiana	*2*
	S. eriophora	*26*
	S. lanata	*163*
	S. lanigera	*200*
	S. lavanduliefolia	*156*
	S. moorcraftiana	*223*
	S. officinalis	*195*
	S. pratensis	*157, 215*
	S. tchihatchiffi	*167*
	S. tomentosa	*215*
	S. verbenaca subsp. *clandestina*	*4*
12-Deoxy-6,7-dehydroroyleanone **(344)**	*S. nutans*	*208*
12-Deoxy-6-hydroxy-6,7-dehydroroyleanone **(345)**	*S. nutans*	*208*
12-Methoxy-5,6-dehydrohorminone **(346)**	*S. multicaulis*	6
	S. recognita	*126*
12-Methoxy-5,6-dehydroacetylhorminone **(347)**	*S. multicaulis*	*6*
Bractealine **(348)**	*S. bracteata*	*16*
	S. multicaulis	*6*
Canariquinone **(349)**	*S. canariensis*	*9*
Atuntzensin **(350)**	*S. officinalis*	*15*
6α,12,19-Trihydroxy-11,14-dioxo-abieta-8,12-dien-20,7β-olide **(351)**	*S. gilliessi*	*224*
Columbaridione **(352)**	*S. columbariae*	*184*
	S. officinalis	*15*
Reglin **(353)**	*S. regla*	*65*
Sessein **(354)**	*S. regla*	*67, 220*
Deactylsessein **(355)**	*S. regla*	*63, 209*
12-Hydroxy-11,14-dioxoabieta-6,8,12-trien-19,20-olide **(356)**	*S. gilliessi*	*224*
7,20-Epoxyroyleanone **(357)**	*S. candidissima*	*40*
	S. napifolia	*120*
Conacytone **(358)**	*S. anastomans*	*225*
	S. ballotaeflora	*226*

Compound	Species	References
	S. candicans	*220*
	S. pubescens	*137*
Methylconacytone **(359)**	*S. candicans*	*220*
	S. pubescens	*227*
Isomere of conacytone **(360)**	*S. candicans*	*220*
19-Methoxy of **360** **(361)**	*S. candicans*	*220*
8,9-Epoxy-7-oxoroyleanone **(362)**	*S. pratensis*	*1571*
Trilobiol **(363)**	*S. triloba*	*222*
Candesalvone A **(364)**	*S. candelabrum*	*131*
2-Oxo-candesalvone A **(365)**	*S. palaestina*	*134*
19 (4→3) Abeo-O-demethylcryptojaponol **(366)**	*S. pubescens*	*137*
11,12,16-Trihydroxy-20(10→5)-abeo-abieta-1(10),6,8,11,13-pentaene **(367)**	*S. apiana*	*140*
Sageone **(368)**	*S. mellifera*	*178*
	S. officinalis	*44*
16-Hydroxysageone **(369)**	*S. chinopeplica*	*182*
	S. mellifera	*116*
Arucadiol **(370)**	*S. apiana*	*140*
	S. argentea	*118*
	S. columbariae	*184*
	S. lanigera	*185*
	S. milthiorrhiza	*161, 228*
	S. prionitis	*149, 229*
Arucatriol **(371)**	*S. canariensis*	*158*
Salvirecognone **(372)**	*S. recognita*	*126*
Salvirecognine **(373)**	*S. recognita*	*126*
1,10-Epoxy-5α-hydroxyabieta-8, 11,13- trien -7-one **(374)**	*S. moorcraftiana*	*212*
Miltirone **(375)**	*S. drobovi*	*230*
	S. milthiorrhiza	*145, 231*
	S. officinalis	*15*
	S. prionitis	*232*
1(R)-Hydroxymiltirone **(376)**	*S. argentea*	*118*
Tanshinone V **(377)**	*S. milthiorrhiza*	*146*
Δ^1 Dehydromiltirone **(378)**	*S. milthiorrhiza*	*146, 161*
Saligerone **(379)**	*S. lanigera*	*233*
Neocryptotanshinone **(380)**	*S. milthiorrhiza*	*234, 235*
Oleoylneocryptotanshinone **(381)**	*S. milthiorrhiza*	*236*
Miltionone I **(382)**	*S. milthiorrhiza*	*237*
Deoxyneocryptotanshinone **(383)**	*S. lanigera*	*217*
	S. milthiorrhiza	*235*
Sanigerone **(384)**	*S. lanigera*	*233*
7-Hydroxy-12-methoxy-20-nor-abieta-1,5(10);7,9,12-pentaene-6,14-dione **(385)**	*S. cilicica*	*38*
Salvidorol **(386)**	*S. dorrii*	*199*
Multicaulin **(387)**	*S. blepharochlyna*	*5*
	S. multicaulis	6
Demethylmulticaulin **(388)**	*S. multicaulis*	*6*
Multiorthoquinone **(389)**	*S. blepharochlyna*	*5*
	S. multicaulis	5
2-Demethylmultiorthoquinone **(390)**	*S. blepharochlyna*	5
	S. multicaulis	6
Kronenquinone **(391)**	*S. kronenburgui*	238
18,20-Dinorabieta-1,3,5(10),6,8,13-hexaene-11,12-dione **(392)**	*S. glutinosa*	213
	S. milthiorrhiza	145, 238
Tanshinone VI **(393)**	*S. milthiorrhiza*	145

Compound	Species	Ref
Methylene miltirone **(394)**	*S. milthiorrhiza*	161, 239
Danshenxinkun B **(395)**	*S. glutinosa*	213
	S. milthiorrhiza	161, 235
	S. prionitis	232
	S. przewalskii	240
12-deoxydanshenxinkun B **(396)**	*S. glutinosa*	213,
Danshenxinkun A **(397)**	*S. milthiorrhiza*	161, 235
	S. przewalskii	240
Oleoyldanshenxinkun A **(398)**	*S. milthiorrhiza*	236
Salvinone **(399)**	*S. milthiorrhiza*	161, 239
Neocryptotanshinone II **(400)**	*S. milthiorrhiza*	193
Norsalvioxide (Przewalskin) **(401)**	*S. milthiorrhiza*	228
	S. przewalskii	241
Danshenxinkun C **(402)**	*S. milthiorrhiza*	161
Salvigerone **(403)**	*S. lanigera*	185
Salvipalestinoic acid **(404)**	*S. palaestina*	134
12,14-dihydroxy-7-oxo-2,3-seco- 19(4→3)abeo-abieta-4(18),8,11,13-tetraen-2,11-olide **(405)**	*S. palaestina*	242
Candesalvone B **(406)**	*S. candelabrum*	131
	S. palaestina	134
12-O-methylcandesalvone B **(407)**	*S. candelabrum*	131
Candesalvone B methyl ester **(408)**	*S. candelabrum*	131
Candesalvoquinone **(409)**	*S. candelabrum*	131
Limbinol **(410)**	*S. limbata*	41
	S. monbretii	130
	S. prionitis	243
Acetyllimbinol **(411)**	*S. limbata*	42
Salvonitin **(412)**	*S. prionitis*	*7*
De-O-ethylsalvonitin **(413)**	*S. prionitis*	*244*
Salprionin **(414)**	*S. prionitis*	*244*
Salvilimbinol **(415)**	*S. eriophora*	26
	S. limbata	41
	S. monbretii	130
	S. prionitis	243
4-Dehydrosalvilimbinol **(416)**	*S. limbata*	41
	S. syriaca	22
Aethiopinone **(417)**	S. *aethiopis*	34, 245
	S. argentea	118, 141
	S. candidissima	54
	S. ceratophylla	119
	S. cyanescence	52
	S. eriophora	26
	S. hypargeaie	142
	S. sclarea	50
	S. viridis	21
1-Oxo-aethiopinone **(418)**	*S. argentea*	118, 141
	S. candidissima	112
	S. monbretii	130
	S. sclarea	50
Saprorthoquinone **(419)**	*S. hypargeaie*	142
2-Hydroxysaprorthoquinone **(420)**	*S. monbretii*	130
4-Hydroxysaprorthoquinone **(421)**	*S. prionitis*	195
	S. limbata	41
	S. prionitis	243
4,14-Dihydroxysaprorthoquinone **(422)**	*S. eriophora*	26
Sclareapinone **(423)**	*S. prionitis*	243

Compound	Species	Ref
	S. sclarea	45
Salvisyrianone **(424)**	*S. syriaca*	22
Salvipisone **(425)**	S. *aethiopis*	139
	S. argentea	118, 141
	S. candidissima	40, 112
	S. ceratophylla	119
	S. cyanescence	52
	S. eriophora	26
	S. sclarea	45, 47
1-Oxosalvipisone **(426)**	*S. candidissima*	112
3-Oxosalvipisone **(427)**	*S. candidissima*	53
Acetylsalvipisone **(428)**	*S. sclarea*	47
12-Isopentenyl-3-oxosalvipisone **(429)**	*S. cyanescence*	52
2,3-Dehydrosalvipisone **(430)**	*S. argentea*	141
	S. candidissima	40
	S. sclarea	45, 47
Prineoparaquinone **(431)**	*S. prionitis*	8
12-Hydroxysapriparaquinone **(432)**	*S. eriophora*	26
	S. limbata	41
	S. prionitis	246
Limbinal **(433)**	*S. limbata*	42
3, 12-Dihydroxysapriparaquinone **(434)**	*S. limbata*	41
4, 12-Dihydroxysapriparaquinone **(435)**	*S. eriophora*	26
	S. prionitis	195
3-Oxo-sapriparaquinone **(436)**	*S. prionitis*	149
3, 12-Dihydroxysapriparaquinone-1-ene **(437)**	*S. eriophora*	26
	S. limbata	41
Rosmadial **(438)**	*S. columbariae*	*177*
	S. limbata	*41*
	S. mellifera	*172, 177*
	S. officinalis	*15*
16-Hydroxyrosmadial **(439)**	*S. mellifera*	*173*
2α,11,12-Trihydroxy-6,7-seco-abieta-8,11,13-Triene-6,7-dial **(440)**	*S. texana*	*192*
		147
11,12-Dihydroxy-6,7-seco-abieta-8,11,13-triene-6,7-dial-11,6-hemiacetale **(441)**	*S. munzii*	
	S. texana	*192*
Epimer of **440 (442)**		
7,8-Seco-paraferruginone **(443)**	*S. prionitis*	*243*
Tanshinone IIA **(444)**	S. *aeria*	*141*
	S. constanea	*141, 247*
	S. glutinosa	*213*
	S. hians	*248*
	S. milthiorrhiza	*145, 249*
		240, 241
	S. przewalskii	*141, 250*
	S. trijuga	*251*
	S. triloba	*141*
	S. yunnanensis	*161, 231,*
	S. milthiorrhiza	*252*
1-Hydroxytanshinone IIA **(445)**	*S. przewalskii*	*241*
	S. milthiorrhiza	*253, 254*
3α-Hydroxytanshinone IIA **(446)**	*S. trijuga*	*250*
	S. hians	*248*
3α,17-Dihydroxytanshinone IIA **(447)**	*S. milthiorrhiza*	*249, 254*
Tanshinone IIB **(448)**	*S. przewalskii*	*240, 241*
	S. trijuga	*249*

Compound	Species	Ref
Przewaquinone A **(449)**	*S. constanea*	*141*
	S. milthiorrhiza	*255*
	S. przewalski	*241, 256*
Methyltanshinonate **(450)**	*S. milthiorrhiza*	*252, 254*
	S. przewalskii	*240*
	S. trijuga	*250*
Tanshindiol B **(451)**	*S. milthiorrhiza*	*253, 254*
	S. przewalskii	*257*
Tanshindiol C **(452)**	*S. milthiorrhiza*	*161, 253*
	S. przewalskii	*257*
Przewaquinone C **(453)**	*S. milthiorrhiza*	*258*
	S. przewalskii	*259*
Δ^1-Dehydrotanshinone IIA **(454)**	*S. milthiorrhiza*	*145, 161*
Cryptotanshinone **(455)**	*S. apiana*	*140*
	S. axillaris	*260*
	S. drobovi	*232*
	S. glutinosa	*213*
	S. karabachensis	*197*
	S. mellifera	*116*
	S. milthiorrhiza	*235, 249*
	S. munzii	*147*
	S. przewalskii	*240*
	S. trautivettri	*230*
	S. trijuga	*250*
6-Methylcryptotanshinone **(456)**	S. *aegyptiaca*	34
Trijuganone C **(457)**	*S. milthiorrhiza*	*261*
	S. trijuga	*151*
17-Hydroxcryptotanshinone **(458)**	*S. munzii*	*147*
Tanshinalehyde **(459)**	*S. milthiorrhiza*	*252, 258*
	S. przewalskii	*241*
Tanshindiol A **(460)**	*S. milthiorrhiza*	*161, 253*
Miltionone II **(461)**	*S. milthiorrhiza*	*257*
Danshenol B **(462)**	*S. milthiorrhiza*	*164, 262*
Lanugon Q **(463)**	*S. apiana*	*140*
6-Deoxo-5,6-dehydrolanugon Q **(464)**	*S. apiana*	*142*
Tanshinone I **(465)**	S. *aeria*	*141*
	S. constanea	*141, 263*
	S. drobovi	*230*
	S. glutinosa	*213*
	S. karabachensis	*239*
	S. milthiorrhiza	*235, 249*
	S. przewalskii	*240, 241*
	S. trijuga	*140*
	S. triloba	*264*
	S. yunnanensis	*140*
Przewaquinone B **(466)**	*S. constanea*	*140*
	S. milthiorrhiza	*255*
	S. przewalskii	*256*
Formyltanshinone **(467)**	*S. milthiorrhiza*	*161*
15,16-Dihydrotanshinone I **(468)**	*S. glutinosa*	*213*
	S. milthiorrhiza	*249*
	S. nipponica	*203*
	S. przewalskii	*240*
	S. trijuga	*250*
Danshenol A **(469)**	*S. glutinosa*	*213*
	S. milthiorrhiza	*164, 262*

Compound	Species	Ref
15-epi-danshenol A **(470)**	*S. glutinosa*	*213*
Trijuganone B (1,2,15,16, terahydrotanshiquinone) **(471)**	*S. milthiorrhiza*	*161, 231*
	S. trijuga	*264*
1,2-dihydrotanshinquinone **(472)**	*S. milthiorrhiza*	*161*
Methylenetanshiquinone **(473)**	*S. milthiorrhiza*	*161, 248*
	S. trijuga	*250*
	S. triloba	*265*
3-Hydroxymethylenetanshiquinone **(474)**	*S. milthiorrhiza*	*161, 252*
	S. trijuga	*250*
Nortanshinone **(475)**	*S. milthiorrhiza*	*231, 253*
	S. przewalskii	*240*
Przewaquinone F **(476)**	*S. przewalskii*	*257*
Dihydronortanshinone **(477)**	*S. milthiorrhiza*	*261*
Methylene dihydrotanshinquinone **(478)**	S. milthiorrhiza	*161*
Isotanshinone I **(479)**	*S. glutinosa*	*213*
	S. milthiorrhiza	*155*
Dihydroisotanshinone I **(480)**	*S. glutinosa*	*204*
	S. milthiorrhiza	*161, 231*
	S. prionitis	*229*
Trijuganone A **(481)**	*S. trijuga*	*265*
Isotanshinone IIA **(482)**	*S. milthiorrhiza*	*161*
Isotanshinone IIB **(483)**	*S. milthiorrhiza*	*161, 234*
Isocryptotanshinone **(484)**	*S. mellifera*	*128*
	S. milthiorrhiza	*161*
Isotanshinone II **(485)**	*S. glutinosa*	*213*
Dihydroisotanshinone II **(486)**	*S. glutinosa*	*213*
Tanshinlactone **(487)**	S. milthiorrhiza	*259, 266*
(488)	*S. milthiorrhiza*	*161*
(489)	*S. milthiorrhiza*	*161*
(490)	*S. milthiorrhiza*	*161*
Prioketolactone **(491)**	*S. prionitis*	*229*
Danshenxinkun D **(492)**	*S. milthiorrhiza*	*234, 267*
	S. prionitis	*232*
Cryptoacetalide **(493)**	*S. milthiorrhiza*	*268*
6-Methylcryptoacetalide **(494)**	S. *aegyptiaca*	*34*
Epicryptoacetalide **(495)**	*S. milthiorrhiza*	*268*
6-Methylepicryptoacetalide **(496)**	S. *aegyptiaca*	*34*
Danshenspiroketallactone **(497)**	*S. milthiorrhiza*	*161, 258*
Epidanshenspiroketallactone **(498)**	*S. milthiorrhiza*	*258*
Neoprzewaquinone A **(499)**	*S. przewalskii*	*269*
Icetexone **(500)**	*S. anastomans*	*225*
	S. ballotaeflora	*226, 270*
	S. candicans	*220*
	S. pubescens	*227*
5-Epi-icetexone **(501)**	*S. gilliessi*	*224*
19-Deoxy-icetexone **(502)**	*S. ballotaeflora*	*259*
19(R) Acetoxy-19-deoxo-icetexone **(503)**	*S. pubescens*	*227*
19-hydroxy-19-deoxo-icetexone **(504)**	*S. pubescens*	*271*
19-Deoxyisoicetexone **(505)**	*S. ballotaeflora*	*259*
Romulgarzone **(506)**	*S. ballotaeflora*	*270*
Fruticulin A **(507)**	*S. fruticulosa*	*207, 273*
Demethylfruticulin **(508)**	*S. fruticulosa*	*213*
	S. arizonica	*274*
Salvicanol **(509)**	*S. apiana*	*139*

Compound	Species	Ref
	S. canariensis	*135*
	S. columbariae	*177*
	S. mellifera	*172, 173*
Demethylsalvicanol **(510)**	*S. aspera*	*162*
	S. broussonetii	*168*
	S. mellifera	*172, 173*
	S. munzii	*147*
Icetexane coulterone **(511)**	*S. coulteri*	*161*
Lanigerol **(512)**	*S. lanigera*	*12*
Salviasperanol **(513)**	*S. aspera*	*162*
5,6-Dihydro-6α-hydroxysalviasperanol **(514)**	*S. aspera*	*162*
Anastomosine **(515)**	*S. anastomans*	*225*
	S. ballotaeflora	*259*
	S. candicans	*220*
7,20-Dihydroanastomosine **(516)**	*S. ballotaeflora*	*259*
Derivative anastomosine **(517)**	*S. candicans*	*220*
Salviolone **(518)**	*S. milthiorrhiza*	*161, 225*
Brussonol **(519)**	*S. brussonetti*	*168*
Multipolone **(520)**	*S. milthiorrhiza*	*274*
Tilifolidione **(521)**	*S. tiliaefolia*	*144*
	S. thymoides	*275*
3-Hydroxytilifolidione **(522)**	S. thymoides	*275*
3-Oxotilifolidione **(523)**	S. thymoides	*275*
Nor derivative of **523 (524)**	S. thymoides	*275*
Fruticulin B **(525)**	*S. fruticulosa*	*273*
Aegyptionone A **(526)**	S. *aegyptiaca*	*276*
Aegyptionone B **(527)**	S. *aegyptiaca*	*276*
Dichroanal A **(528)**	*S. dichroanta*	*160*
Dichroanal B **(529)**	*S. dichroanta*	*160*
Dichroanone **(530)**	*S. dichroanta*	*160*
Salvimultine **(531)**	*S. multicaulis*	*277*
Salvicanaric acid **(532)**	*S. canariensis*	*278*
	S. monbretii	*130*
	S. munzii	*147*
Salviacanaraldehyde **(533)**	*S. munzii*	*147*
	S. sclarea	*46*
2α-Hydroxysalvicannaric acid **(534)**	*S. texana*	*203*
Safficinolide **(535)**	*S. officinalis*	*14*
Paramiltioic acid **(536)**	*S. paramiltiorhiza*	*279*
Microstegiol **(537)**	*S. candidissima*	*40, 112*
	S. hypargeaie	*143*
	S. jaminiana	*2*
	S. lanigera	*144*
	S. microstegia	*24*
	S. monbretii	*124*
	S. napifolia	*130*
	S. prionitis	*8*
	S. sclarea	*45, 47*
	S. staminea	*27*
	S. verbenaca subsp. *clandestina*	*4*
	S. viridis	*21*
Salvibretol **(538)**	*S. monbretii*	*130*
1-Oxo-salvibretol **(539)**	*S. candidissima*	*40*
	S. monbretii	*130*
	S. staminea	*27*
Candidissiol **(540)**	*S. candidissima*	*40, 52*

Compound	Species	Ref
	S. ceratophylla	*119*
	S. monbretii	*130*
	S. sclarea	*45, 46, 47*
	S. syriaca	*22*
Saprionide **(541)**	*S. prionitis*	*234*
Sapriolactone (Salpriolactone) **(542)**	*S. prionitis*	*280*
Saprireanin **(543)**	*S. prionitis*	*244*
Salvilenone **(544)**	*S. milthiorrhiza*	*281*
	S. prionitis	*149*
	S. moorcraftiana	*282*
3-hydroxysalvilenone **(545)**	*S. prionitis*	*149*
Prionitin **(546)**	*S. prionitis*	*283*
Salvinolactone **(547)**	*S. prionitis*	*194*
Mattenoquinone **(548)**	S. melissodora	*68*
Sempervirol **(549)**	*S. multicaulis*	*6*
6,7-Dihydrosempervirol **(550)**	*S. apiana*	*139*
	S. munzii	*150*
	S. officinalis	*14*
Verbanicine **(551)**	*S. verbenaca*	*51*

CONCLUSION

More than 550 diterpenoids have been isolated from *Salvia* species widely distributed in Mexico, Turkey, Spain and China. We've recently isolated five abietane diterpenoids from each of the endemic species *S. jaminiana* and *S. verbenaca* subsp. *jaminiana* and seven diterpenoids, from which three are new, from the endemic species *S. barrelieri*. *Salvia* diterpenoids could be divided into 2 categories. The first category includes one monocyclic and bicyclic diterpenoids containing labdanes, clerodanes, neo-clerodanes, seco-clerodanes and other rearranged clerodanes. The second category concerns tricyclic and tetracyclic diterpenes represented by pimaranes and abietanes including quinone abietanes, nor-abietanes, rearranged nor-abietanes, dinor-abietanes, seco-abietanes, tanshinones and other particular rearranged abietanes. Clerodane-type diterpenoids are essentially found in american species while most abietane-type are reported from european and asiatic species. The diterpenoids found in the three algerian species present an abietane-type squeleton. Except inuroyleanol or 14-hydroxycryptojaponol (11,14-dihydro-12-méthoxy-7-oxoabieta-8,11,13-triene), all the diterpenoids isolated from *S. barrelieri* possess a royleanone squeleton or a para-quinone

ring. A great number of the described *Salvia* diterpenoids exhibited interesting biological activities *e.g* anti-tuberculous, antitumour, antimicrobial, antibacterial, antileishmanial and antispasmolytic. The roots of *S. jaminiana* and *S. verbenaca* subsp. *jaminiana* exhibited a very good antibacterial activity against the tested microorganisms while the diterpenoids isolated from *S. barrelieri* showed a good antioxidant activity.

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BIOLOGICALLY ACTIVE COMPOUNDS OF SEMI-METALS

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Abstract: Semi-metals, viz. boron, silicon, arsenic, selenium, tellurium and astatine form organometal compounds, some of which are found in nature and have striking effects on the physiology of living organisms. Representatives of these compounds are, e.g., four boron-containing antibiotics (aplasmomycin, borophycin, boromycin, and tatrolon). Silicon compounds, frequently present in "silicate" bacteria, which appear to be members or relatives of the genus *Bacillus*, are known to release silicon from aluminosilicates through the secretion of organic acids. Microbes and microbial enzymes have been used in the biotransformation of organosilicon compounds, and biodegradation of organosilicon under aerobic and/or anaerobic conditions has also been described. Arsenic can be incorporated into complex organic molecules such as arsenosugars and arsenobetaines by, e.g., marine algae and invertebrates, and fungi and bacteria can produce volatile methylated arsenic compounds. Arsenate is typically taken up by cells through phosphate transport systems, whereas arsenite is often acquired via aquaglyceroporins. Additionally, some prokaryotes can use arsenate as a terminal electron acceptor during anaerobic growth and others can utilize arsenite as an electron donor to generate energy. Organoarsenic compounds are also added to chicken feed as antibiotics. Selenium is incorporated into the amino acid selenocysteine that is found in some proteins. Selenide undergoes biomethylation to produce methylselenide and dimethylselenide. Selenium compounds are often used as analogues of sulfur compounds and have important pharmacological effects (e.g. selenium analogues of amino acids, antitumor, antibacterial, antifungal, antiviral, anti-infective drugs, and others). Tellurium is considered to be toxic to most organisms. Although genes correlated with tellurium resistance have been identified, the mechanisms of tellurium resistance are not completely understood. Tellurium-resistant fungi grown in the absence of sulfur and in the presence of tellurite ions incorporate tellurium into amino acids and proteins.

INTRODUCTION

Semi-metals or metalloids are a very small group of elements found in the periodic table of elements along the zig-zag line that distinguishes metals from non-metals and is drawn from between boron and aluminum to the border between polonium and astatine. Elements to the upper right of this line are nonmetals while metals are to the lower left. Together with metals and nonmetals, metalloids form one of the three categories of chemical elements as classified by ionization and bonding properties [1, 2].

Metalloids manifest some of the qualities of the metals and some of the qualities of the nonmetals. Because of their diverse nature, it is very difficult to establish a unique set of physical or chemical characteristics for these materials. There is no unique way of distinguishing a metalloid from a true metal but the most common is that metalloids are usually semiconductors rather than conductors. Also, unlike the metals which become better conductors at low temperatures, the metalloids become better conductors at higher temperatures.

Because of their unique electrical conductivity, they have received intensive attention from the computer and electronics industries. Their relative abundance in the environment is summarized below.

Table 1. Relative abundance of metalloids in the environment

Element	in Earth's crust mg kg^{-1}	in sea water mg L^{-1}
As	1.8 - 5.0	0.003
B	3 - 10	4.6
Se	0.05 - 0.09	0.000 09
Si	257 000 - 282 000	3
Te	0.001 - 0.005	0
At	0	0

Semi-metallic behavior is not confined to the elements, but is also found in alloys and compounds. When involved in chemical bonding, the metalloids again exhibit intermediate qualities. They are capable of taking electrons from most metals and will readily lose electrons to most nonmetals. Their electronegativity values are also mid-range. Consequently, it is unlikely for them to be involved in ionic bonding; when found in compounds, they will usually establish covalent bonding.

The reactivity of the metalloids depends on the element with which they are reacting. For example, boron acts as a nonmetal when reacting with sodium yet as a metal when reacting with fluorine. In organic compounds, they can substitute other elements such as sulfur. Boron and arsenic form compounds with, e.g., lipids, sugars, phenols, organic acids and some polymers. Arsenic compounds, especially organic ones, are integral components of the food chains of many organisms including humans and may play potentially important biological roles in these organisms. Apart from its soluble inorganic forms, selenium is found in living organisms as protein-bound and free seleno-amino acids and volatile organoselenium compounds. With few exceptions, all small organic selenium compounds in bacteria, yeast, plants, or animals are isologues of sulfur amino acids or their derivatives. The interactions of silicon with living matter are the least understood, but they are vitally important for some organisms such as bacteria, which are known to release silicon from aluminosilicates through the secretion of organic acids, or for unicellular algae – diatoms for which it serves as the basic building block of their bodies. Unlike all the above elements, tellurium is highly toxic and is not thought to be required by biological systems; the highly radioactive astatine is so rare in nature that the existence of astatine compounds with any possible biological significance is highly improbable. All in all, many metalloid compounds, both organic and inorganic, have profound effects on living organisms, whether beneficial or adverse; these are the subject of this review.

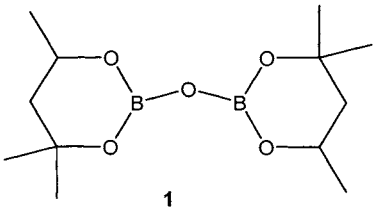
Boron

Boron is a ubiquitous element in rocks, soil, and water. Most of the Earth's soils have <10 ppm boron but large areas of the world are boron deficient. Boron concentrations in rocks range from 5 ppm in basalts to 100 ppm in shales, the average being 10 ppm in the Earth's crust overall. Soils have boron concentrations of 2 to 100 ppm. Seawater contains an average of 4.6 ppm boron, the range being from 0.5 to 9.6 ppm. Freshwaters normally range from 0.01 to 1.5 ppm, with higher concentrations in regions of high boron soil levels [3].

Simple alcohols react with boric acid to give esters. In aqueous solution, this equilibrium usually lies far to the left.

$B(OH)_3 + 3ROH \leftrightarrow B(OR)_3 + 3H_2O$

The partially esterified species $(RO)_2BOH$ and $ROB(OH)_2$ are probably also involved [4]. Polyhydric alcohols form cyclic esters with boric acid. Both compounds (**1**) and (**2**) are mixed to give the jet fuel microbiocide Biobor JF. This additive prevents the growth of *Cladosporium resinae* and *Pseudomonas aeruginosa* in fuel tanks by distributing itself between the water and fuel phases. This hydrocarbon-metabolizing fungus and bacterium grow at the fuel-water interface and can cause corrosion and, e.g., foul fuel filters [5].



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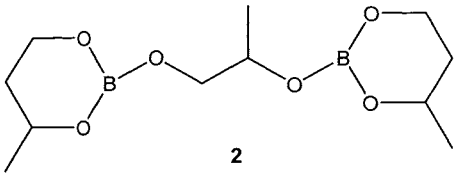


2

Tetrahedral borate or boronate complexes have been shown to be involved in enzyme inhibition. Serine proteases were proposed to be inhibited by boric acid [6], and simple borates have been patented as protease stabilizers in liquid detergent formulations [7, 8].

Reactions such as acylation and deacylation are catalyzed by enzymes through lowering of the energy of activation. Binding and stabilization of the near tetrahedral oxyanion transition state by the active site of the enzyme is how this is accomplished. Thus, a transition state analogue inhibitor is a species, which binds the active site in a way similar to that

of the substrate. Serine hydrolase enzymes react with various borates, boronates, and borinates by forming a tetrahedral complex between the serine hydroxyl group and the boron atom. Hydrogen bonding to the imidazole ring of an adjacent histidine adds further stabilization (Fig. 1).

X-ray structures have been worked out for the benzeneboronic and 2-phenyl-ethaneboronic acid (PEBA) complexes of subtilisin [9] and for the PEBA complex of α-chymotrypsin (α-CHT) dimer [10]. Further stabilization of the hydroxyls on boron is gained by hydrogen bonding to other amido groups lining the oxyanion hole.

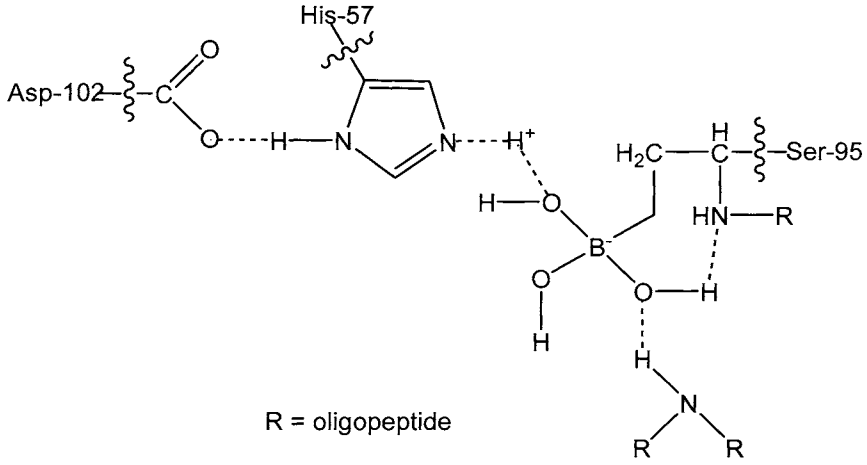
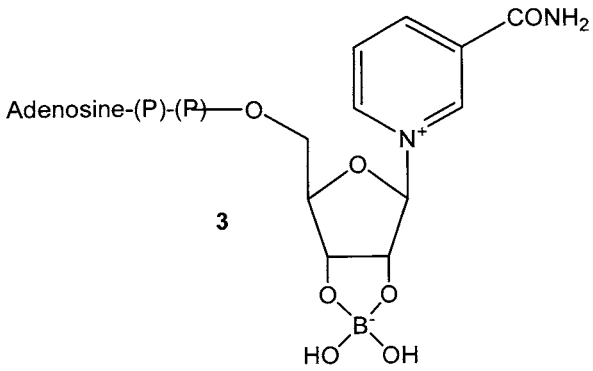


Fig. 1. Complex formation between a peptide and boron atom.

Subtilisin [11] and cholesterol esterase [12, 13] are also inhibited by a range of aliphatic and aromatic boronic acids. Work on inhibiting α-CHT and cell replication with a variety of boronic acids concluded that the α-CHT activity was associated with chromatin in normal and tumorous tissue of mice [14]. Certain boronic acids were demonstrated to inhibit protease activity in rat liver chromatin [15]. It was concluded that good boronate inhibitors of CHT, like PEBA, inhibit cell replication and that this effect is expected to be higher in rapidly proliferating cancer cells than in normal tissue [14].

Complexing of the ribose group of nicotinamide adenine dinucleotide (NAD) (**3**) is preferred electrostatically over that of reduced nicotinamide adenine dinucleotide (NADH), leading to inhibition of this coenzyme system [16].

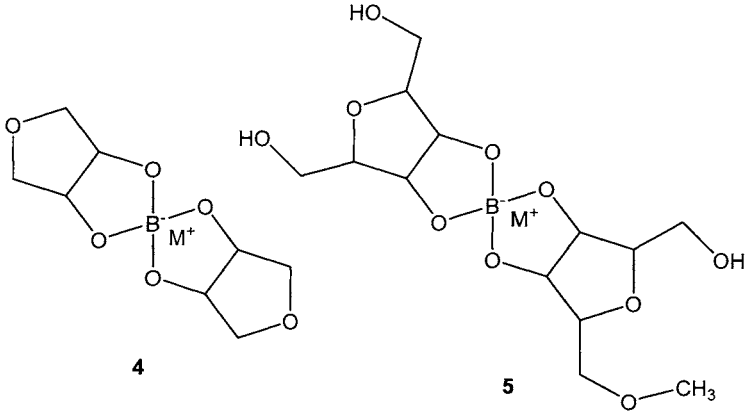


3

Compounds capable of complexing with boric acid include sugars or their derivatives, phenols, organic acids, and some polymers [17, 18]. Yamaguchi et al. [19] reported that boron-polysaccharide complexes were solubilized from cell walls of tomato leaves. Later these complexes were isolated from radish (*Raphanus sativus*) roots and characterized [20]. One of the complexes (BR-II) had a molecular weight of 7.5 kDa and contained boron (23%), uronic acid (52%) and neutral sugars (32%). According to ^{11}B-NMR analysis, it is suggested that the boron was present as a tetravalent 1:2 borate-diol complex [21]. ^{11}B-NMR was used to establish that BR-II boric acid links with two rhamnogalacturonan-II chains, which include apiose, aceric acid (3-*C*-carboxy-5-deoxy-L-xylose), 2-*O*-methyl-fructose, 3-deoxy-D-manno-2-octulosonic acid (Kdo), rhamnose, galactose, arabinose, 2-*O*-methyl-L-fucose, fucose, 2-*O*-methyl-D-xylose, galactouronic acid, and glucouronic acids residues [21]. Partially acid hydrolyzed BR-II complex from sugar beet (*Beta vulgaris*) was characterized and consisted of two disaccharide moieties: α-L-Rhap-(1→5)-D-Kdo and α-L-Araf-(1→5)-Dha (3-deoxy-D-*lyxo*-heptulosaric acid), an aceric acid-containing oligosaccharide [22]. The monosaccharide constituents of BR-II complexes are interconnected by at least 100 different glycosidic linkages [23]. Chemical fragmentation of the BR-II isolated from the walls of suspension-cultured sycamore (*Acer pseudoplatanus*) cells led to the isolation and structural characterization of two 3-deoxysugar containing disaccharides, i.e., α-L-Rhap-(1→5)-D-Kdo [24] and β-L-Araf-(1→5)-D-Dhap [25].

Benner and Klufers [26] used a combined X-ray and NMR method and reported for the first time two structures of furanoidic cis-1,2-diol borate

esters: 1,4-anhydroerythritol (**4**) and methyl β-D-ribofuranoside (**5**) boron-containing complexes.

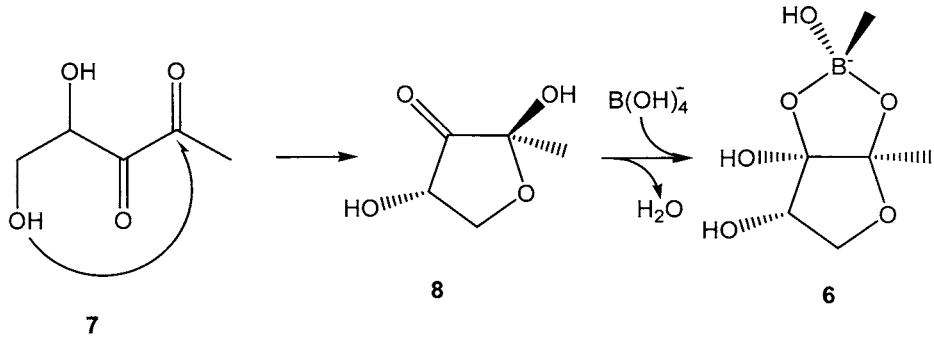


4 **5**

The NMR spectroscopic analysis of autohydrolysate fragments obtained from the gel-forming water-soluble cell-wall sulphated polysaccharides from the green seaweed *Ulva* sp. allowed the identification of two major repeating units in ulvan. These were the aldobiuronic acids (1→4)-β-D-GlcpA-(1→4)-α-L-Rhap 3-sulphate and (1→4)-α-L-IdopA-(1→4)-α-L-Rhap 3-sulphate that were given the name ulvanobiuronic acid 3-sulphate A and B, respectively. The results indicate that the ring hydroxyl groups of the major repeating structures, which contain the iduronic acid rarely encountered in plant polysaccharides, are probably not involved in the boric acid fixation step during the gelation of ulvan [27]. Free and complex forms of accumulated borate in marine green (*Ulva fasciata* and *U. pertusa*), brown (*Laminaria japonica* and *Undaria pinnatifida*) and red algae (*Gloiopeltis tenax* and *Grateloupia turuturu*) were identified by ^{11}B-NMR [28]. Boron-containing complexes with compounds containing vicinal hydroxy groups, such as mannitol, laminarin, and alginic acid were also reported [28].

A novel AI-2 furanosyl borate diester complex (**6**) was originally identified in the bioluminescent marine bacterium *Vibrio harveyi* as one of two autoinducers that regulate light production in response to cell density [29, 30]. AI-2 is produced from S-adenosylmethionine in at least three enzymatic steps [31, 32]. In the final stage 4,5-dihydroxy-2,3-pentanedione (DPD) (**7**) is converted to the cyclic form of DPD, which is named pro-AI-2 (**8**), and can react with borate to form a cyclic borate

diester AI-2 (**6**). AI-2 has been proposed to serve as a 'universal' bacterial quorum-sensing boron-containing signal for inter-bacterial community communication [33].



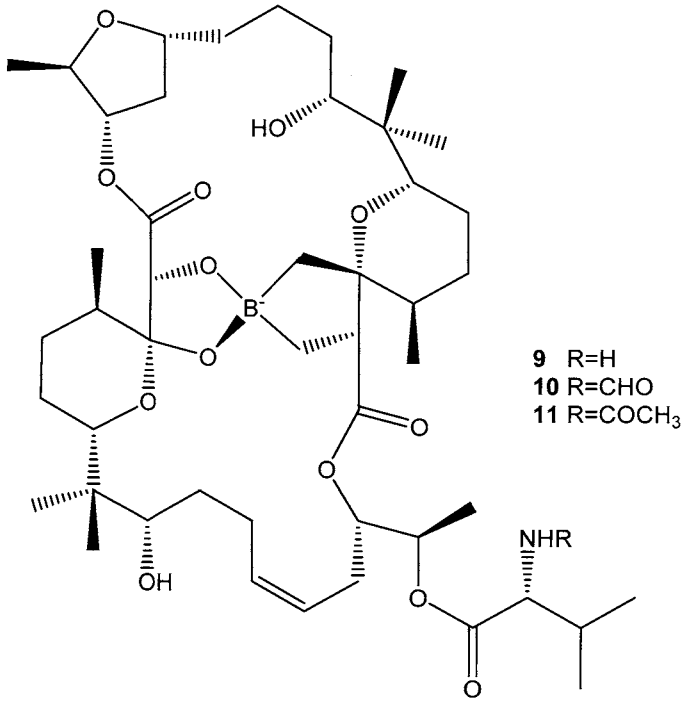
Boromycins

A new strain of *S. antibioticus* was isolated from the soil of Ivory Coast and it was observed that this species contained a novel antibiotic, boromycin, which appears to be the first well-defined boron containing compound to have been found in nature [34]. Boromycin (**9**) shows a growth impeding effect against Gram-positive bacteria, see Table 2. In the concentration 10 μg/ml it has no effect on the following microorganisms: *Escherichia coli* 2018, *Proteus vulgaris* ATCC 10145, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Aspergillus elegans*, *Trichophyton interdigitale,* and *T. mentagrophytes*.

Table 2. Biological activity of boromycin

Strain	MIC concentrations (in μg/mL)
Staphylococcus aureus SG 511	0.4
Streptococcus mitis	0.08
Bacillus subtilis ATCC 6633	0.3
Bacillus megatherium	0.3
Candida albicans	2
Rhodotorula rubra	1
Paecilomyces varioti	1
Mycobacterium bovis	10

The structure of boromycin was elucidated by X-ray analysis and NMR spectra [35]. The two minor products (**10, 11**) (*N*-formyl of **9**, *N*-acetyl of **9**) of boromycin fermentation described by Lee et al. [36] differ in that acylation has occurred not on the 9-hydroxyl group but on the nitrogen of the valine moiety.



The strain *S. antibioticus* was cultivated at 28 °C on soybean flour and mannitol and its pH was adjusted to 7.8 [37]. The culture was stirred at ~800 rpm for 72 hours with aeration (volumetric ratio 1:20). The yield of boromycin was 0.167 g/L from a 3200 L fermentor. The test with *Botrytis cinerea* was used to test microorganisms for the presence of boromycin. The following Table 3 shows the various concentrations at which the boromycin inhibits different microorganisms in the plate diffusion test. The numbers give inhibition zones for different concentrations.

Table 3. The plate diffusion test of boromycin

	Zone diameter in mm at an antibiotic concentration of		
Microorganism	1 mg/mL	0.1 mg/mL	0.01 mg/mL
Bacillus subtilis	33	18	10
Candida vulgaris	10	-	-
Saccharomyces cerevisiae	10	-	-
Paecilomyces varioti	28	15	9
Spicaria sp.	21	18	11

The action against protozoa of the genera plasmodiae and babesiae has also been demonstrated [37]. The ED_{50} of boromycin for *Plasmodium berghei* was 2.2 mg/kg in albino mice while the corresponding ED_{50} for the routinely used drug [7-chloro-4-(4-diethylamino-1-methyl-butylamino)quinoline-diphosphate)] was 2 mg/kg. A similar test with chicks infected with *P. gallinaceum* causing avian malaria was performed and ED_{50} was ~7 mg/kg. Another test was done again with albino mice infected with *Babesia rodhaini*; the ED_{50} was 2.4 mg/kg and LD_{50} was approximately 15 mg/kg.

A US patent [38] described the use of boromycin for the treatment and prevention of coccidiosis in susceptible poultry. Coccidiosis is a common and widespread bird disease caused by several species of protozoan parasites of the genus *Eimeria* (*E. tenella, necatrix, acervulina, maxima, hagani, brunette, meleagridis*, and *adenoides*). *Eimeria* is the causative agent of a severe and often fatal infection of the caeca, which is manifested by severe and extensive hemorrhage, and accumulation of blood in the caeca. When left untreated, severe forms of coccidiosis lead to poor weight gain, reduced feed efficiency and mortality of the poultry. Boromycin is usually used as a feed supplement in different materials such as corn or soybean meals. The compound is administered in an amount equal to about 0.006 to 0.0125% finished feed by weight. Higher amounts approximately up to about 0.05% by weight of the consumed feed are used for therapeutic treatment of an established coccidia infection.

Boromycin was found to influence the Ca^{2+} homeostasis in both excitable and non-excitable cells [39]. This could be explained by the indirect interaction of boromycin with both Ca^{2+} and Na^{+} transport systems via transmembrane ionic gradients of monovalent cations.

Differences in the effects of boromycin on "resting" transport of Ca^{2+} could potentially be used for determining whether the cells belong to excitable or non-excitable cells.

The DNA-damaging agent bleomycin arrests the cell cycle of Jurkat cells defective in the G1 checkpoint in the G2 phase, and microtubule-affecting colchicine arrests it in the M phase [40]. Boromycin showed no effect on the cell cycle status of Jurkat cells at least up to 340 nM but potentiated anti-tumor activity of bleomycin in SCID mice inoculated with Jurkat cells. These data suggest that boromycin disrupts the cell cycle at the G2 checkpoint of cancer cells selectively, leading to sensitization of cancer cells to anti-cancer reagents.

Boromycin was found [41] to strongly inhibit the replication of a clinically isolated HIV-1 (Table 4) strain as well as a cultured strain in *in vitro* laboratory experiments. The mechanism for the anti-HIV activity of boromycin is suggested to involve blocking the later stage of HIV infection, and probably the maturity step for replication of the HIV molecule.

Table 4. Anti-HIV activity and cytotoxicity of boromycin

Virus	Strain	Cell	Day of analysis	Anti-HIV activity		Cytotoxicity	SI
				IC_{50} (μM)	IC_{90} (μM)	CC_{50} (μM)	
HIV-1	LAV-1	MT-4	6	0.008	0.027	0.22	28
	RF	MT-4	5	0.11	0.18	0. 17	15
	KK-1	MT-4	6	0. 14	0. 31	0.22	16
	KK-1	PBMC	10	<0.1	<0.1	4.3	>43
	KK-1	PBMC	10	1.8	4. 8	4.3	2.4
HIV-2	LAV-2	MT-4	6	0.007	0.019	0.22	31

Boromycin at a concentration of 0.05 μg/mL inhibits the synthesis of protein, RNA and DNA in whole cells of *Bacillus subtilis* [42]. It is being antagonized by surface active compounds and is bound to lipoprotein. Binding of boromycin within the cell takes place especially at the cytoplasmic membrane.

The inhibitory effect on *Bacillus subtilis* is being reversed by high concentration of potassium salts (e.g. 0.2 M KCl). The reversion is specific for potassium salts. Addition of boromycin is followed by a discharge of potassium ions from the cells. The K^+, Na^+-activated ATPase of the cytoplasmic membrane is not influenced by boromycin. The degradation of boromycin through alkaline and acid hydrolysis leads to a loss of antibiotic activity, due to the boric acid splitting off from the molecule.

Aplasmomycins

A new boron containing antibiotic, aplasmomycin (**12**), was obtained from a strain of *Streptomyces griseus* isolated from shallow sea sediment in Sagami Bay, Japan [43, 44].

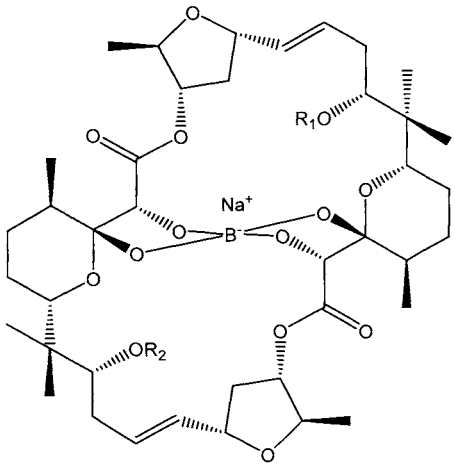
The strain was isolated from marine sediment which is poor in nutrients and fairly high in salt concentration; the effect of NaCl and nutrient concentrations on the production of aplasmomycin was studied in a basal medium containing yeast extract 0.4%, malt extract 1.0% and glucose 0.4%, at pH 7.4. The antibiotic was produced in a medium with diluted nutrients and high salt concentration [45]. The maximum yield (50 μg/mL) of aplasmomycin was obtained in medium diluted to 1/16 with deionized water supplemented with 3.0% NaCl and at 27 °C. The production of aplasmomycin in Kobu-Cha medium (with powdered *Laminaria* sp.) was also examined. This medium was used because the Streptomyces was isolated from sea mud containing organic residues arriving mostly from algae. Maximum production (125 μg/mL) was obtained on supplementation with 1.5% NaCl.

Aplasmomycin inhibits Gram-positive bacteria including mycobacteria *in vitro*, see also Table 5. Male mice were infected intraperitoneally by *Plasmodium berghei* and when aplasmomycin in peanut-oil was administered orally, the number of plasmodium-containing red cells decreased and all treated mice survived. It was on the basis of this potent antiplasmodium activity that aplasmomycin received its name. The acute toxicity in mice was 125 mg/kg by intraperitoneal injection.

Table 5. Biological activity of aplasmomycin

Organism	MIC (μg/mL)
Staphylococcus aureus FDA 209 P	1.56
Staphylococcus aureus Smith	1.56
Staphylococcus aureus 193	3.12
Staphylococcus aureus EMf	1.56
Staphylococcus aureus BBC	0.78
Staphylococcus aureus Terajima	3.12
Staphylococcus aureus MS 8800	3.12
Micrococcusfiavus FDA 16	1.56
Sarcina lutea PCI 1001	1.56
Bacillus anthracis	0.78
Bacillus subtilis PCI 219	1.56
Corynebacterium bovis 1810	0.78
Mycobacterium smegmatis 607	6.25
Mycobacterium phlei	6.25
Escherichia coil K-12	>100
Salmonella typhi T-63	>100
Candida albicans	>100

The structure of the Ag-salt of aplasmomycin was solved by an X-ray crystallographic analysis [46] and the structure of the aplasmomycin was further proved by ^{1}H and ^{13}C NMR spectra [47]. It was found than the conformation of aplasmomycin in $CDCl_3$ solution is identical to that in the solid state and that removal of the boron atom from aplasmomycin results in only a slight conformational change of the molecule.



12 $R_1 = R_2 = H$
13 $R_1 = Ac$, $R_2 = H$
14 $R_1 = R_2 = Ac$

The strain *Streptomyces griseus* has produced two other minor components, aplasmomycins B and C, under the cultural conditions described previously [48]. Recently, an aplasmomycin C-producing actinomycete was isolated from a sandy sediment sample in California [45].

The three aplasmomycins (**12-14**) were compared in terms of antibacterial activity (Table 6), alkali metal ion selectivity (Table 7) and K^+ transport ability. The results summarized below show that the antibacterial activity of aplasmomycin B (**13**) was nearly equal to that of aplasmomycin, while aplasmomycin C (**14**) showed a weaker activity. It was also noted that the ability to form complexes with other metals does not directly correspond with antibacterial activity. Cation selectivity decreased in the order Rb > K > Cs = Na > Li. The three aplasmomycins did not show any affinity towards divalent cations.

Table 6. Biological activity of aplasmomycins

Compounds	*Bacillus subtilis*[a]	*Staphylococcus aureus*
12	21.5	22.0
13	22.0	22.0
14	11.0	10.5

[a] Diameter of inhibition zone with **12-14** (500 μg/mL) determined by the disk diffusion method.

Table 7. Relative affinity of aplasmomycins for various cations

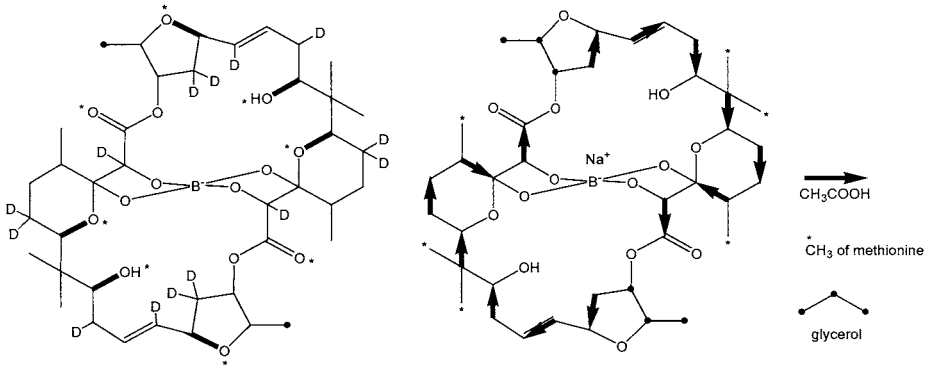
Cations	12	13	14
Na	0.27	0.40	0.45
K	1	1	1
Rb	1.1	1.4	1.5
Cs	0.9	0.6	0.8
Li	0.12	0.15	0.11
Mg	10^{-2}	$<10^{-2}$	$<10^{-2}$
Ca	10^{-2}	$<10^{-2}$	$<10^{-2}$

Labeled precursors, i.e. ([1-^{13}C, 2-^{13}C, 1,2-^{13}C]acetates and L-[methyl-^{13}C]methionine), were added to shaken cultures of *Streptomyces griseus* strain SS-20 and fermented. The antibiotics thus obtained were analyzed by ^{13}C NMR spectroscopy. The biosynthetic origin of aplasmomycin can therefore be summarized as shown in Fig. 2. Each half of the macrocyclic lactone ring is formed from one glycerol, seven acetate units, and three methyl groups of methionine. Surprisingly, propionate is not incorporated intact, but is converted, with decarboxylation, into acetate via symmetrical intermediates, i.e. succinate and the Krebs cycle. The starter unit of the polyketide thus does not originate from propionate [49].

At least two unusual features characterize this biosynthesis. One is the origin of the methyl branches in the polyketide chain from the methyl group of methionine. This is in contrast to the biosynthesis of most macrolide antibiotics in which chain branches are formed by utilization of the appropriate homologs in place of acetate chain extension units, i.e., propionate units. The second unusual feature of this biosynthesis is the mode of incorporation of glycerol and particularly the fact that it is a specific precursor of the three-carbon polyketide chain starter unit [50].

The following elucidation of aplasmomycin biosynthesis was done with isotopically (D, T, ^{13}C and ^{18}O) labeled precursors. In the biosynthesis of aplasmomycin by *Streptomyces griseus,* seven atoms of deuterium from C-2 of acetate and 4 atoms of oxygen from C-1 of acetate were incorporated into the molecule. The two hydrogens of the *pro-R*-hydroxymethyl group of glycerol are incorporated into carbon-17, giving rise to a chiral methyl group of *S* configuration when (1*R*, 2*R*)-[1-D, 1-T]glycerol is used as substrate. The three methionine-derived C-methyl groups per chain are transferred stereospecifically with inversion of

configuration, but racemization is observed during the formation of the methionine methyl group from stereospecifically labeled [3-D, 3-T]serine. The stereochemical and precursor feeding experiments point to phosphoglyceric acid or phosphoenolpyruvate as the glycerol-derived polyketide chain starter unit, ruling out serine, methylglyoxal, and pyruvate and compounds derived from them [51].



D = deuterium from 2-^{13}C,D_3-acetate; O* ^{18}O from 1-^{13}C,$^{18}O_2$ -acetate. Heavy lines connect the ^{18}O to the carbon from which it originated. `● = deuterium from (2*R*)-1,1-D_2-glycerol

Fig. 2. Biosynthetic origin of aplasmomycin

A US patent [52] describes the use of aplasmomycins for modifying rumen metabolism in domestic ruminant animals (cattle, sheep, goats, and/or deer), by reducing the proportion of methane formed, and increasing the proportion of propionate at the expense of methane and/or acetate. This modification of rumen metabolism is believed to improve growth in ruminant animals. It describes compositions that may be used, an improved process for the production of aplasmomycin (which is called in this patent aplasmomycin A), and two novel analogues B and C together with processes for their manufacture.

A fermenter containing 30 L of sterilized medium (soya bean meal, Difco Bacto Casitone, glucose, calcium carbonate, and sodium nitrate) was stirred at 27 °C for 88 hours and aerated at a rate of 7.5 L per minute. The yield of aplasmomycin A was 4.7, B 8.1, and C 2.4 mg/L.

The ability of aplasmomycins to inhibit the production of methane in the rumen of ruminant animals, and to increase the proportion of

propionate (Ac/Pr) and butyrate (Ac/Bu) without depressing at the same time the overall digestive process is shown in Table 8.

Table 8. Effect of aplasmomycins on the methane production and the proportion of volatile acids in ruminants

aplasmomycins	concentration	% inhibition production of CH_4[a]	Ac/Pr[a]	Ac/Bu[a]
12	3ppm	69	-40	-49
13	3ppm	76	-45	-18
14	3ppm	44	-41	+22
12	1ppm	57	-41	-36
13	1ppm	63	-48	+21
14	1ppm	15	-33	+18

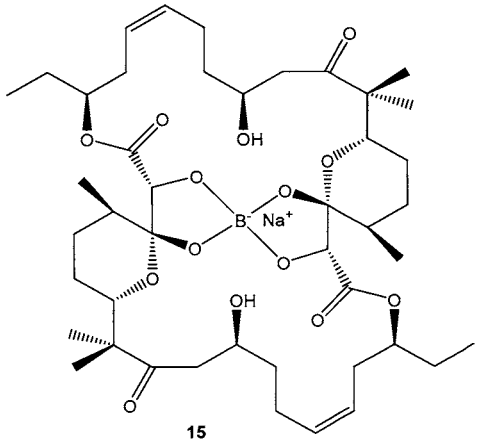
[a] percentages of the values obtained in untreated controls

The animals were treated with an appropriate amount of aplasmomycins per day.

Borophycin

Borophycin **(15)** was found to display appreciable cytotoxicity against LoVo cells (MIC 0.066 μg/mL) and KB cells (MIC 3.3 μg/mL). It was isolated from the lipophilic extract of a marine strain of the blue-green alga *Nostoc linckia* [53]. Borophycin is made up of two identical halves with an overall structure reminiscent of other boron-containing antibiotics. The C3 starter unit for the biosynthesis of **15** is derived from acetate and methionine, but not propionate.

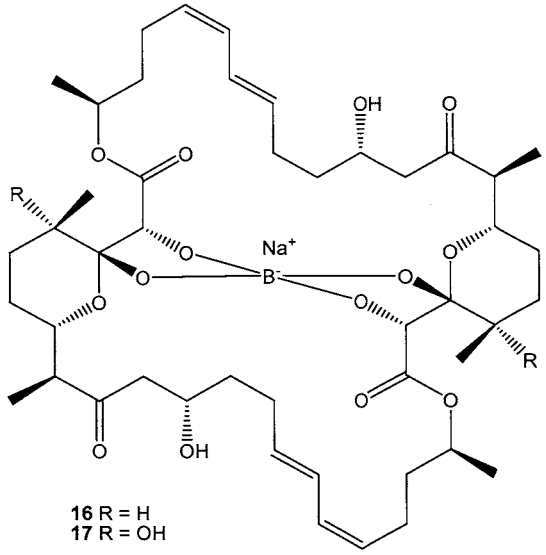
Borophycin and four new cyclic hexapeptides containing no boron, tenuecyclamides A-D, were also isolated from the methanol extract of *Nostoc spongiaeforme* var. *tenue* collected in the Volcani Center, Israel. The yield was 0.1% on the basis of the dry weight of the blue-green alga [54].



15

Tatrolons

In a screening of myxobacteria for antibacterial metabolites, the *Sorangium cellulosum* strain So ce678 was selected for its activity against *Staphylococcus aureus* [55]. The active compounds were produced as two rather lipophilic active compounds, i.e. tartrolons B (**16**) and C (**17**). Tartrolon B was identified as a boric acid ester of tartrolon A3, whereas tartrolon A1 and tartrolon A2 are stereoisomers of tartrolon A3. All components could be chemically converted into each other.



16 R = H
17 R = OH

A large difference was observed between the productions of the tartrolons in shaking flasks, with tartrolon B as the main product, or large-scale fermentation in steel fermenters with tartrolon B as the minor component. Table 9 shows a good correlation between the borate concentration in the medium and the production rate of tartrolon B. However, an increased boron supply by the addition of up to 0.5 g/L of sodium tetraborate showed no influence on the total amount of tartrolons formed.

Table 9. Production of tartrolons A1, A2, A3, and B in relation to the boron supply

Container material for sterilization[a]/incubation[b]	H_3BO_3 [µmol/L] without inoculation	Tartrolon A1-A3 (µmol/L)	Tartrolon B (16) (µmol/L)
Glass/glass	55	0.6	13.8
Glass/polypropylene	40	0.8	5.7
Polypropylene/glass	25	3.0	11.9
Polypropylene/polypropylene	7	10.7	2.3

[a] 121°C for 20 min; [b] 30°C for 7 days

A new member of the tartrolon series of macrodiolides, tartrolon C (**17**), was isolated from a *Streptomyces* species on the basis of its insecticidal activity [56]. The producing organism was isolated in 1990 from a soil sample collected near Braunschweig, Germany. Tartrolon C was active on the beet army worm and tobacco bud worm, with minimum emergent larvicide concentration of 125 ppm on both insects, approximately 40 x and 310 x less active than a standard of spinosyn A.

A partial 16S ribosomal-RNA sequence of CP1130 matched the genus *Streptomyces*, most closely the species *verticillus*. Therefore, this represents the first report of tartrolons from a *Streptomyces* species, since their only previously reported occurrence was in the myxobacterium *Sorangium cellulosum* [55, 57, 58].

Sorangium cellulosum was fermented in 65 liters at 30 °C with an aeration of 0.15 m^3 air per hour and stirrer speed 150 rpm [4]. The yields of tartrolons A and B are given in Table 10. The main product was tartrolon A whereas in flask culture tartrolon B prevailed. Its proportion was increased on supplying the culture medium with borate.

Table 10. Influence of the fermentation vessel on the formation of tartrolons A and B

	Tartrolon (µg/mL)	
Fermentation vessel	A	B
Stainless steel bioreactor	42	2
Glass flask	5.6	38.7
Glass flask+0.005% $Na_2B_4O_7$	4.7	41

The antibiotic spectrum of the tartrolons is given in Table 11. Both tartrolons acted against Gram-positive bacteria with similar MIC values. Gram-negative bacteria, yeasts, and fungi were insensitive, but mammalian cells were strongly inhibited, especially by tartrolon B.

Table 11. Antibiotic spectrum of tartrolons A and B

Test strain	Inhibition zone[a] (mm)		MIC (µg/mL)	
	A	B	A	B
Arthrobacter simplex	13	9	-	-
Bacillus megaterium	13	9	-	-
Bacillus subtilis	15	11	0.62	0.31
Micrococcus luteus	17	11	1.25	1.25
Mycobacterium lacticola	13	10	1.56	1.56
Staphylococcus aureus	16	11	0.62	0.62
Nocardia corallina	15	12	0.62	0.62
Streptococcus faecalis	12	11	>40	10
Escherichia coli	0	0	>40	>40
Escherichia coli tol C[b]	11	0	>40	>40
Pseudomonas fluorescens	0	0	-	-
Salmonella typhimurium	0	0	-	-
Serratia marcescens	0	0	-	-
Candida albicans	0	0	-	-
Saccharomyces cerevisiae	0	0	-	-
Rhodotorula glutinis	0	0	-	-
Hansenula anomala	0	0	-	-
Schizosaccharomyces pombe	0	0	-	-
Mucor hiemalis	0	0	-	-
Aspergillus niger	0	0	-	-
Trichoderma harzianum	0	0	-	-
Mouse fibroblast cells L929	-	-	0.46	0.017

[a] With paper disks of 6 mm diameter and 10 µg tartrolon per disk.

[b] Mutant with damaged outer membrane.

In *Staphylococcus aureus* the syntheses of several important cellular macromolecules were inhibited immediately after the addition of tartrolon B. Isolated RNA polymerase and DNA polymerase from *Escherichia coli* were not influenced by the antibiotic. This could mean that either tartrolon acts specifically on enzymes of Gram-positive bacteria or, more probably, that the antibiotic interferes with energy delivery or membrane integrity.

Experiments with feeding the production strain *Sorangium cellulosum* with sodium [1-^{13}C]acetate, [$^{13}CH_3$]methionine, and sodium [1,2-$^{13}C_2$]acetate were performed to investigate the biosynthesis of the tartrolons [58] (Fig. 3). The ^{13}C-NMR spectrum of [1-^{13}C]acetate-derived tartrolon B shows nine enhanced carbon signals. However, the three-carbon starter unit C-19 to C-21 was not derived from either acetate, propionate, or methionine. This result is in good agreement with boromycin and aplasmomycin where C-1 to C-14 are derived from seven acetate units and with borophycin (see above) where C-1 to C-16 are derived from eight acetate units. Whereas the three-carbon starter unit, C-15 to C-17 in boromycin and aplasmomycin, is derived from glycerol, C-17 to C-19 in borophycin are derived from acetate and methionine. Thus, regarding the biosynthesis, tartrolon is closely related to boromycin and aplasmomycin whereas borophycin with a propionyl starter unit derived from acetate and methionine exhibits a more distant relation.

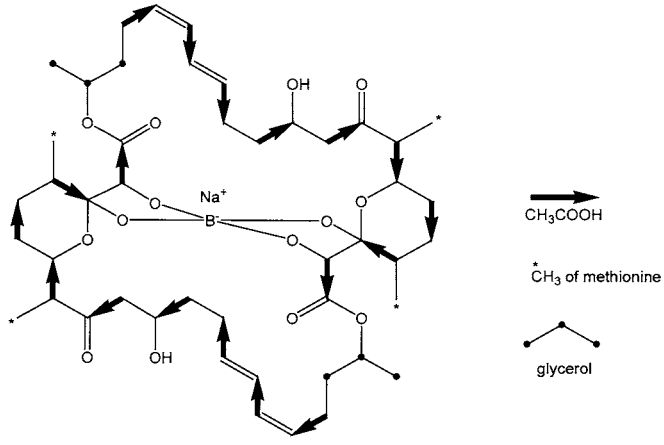


Fig. 3. Biosynthesis of tartrolon.

Tartrolon B and also previously isolated boromycin, aplasmomycin, and borophycin are polyketides and have the same boron-binding substructure C1-C7 in each half of the symmetric molecules (Fig. 4).

CONCLUSION

Boron is an essential trace element for plants and is beneficial for animals and humans. Dietary boron obviously plays a role in immune functions. Among the best-known natural boron-containing compounds are polyketide antibiotics such as boromycin, aplasmomycins, borophycin and tatrolons. Attempts are underway to incorporate boron into different biologically active molecules, particularly for medicinal application, e.g. for boron neutron capture therapy of brain tumors. Some boron-containing biomolecules may apparently act as signaling molecules that interact with cell surfaces.

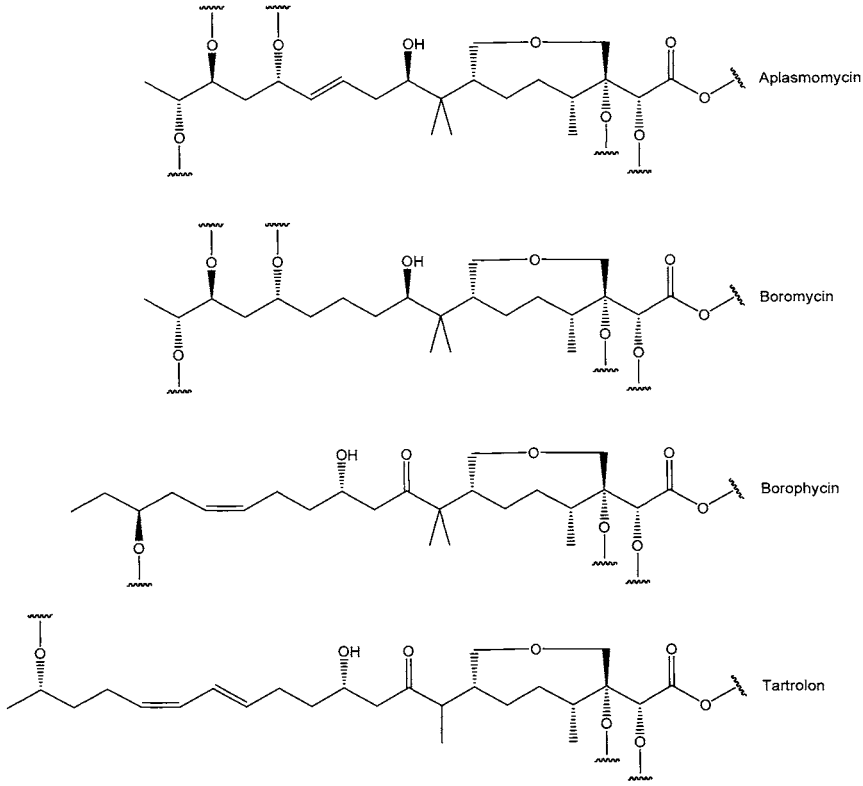


Fig. 4.The boron binding substructures of different boron-containing antibiotics.

Silicon

Silicon is a tetravalent metalloid and it is less reactive than its chemical analogue carbon. It is the second (after oxygen) most abundant element in the Earth's crust, making up 25.7% of it by weight. Elemental silicon is not found in nature. It occurs mainly in the form of silicon dioxide (also known as silica) and silicates (compounds containing silicon, oxygen, and metals). Amethyst, agate, quartz, rock crystal, flint, jasper, and opal are some of the forms in which the oxide appears. Granite, asbestos, feldspar, clay, hornblende, and mica are a few of the many silicate minerals. Its concentration in seawater is relatively low, only 3 mg/L [59].

Silicon has many similar chemical properties to carbon but its has a number of handicaps as a carbon analogue, however. Because silicon atoms are much bigger, they have difficulty forming double or triple bonds. Silanes (analogues of the alkanes) are highly reactive with water, and long-chain silanes spontaneously decompose. Molecules incorporating Si-O-Si bonds instead of Si-Si bonds are much more stable; ordinary sand is one such example [60].

Silicon is a biogenic element, although its content in the tissues of living organisms is not very high. The body of an adult human contains about 1 g silicon, mostly in bones, cartilages and tooth enamel; silicon is indispensable for normal growth and development of these tissues. In plant cells, increased silicon content can be found, e.g., in horse-tail or shave grass, and in stimuli of stinging nettles.

Some marine organisms have evolved the ability to biosynthesize exquisitely structured silicate shells. The "silicate" bacteria, which appear to be members or relatives of the genus *Bacillus*, are known to release silicon from aluminosilicates through the secretion of organic acids [61].

Silicon is extremely important as a building block of unicellular algae - diatoms. The main building material of the frustule (the finely sculptured protective surface layer of diatoms) is a float-stone, an opal-like water-containing polymer of silica: Diatoms are the only group of organisms whose development is totally dependent on the presence of soluble forms of silica in the environment. When silicon sources run out, DNA replication stops.

Microbes and microbial enzymes have been used in the biotransformation of organosilicon compounds. Biodegradation of

organosilicon under aerobic [62] and anaerobic [63] conditions has also been described.

Conceivably, some strange life forms might be built from silicon-containing substances were it not for an apparently fatal flaw in silicon's biological credentials. This is its powerful affinity for oxygen. When carbon is oxidized during the respiratory process of a terrestrial organism, it becomes the gas carbon dioxide, a waste material that is easy for a creature to remove from its body. The oxidation of silicon, however, yields a solid because, immediately upon formation, silicon dioxide organizes itself into a lattice in which each silicon atom is surrounded by four oxygens. Disposing of such a substance would pose a major respiratory challenge.

Life forms must also be able to collect, store, and utilize energy from their environment. In carbon-based biota, the basic energy storage compounds are carbohydrates and lipids in which the carbon atoms are linked in most cases by single bonds into a chain. Carbohydrates or lipids are oxidized to release energy and the waste products water and carbon dioxide in a series of controlled steps using enzymes, which catalyze specific reactions because of their shape and "handedness." A feature of carbon chemistry is that many of its compounds can take right and left forms, and it is this handedness, or chirality, that gives enzymes their ability to recognize and regulate a huge variety of processes in the body. Silicon's failure to give rise to many compounds that display handedness makes it hard to see how it could serve as the basis for the many interconnected chains of reactions needed to support life [64].

Even so, as has been pointed out, silicon may have had a part to play in the origin of life on Earth. A curious fact is that terrestrial life forms utilize exclusively right-handed carbohydrates and left-handed amino acids. One theory to account for this is that the first prebiotic carbon compounds formed in a pool of "primordial soup" on a silica surface having a certain handedness. This handedness of the silicon compound determined the preferred handedness of the carbon compounds now found in terrestrial life. An entirely different possibility is that of artificial life or intelligence with significant silicon content.

In the realm of science fiction there have occasionally been forms of life proposed that, while often highly speculative and unsupported by rigorous theoretical examination, are nevertheless interesting and in some

cases even somewhat plausible. One of the major sentient species in Terry Pratchett's Discworld universe is Trolls. Their being mineral-based has various interesting effects on their physiology and culture. Other silicon-based life forms are said to exist there but few appeared in the books. In the Star Wars universe, at least two life forms are based on silicon, and they live in space: the Mynocks and the Space slugs. A silicon-based life form, the Horta, appears also in the Star Trek.

As demonstrated in recent studies [65-68], organic molecules have a crucial role in the formation of biosilica owing to the specificity of interactions at the organic-inorganic interface. Biosilicification has been studied most extensively in diatoms and sponges. The proteins involved in biosilicification in these different marine species will be reviewed here, along with the mechanistic basis for their function.

As mentioned above, dissolved silicon in seawater occurs mostly as the undissociated orthosilicic acid, $Si(OH)_4$, whose total content in the world's oceans amounts to about 9.6 teratons. A variety of marine organisms, such as diatoms, silicoflagellates, radiolarians, and sponges, contain silica skeletons (SiO_2 x nH_2O) that are built up by taking up orthosilicic acid from seawater. The diatom cell wall (frustule) is made of nanostructured amorphous silica that is associated with polysaccharides and proteins. New cell walls are produced in a silica deposition vesicle (SDV); soluble silicon is taken up from the environment and concentrated in SDV, and here the insoluble silica is formed and subsequently secreted. Recently, the first data were reported that correlate distinct silica elements with specific proteins within the diatom cell wall. Three families of proteins have been identified in the organic matrix of the cell wall of the diatom *Cylindrotheca fusiformis*: frustulins, pleuralins, and silaffins. Frustulins were extracted from the cell wall with ethylenediaminetetraacetate (EDTA), and consisted of four (α, β, γ, δ) Ca^{2+} binding glycoproteins with molecular weights ranging from 75 to 200 kDa.

Three types of pleuralins, formerly known as HF-extractable proteins - pleuralin 1 (200 kDa), pleuralin 2 (180 kDa), and pleuralin 3 (150 kDa) were discovered. They are involved in the formation of new theca (one of the halves in the cell wall) when one parental diatom is divided into two daughter diatoms.

The silaffins induced and regulated silica precipitation at ambient temperature and pressure. Native silaffins (natSil) including natSil-1A (6.5 kDa), natSil-1B (10 kDa), and natSil-2 (40 kDa), were detected by treating the diatom cell wall with NH_4F. Native silaffins are highly post-translationally modified and some functional groups such as phosphate, sulfate, and carbohydrate are lost during the process of dissolving the cell wall with HF, resulting in lower molecular weight proteins: silaffin-1A (4 kDa), silaffin-1B (8 kDa) and silaffin-2 (17 kDa) proteins. A gene *sil 1* has been isolated from a *C. fusiformis* genomic library that encodes a polypeptide of 265 amino acids. Seven repeat sequences were identified in *sil 1* and termed R1 to R7. R1 and R2 peptides correspond to the precursors of silaffin-1B and silaffin-1A2, respectively, whereas R3 to R7 peptides correspond to the precursors of the silaffin-1A1.

The major component, 1-frustulin, is one of a number of isoforms of a 75 kDa protein that is immunologically related to proteins extracted from the cell walls of other diatom species, namely *Navicula pelliculosa, Nitzschia alba, N. angularis* and *Phaeodactylum tricornutum.*

Each frustulin contains at least three of five structural elements: 1) presequence domain, 2) acidic cysteine-rich domain with a highly repetitive structure, its common sequence being C-E/Q-G-D-C-D, 3) proline-rich domains, 4) polyglycine domains and 5) a tryptophan-rich domain.

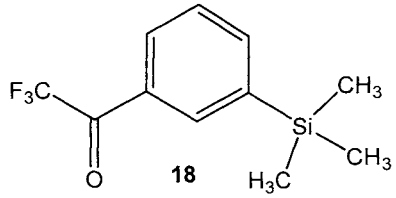
Diatoms were also investigated for the mechanism of silicon transport that is an integral part of the silicification process. As the environmental concentrations of dissolved silicon are rather low, diatoms must have an efficient transport system. Orthosilicic acid must not only be transported into the cell, but also transported intracellularly into the SDV where silica formation occurs. A protein of the diatom *C. fusiformis* was characterized that transports silicon from seawater into the cell. This discovery was accomplished by cloning and characterizing the DNA that codes for this protein. However, silicon transporter proteins of this particular type are not necessarily involved in intracellular transport.

A further major breakthrough in silicon biochemistry came from recent studies with the common marine sponge *Tethya aurantia.* After dissolution of the silica with buffered hydrofluoric acid, these filaments can be isolated, purified, and resolved into three very similar silicon-free subunits, named silicatein (silica protein) α, β, and γ. Analyses of the

amino acids demonstrated that the compositions of silicatein α (29 kDa), β (28 kDa), and γ (27 kDa) are highly similar, the relative proportions in the filaments being α:β:γ 12:6:1. Analysis of the DNA sequence for silicatein α (the principal subunit that comprises about 70% of the mass of the filaments) revealed that this protein is highly similar to members of the cathepsin L and papain family of proteases. These findings gave rise to the fascinating idea that the silicateins might possess an enzyme-like activity and might catalyze the hydrolysis of silicic acid esters. This result clearly indicates the catalytic function of the silicateins (in their native three-dimensional structure) in accelerating the formation of silica and silsesquioxanes. In summary, silicateins can catalyze the formation of silica and organyl-substituted silsesquioxanes from the corresponding silicic acid esters $RSi(OEt)_3$ (R=OEt, Me, Ph) at neutral pH and ambient temperature, and can direct the structures of the resulting products. The complete amino acid sequence and the three-dimensional structure of silicatein α are highly homologous to those of cathepsin L. Thus, it has been proposed that silicatein α catalyzes the hydrolysis of $Si(OEt)_4$ at neutral pH through the activity of the serine and histidine residues. Direct experimental evidence for the proposed role of the specific serine-26 and histidine-165 residues came from site-directed mutagenesis, in which the cloned recombinant DNA that codes for silicatein α was modified in a site-specific manner *in vitro*, and the mutant DNAs were then used as templates to direct the synthesis of the corresponding proteins in bacteria. Two structural variants of the silicatein α protein were produced, in which the serine-26 residue and histidine-165 residue were specifically replaced by an alanine moiety. Quantitative comparison of the catalytic activities of the resulting protein products with the activity of the original protein supports the suggestion that serine-26 and histidine-165 of silicatein α are indeed required for the efficient catalysis of silica formation from $Si(OEt)_4$ at neutral pH. From these findings, a detailed mechanistic model was deduced for the silicatein α-mediated catalysis. Intra-silica proteinaceous materials have been extracted from the branches of primitive plants, *Equisetum telmateia* (great horsetail) and *E. arvense* (common horsetail), and from hairs found on the lemma of the grass *Phalaris canariensis*, where they comprise 0.015 - 0.03 wt % of the silica. The extracts contain protein and carbohydrate components enriched in xylose and glucose. In common with the earlier diatom studies, the amino acid compositions of the matrices contain around 25 mol % serine/threonine and 20 mol % glycine [69].

The bioorganic chemistry of silicon covers two diametrically different areas. One is the formation of silicon compounds in nature – see above – while the other is concerned with organosilicon compounds obtained by synthetic procedures. This latter group of compounds, though not of natural origin, deserves our attention since it can shed light on some of the mechanisms and interactions taking place in the former group. The highly varied, both structurally and pharmacologically, group of compounds is illustrated here on a few of them, in particular those that are in different stages of clinical trials or are already commercially produced [70, 71].

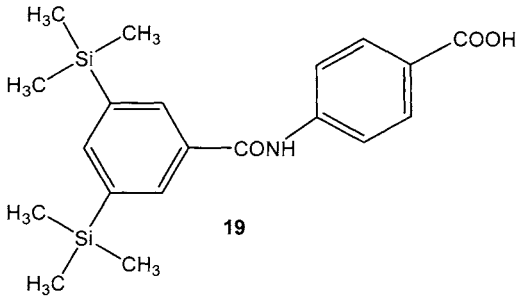
Zifrosilone (**18**) is a novel tight-binding inhibitor of acetylcholinesterase, which is in development as a potential therapeutic compound in the symptomatic treatment of Alzheimer's disease [72]. Pharmacokinetics and pharmacodynamics of the compound were studied in the dogs and rats after single intravenous and subcutaneous administrations. When evaluated in human healthy volunteers, the orally administered drug was well tolerated but displayed a strong dose-related inhibition of red blood cell acetylcholinesterase [73] and its development was consequently halted.



18

TAC-101 (**19**) is a synthetic retinoid with selective binding affinity for RAR-α (retinoic acid receptors) [74, 75]. RAR-α binding by TAC-101 has been shown to inhibit the activity of transcription factors, including activated protein-1, which activates the expression of metastasis-related genes, including urokinase-type plasminogen activator, matrix metalloproteinase-9, and vascular endothelial growth factor. TAC-101 has been demonstrated to inhibit liver metastases caused by intrasplenic or intraportal vein injection of tumor cell lines, including AZ-521 (human gastric adenocarcinoma), A549 (human lung adenocarcinoma), and colon 26-LF (metastatic variant of murine colon cancer). The pharmacokinetics and distribution of TAC-101 have been studied in mice, dogs, and monkeys. TAC 101 might be also of interest for the treatment of Alzheimer's disease and other age-related dementias.

Given the above preclinical observations, patients in a clinical study were monitored closely during treatment (including measurement of testosterone, luteinizing hormone, follicle-stimulating hormone levels, alkaline phosphatase, and serum vitamin A concentrations). This phase I clinical study of TAC-101 was conducted to determine the safety, toxicity, and pharmacokinetics of this agent in patients with advanced cancer. Currently, the drug is in a phase I/II clinical trial for advanced hepatocellular carcinoma.

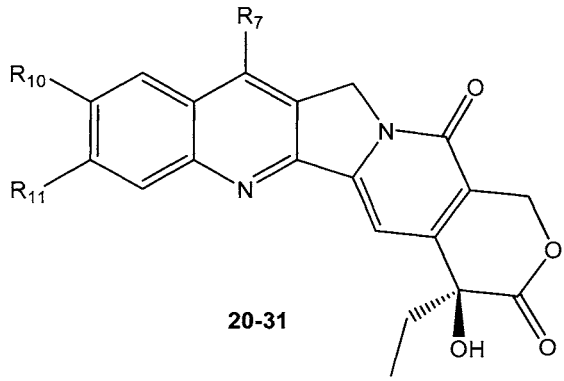


19

Karenitecin (BNP1350) (**20**) is a derivative of camptothecins, a natural alkaloid of *Camptotheca acuminate,* a member of the family Nyssaceae native to China and Tibet [76, 77]. Derivatives of camptothecin are known to have broad anticancer activity, but their use has been limited in part due to common and serious side effects. One of these derivatives, karenitecin, has demonstrated very high potency and broad activity in preclinical studies against human cancers including prostate, pancreas, lung, breast, colon, ovary and head and neck [78]. A phase II trial for karenitecin with non-small cell lung cancer patients has been completed. Karenitecin is currently being evaluated in phase II trials in patients with primary brain tumors, melanoma, and advanced ovarian cancer. A phase I/II trial with orally administered karenitecin was initiated for patients with solid tumors/non-small cell lung cancer.

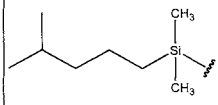
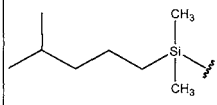
The silatecan (DB-67) (**21**) represents a new generation of camptothecin derivatives (see Table 12) that exhibits a potent *in vitro* DNA topoisomerase I (TOP1)-mediated DNA-damaging activity, improved blood stability, and holds significant promise for the treatment of human cancers [79]. This new agent was found to be 25-times more lipophilic than camptothecin; it incorporates readily into cellular and liposomal bilayers. In addition, its 10-hydroxy functionality enhances drug stability in the presence of human serum albumin. Thus, the net

lipophilicity and the 10-hydroxy moiety together function to promote the enhanced blood stability [80].



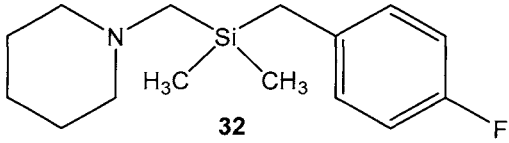
20-31

Table 12. Median effective concentrations for inhibition of the proliferation of five glioma cell lines by a variety of camptothecin derivatives

No	Name	R_7	R_{10}	R_{11}	ED_{50} (ng/mL)				
					U87	A172	SG388	LN-Z308	T98G
20	BNP1350	$(CH_2)_2Si(CH_3)_3$	H	H	-	-	-	-	-
21	DB67	t-Bu$(CH_3)_2$Si	OH	H	2	30	3	40	6
22	CHJ439A	$(CH_3)_3Si$	H	H	2	6	3	500	300
23	CHJ758A	$(CH_3)_3Si$	OH	H	100	8	10	20	80
24	CHJ792A	$(CH_3)_3Si$	NH_2	H	10	20	2	20	30
25	CHJ800A	$(CH_3)_3Si$	H	NH_2	30	100	10	100	200
26	DB3		H	H	200	200	30	>1000	100
27	DB124		OH	H	300	600	300	500	>1000
28	DB148	$Cl(CH_2)(CH_3)_2Si$	H	H	30	30	30	60	60

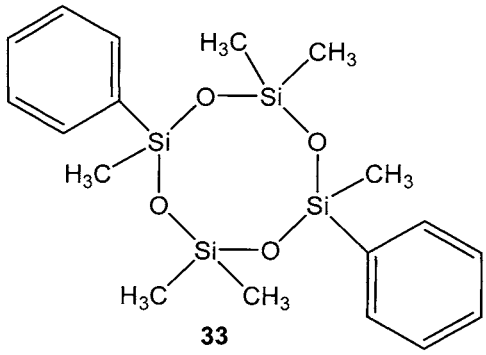
29	DB202	t-Bu(CH_3)$_2$Si	H	H	10	60	20	1000	60
30	DB204	t-Bu(CH_3)$_2$Si	NH_2	H	10	10	200	60	8
31	DB205A	t-Bu(CH_3)$_2$Si	NH_2	F	6	3	20	60	10

Silperisone (**32**) is a sodium channel blocker acting centrally as a muscle relaxant. The compound has been in preclinical animal studies [81, 82] and has been proposed recently to have potential as an antispastic drug in demyelinating diseases such as multiple sclerosis. *In vivo*, silperisone depressed ventral root reflexes and excitability of motoneurons. Whole-cell measurements in dorsal root ganglion cells revealed that silperisone depressed voltage gated sodium channel conductance at concentrations that inhibited spinal reflexes.



32

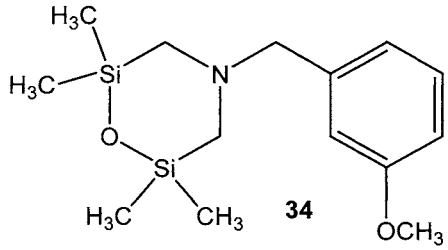
Cisobitan (**33**) has been the first molecule that was investigated as the silicon-containing drug [83]. This drug was shown to possess estrogenic, antigonadotropic [84], and antifertility activities in animals and was evaluated in a pilot and a prospective clinical study as an antiprostate cancer agent. In a limited trial, cisobitan was not adequate for tumour palliation. Cisobitan trials were discontinued because the compound did not provide any significant therapeutic advantage over estrogens [85].



33

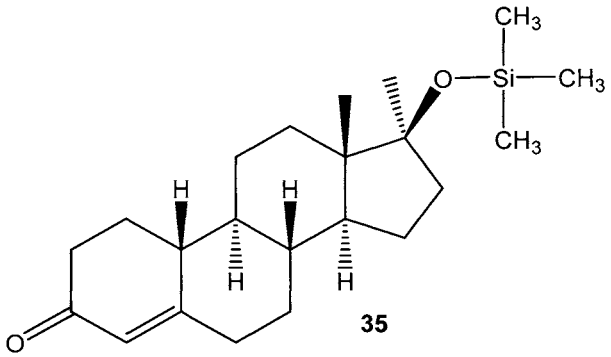
The pharmacokinetics of Sandoz compound 58-112 (**34**), a new chemical entity with a unique myotonolytic effect, was studied [86]. The

metabolic pathways of Sandoz compound 58-112 were evaluated in the rat, dog, and man after a single oral dose [87].



34

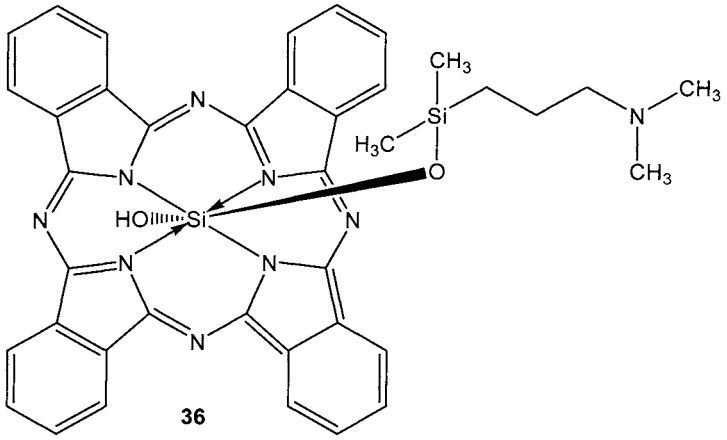
Silabolin (**35**) was used for the treatment of muscle wasting diseases in Russia and eastern Europe. It has often been abused as an anabolic [88].



35

Photodynamic therapy is an innovative treatment for several types of cancer that was developed during the last 30 years. It is based on visible light excitation of a tumor-localized photosensitizer, which, after excitation, transfers energy to oxygen forming reactive oxygen species that further oxidize cellular targets. Pc4 (**36**) (silicon phthalocyanine 4), a synthetic photosensitizer agent containing a large macrocyclic ring chelated with silicon, has been extensively studied in cell models where it was shown to bind preferentially to the endoplasmic reticulum, Golgi complex and mitochondria and to induce rapid apoptosis after exposure to light through mitochondrial oxidative stress. Currently, phase I human photodynamic therapy clinical trials are being conducted with Pc4, for the treatment of respectively skin cancer, cutaneous T-cell lymphoma, actinic keratosis, squamous intraepidermal carcinoma (Bowen's disease), and adult solid tumors, breast cancer, head and neck cancer, hematopoietic and lymphoid cancers, and skin tumors [89, 90]. Pc4 localizes primarily

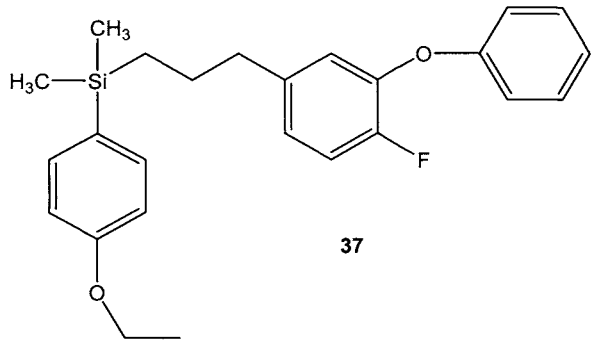
in mitochondrial membranes and, after photoexcitation, forms reactive oxygen species that induce apoptosis [91].



36

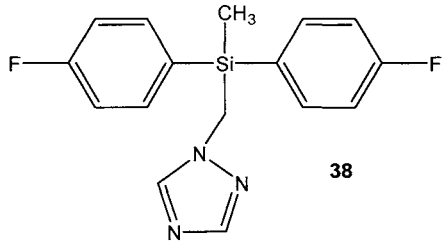
The tumor-promoting activities of different commercial compounds (e.g. the non-ester pyrethroid silafluofen, **37**) used as termiticides were measured by a cell-transformation assay employing Bhas 42 cells [92]. Their initiating activities were also measured by the microsuspension assay employing *S. typhimurium* TA98 and TA100 strains. The termiticidal performance of wood-based composites, hardwood plywood, softwood plywood, particleboard, and oriented strand board was evaluated following treatment with silafluofen, using supercritical carbon dioxide as a carrier solvent [93]. Degradation of termiticides by silafluofen and its effect on performance against Thailand's economically most important subterranean termite, *Coptotermes gestroi* (Isoptera: Rhinotermitidae) were described [94].

The effect of rice cleaning and cooking on the residues of silafluofen (**37**) was described in rice that had been sprayed in a paddy field with this pesticide [95]. Silafluofen was found to remain in the hull of the rice. The residual concentration of the pesticide in polished rice was higher than that in pre-washed rice processed ready for cooking. During the cooking procedure, the reduction of pesticides in polished rice was higher than that in brown rice.



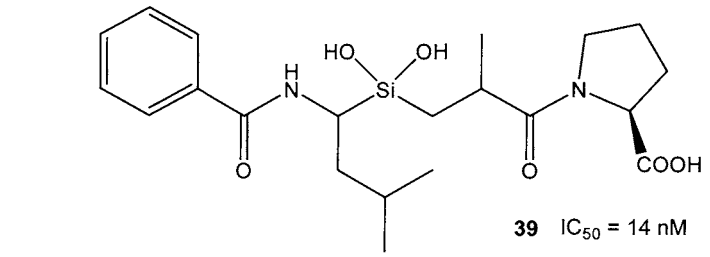
37

Sensitivities (ED_{50}) of 200 monoconidial isolates of *Venturia inaequalis* to the sterol demethylation inhibitor flusilazole (**38**) were determined, based on the inhibitory effect on mycelial growth [96].

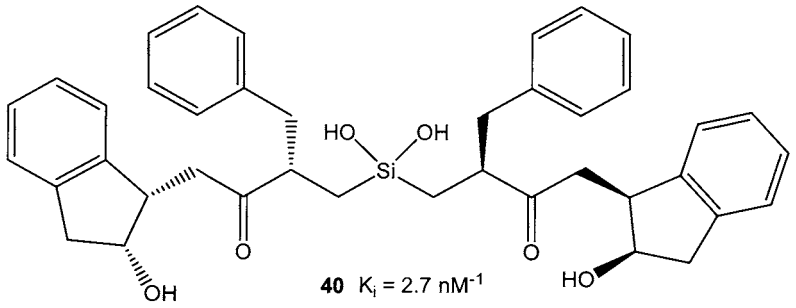


38

Proteases play essential roles in most biological processes and are important therapeutic targets for a multitude of diseases including cancer, viral, parasitic, fungal, and bacterial infections, inflammation, and cardiovascular disease. This is best exemplified by the HIV protease inhibitor drugs. The reaction mechanism of proteases is to convert the carbonyl group of the target peptide bond into an enzyme-stabilized tetrahedral diol transition-state intermediate by addition of water. This intermediate then collapses to yield two peptide products. The enzyme binds the tetrahedral diol intermediate, the specificity being provided by specific contacts for the amino acid side chains. A silicon diol structure, in which the $C(OH)_2NH$ group of the transition state is replaced by a $Si(OH)_2CH_2$ moiety, mimics the tetrahedral transition state and as silicon strongly favors sp^3- over sp^2-hybridisation, silicon diols ($R_2Si(OH)_2$) do not eliminate water to form silicon analogues ($R_2Si{=}O$) of ketones. It has been shown that incorporating such silicon diol moieties into peptide mimetics results in potent and selective inhibitors of proteases, represented e.g. by angiotensin-converting enzyme and HIV protease respectively (compounds **39** and **40**).



39 IC_{50} = 14 nM



40 K_i = 2.7 nM^{-1}

The decapeptides **41-43** were studied [97] *in vitro* in receptor binding and functional assays using recombinant cell lines expressing the human gonadotropin releasing hormone (GnRH) receptor. The C/Si/Ge analogues **41-43** are derivatives of the GnRH antagonist, which bears an (*S*)-tyrosine residue [instead of the (*S*)-Me_3C-Ala, (*R*)-Me_3Si-Ala, or (*R*)-Me_3Ge-Ala residue] in position 5 of its decapeptide backbone. All compounds behaved as potent GnRH antagonists (Table 13), the binding affinities and antagonistic potencies of the three C/Si/Ge analogues being quite similar. Compounds **41-43** were also studied for their *in vivo* activities in male rats after s.c. administration. They produced both a strong testosterone suppression (single-dose treatment, 1.5 mg/kg) and a strong LH (luteinizing hormone) suppression (castrated male rat; single-dose treatment, 0.05 mg/kg). For the silicon- and germanium-containing decapeptides **42** and **43** the testosterone and LH suppression lasted for a significantly longer period of time compared with the effects of the carbon analogue **41**.

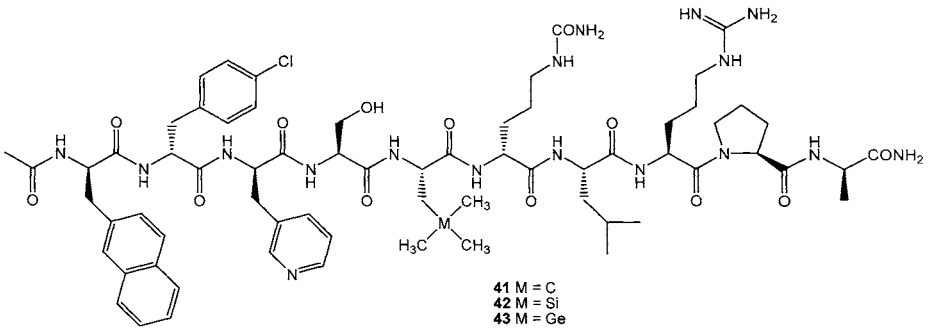


Table 13. Binding affinities (K_A values) and antagonistic potencies (IC_{50} values) of the decapeptides 41-43 and Cetrorelix[a] at the human GnRH receptor

decapeptide	K_A [nM^{-1}]	IC_{50} [nM]
41	2.20	0.93
42	2.82	0.75
43	2.01	0.50
Cetrorelix[a]	5.94	0.25

[a] Cetrorelix is a decapeptide with a sequence derived from luteinizing hormone releasing hormone and subsequently modified. Five of the ten amino acids present are in the D configuration. The peptide is protected from degradation by means of C- and *N*-terminal protective groups and was used as standard.

CONCLUSIONS

Silicon is essential for growth and biological function in a variety of plant, animal, and microbial systems, yet the molecular mechanisms of these interactions are still unknown. The *in vitro* studies of natural systems in the field of silica biosynthesis are complicated. Recently, studies have been conducted to test the ability of homologous enzymes to catalyze the formation and cleavage of siloxane bonds, in order to better understand the role of various proteins in the biosilicification process. Some enzymes, e.g. *Suberites domuncula* silicase, have been observed to hydrolyze an aqueous solution of amorphous silica during the formation of silicic acid. Genetic engineering, in conjunction with biotechnological

methods, offers the prospect of developing new, environmentally benign routes to the synthesis of organyl-substituted siloxanes. It can be expected that further studies of the proteins, genes, and molecular mechanisms controlling silicon metabolism in diatoms and sponges may also help to reveal the mechanisms involved in the essential requirement for silicon for optimal development and growth in many plants and animals.

The synthetic chemistry of organosilicon molecules offers the possibility to produce not only sila-substituted analogues of existing drug molecules, but also new drugs including silicon as a lipophilic bioisostere. Although the sila-substituted molecules have not been found to offer significant advantage in terms of affinity toward cell-membrane receptors relative to their original counterparts, they can provide improved receptor selectivity. The lipophilicity of organosilicon drugs is usually reflected in their easier cell penetration, which results in an improved intracellular activity as enzyme inhibitors or apoptotic inducers. The advantages of organosilicon drugs in terms of pharmacokinetics and pharmacodynamics are further documented by the fact that a number of these compounds are currently undergoing human clinical trials. The introduction of silicon bioisosteres in drugs is not a universally beneficial "miraculous" approach that medicinal chemists can rely on in every situation. In specific instances, however, silicon bioisosteres can lead to significant improvements in the performance of a given drug and they therefore deserve to be considered more often in structure-aided drug design and lead development.

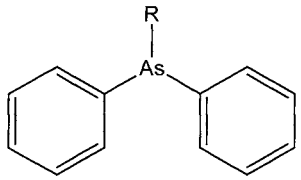
Arsenic

Arsenic is widely distributed in the environment. It ranks twentieth in the abundance of elements in the Earth's crust. In the environment, arsenic is mainly associated with sulfide minerals. Soils contain arsenic usually in the range of 0.5 to 35 mg/kg [98]. Arsenic levels in gold ore deposits in Zimbabwe attain 20 g/kg in soils [99].

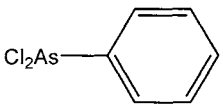
Arsenic contents measured in sediments are between 0.1 and 490 mg/kg while levels up to 1.5 g/kg were found in coals (average: 13 mg/kg). The concentration of arsenic in seawater varies between 0.09 and 24 μg/L (average: 1.5 μg/L), and in freshwater between 0.15 and 0.45 μg/L (maximum: 1 mg/L). In mineral and thermal waters, arsenic was found in concentrations up to a factor of 300 higher than its mean concentration in groundwater [100]. The WHO recommends a maximum contaminant level for arsenic in drinking water 10 μg/L.

The smelting of Cu, Ni, Pb, and Zn ores is the most important anthropogenic arsenic source. Another anthropogenic source of arsenic in the environment is the burning of fossil fuels in households and power plants. The burning of coal leads to the emission of arsenic by volatilization of As_4O_6 which condenses in the flue system [101]. One important anthropogenic source of arsenic contamination of the environment was the use of arsenical fungicides, herbicides, and insecticides in agriculture and wood industry. In World War I organically bound arsenic (diphenylchloroarsine (**44**), diphenylcyanoarsine (**45**), phenyldichloroarsine (**46**)) was used as warfare. Dimethylarsinic acid (**47**) (agent blue, cacodylic acid) was used during the Vietnam War for defoliation for military purposes [102]. Until the discovery of antibiotics, arsenic compounds were widely used in medicine for the treatment of a variety of illnesses. A solution containing 1% of potassium arsenite (Fowler's solution) was used for the treatment of leukaemia and psoriasis or for fortifying. Donovan's solution (arsenic iodide) and Valagin's solution (arsenic trichloride) were recommended for the treatment of rheumatism, arthritis, asthma, malaria, trypanosomiasis, tuberculosis, and diabetes. Sodium arsenate was used for the treatment of chronic skin diseases, some parasitic diseases, and anaemia. An organically bound arsenic compound with 32 % arsenic (Salvarsan=Arsphenamin (**48**) and Neosalvarsan (**49**)) were used for the treatment of syphilis [102]. Today the reuse of arsenic is discussed for the treatment of cancer [103, 104].

Arsenic is considered to be an essential element but a lot of arsenic compounds are toxic [105]. Organic arsenic compounds are less toxic than inorganic ones. AsH_3 is the most toxic compound. The fatal dose is 250 mg/m^3 at an exposure time of 30 minutes [106]. The lethal dose (LD_{50}) for arsenic trioxide is 34.5 mg/kg, sodium arsenite 4.5 mg/kg, sodium arsenate 18 mg/kg, monomethylarsonic acid (**50**) 1800 mg/kg, dimethylarsinic acid (**47**) 1200 mg/kg, and trimethylarsine (**51**) 8000 mg/kg [107].



44 R = Cl
45 R = CN



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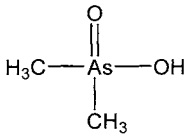

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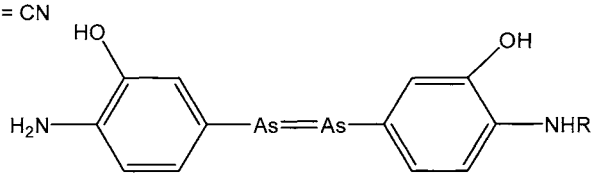


48 R = H
49 R = CH_2OSONa

Arsenic can cause acute and chronic poisoning. Acute arsenic poisoning may cause vomiting, dryness of the mouth and throat, muscle cramps, colicky abdominal pain, tingling of the hands and feet, circulatory disorders, and nervous weakness. Cold and clammy skin, hallucinations, delirium, and diarrhea appear. If death does not occur within 24 h, irreversible organ disorders occur.

Chronic arsenic poisoning involves non-specific symptoms such as chronic weakness, loss of reflexes, weariness, gastritis, colitis, anorexia, weight loss, and hair loss. Long-term exposure through food or air results in hyperkeratosis, hyper pigmentation, cardiovascular diseases, disturbance in the peripheral vascular and nervous systems, circulatory disorders, brittle loose nails with transverse white bands across the nails called Mees lines, eczema, liver and kidney disorders.

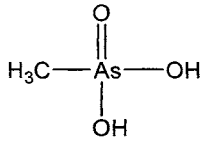
To a certain amount the human body can detoxify the inorganic arsenic compounds As(III) and As(V) by methylation, so that the affinity of arsenic for the tissue is reduced. The possibility of methylation of arsenic

is limited to an arsenic uptake of about 500 μg/day [108]. Arsenic is genetically harmful because it inhibits the repair of DNA damage and is also carcinogenic. Uptake of arsenic by air causes lung cancer. Oral uptake causes bladder cancer, renal cancer, liver cancer, and skin cancer [109-112].

The arsenic compounds described in this chapter were arranged according to their source. This was motivated above all by the fact that nearly all organisms included in this review contain individual organic arsenic compounds. To organize the chapter according to the compounds would mean listing, with each compound, not only the names of the organisms but also citing all relevant literature sources; this would considerably detract from the readability of the text.

The term hyperaccumulators was first used by Brooks et al. [113] to describe the plants that take up and accumulate more than 1000 μmoles As/g dry weight. A report about an arsenic-hyperaccumulating fern species additionally discussed the phytoremediation potentials of such plants [114]. Recent investigation has shown that the arsenic compounds in terrestrial and aquatic plants, fungi, and lichen species are also interesting natural products [115, 116].

A study of plants and soil collected near an ore vein in Gasen (Austria) gave the concentration of total, inorganic, and organoarsenic species at this site [117]. The arsenic concentration in the soil ranged from 700 to 4000 mg/kg on dry weight, and arsenic concentrations in plant species ranged from 0.5 to 6 mg/kg on dry weight. The extracts of the plants, *Trifolium pratense, Dactylis glomerata,* and *Plantago lanceolata*, contain inorganic arsenic such as As(III), As(V), besides the simple methylated compounds **47**, **50**, and **52-55**. The main organoarsenic detected in *Dactylis glomerata* and *Plantago lanceolata* was **51**. Small amounts of **52-54** were present in all three species, whereas **55** was only detected in *Plantago lanceolata.*



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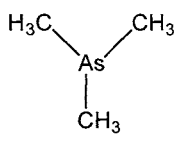

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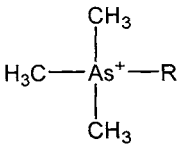


52 R = O^-
53 R = CH_3
54 R = CH_2COO^-
55 R = CH_2CH_2OH
62 R = $CH_2CH_2COO^-$

Twelve green plants, namely *Achillea millefolium, Alnus incana, Asplenium viride, Dryopteris dilata, Deschampsia cespitosa, Equisetum pratense, Fragaria versa, Larix decidua, Picea abies, Rubus idaeus, Vacciniun myrtilis*, and *Vaccinium vitis idaea* as well as two lichen species, *Alectoria ochroleuca* and *Usnea articulata* have been examined for the presence of organoarsenic compounds [118]. All green plants and lichens contain **47, 50**, and **54-56**. The maximum was determined in the lichen *A. ochroleuca*, i.e. 77 μg/kg dry weight. Acid **50** was found in *Agrostis scabra* (0.6%) and *Hordeum jubatum* (3.6%) from Yellowknife, Northwest Territories in Canada [118]. Terrestrial grasses, *Bidens cernua*, *Carex* sp., *Equisedum fluviatile* and *Typha latifolia*, as well as terrestrial plants, contain also compound **50**. Arsenosugars such as glycerol-ribose **56** and phosphate-ribose **57** as well as acid **50** were found in aquatic plants (submergents) such as *Lemna minor*, *Myriophyllum* sp. and *Sparganium augustifolium*.

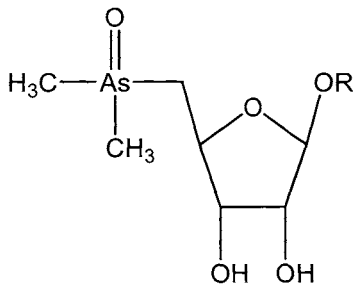
Levels of total arsenic and arsenic compounds were determined in terrestrial fungi, *Paxillus* sp., *Psathyrella* sp., *Leccinum* sp., *Coprinus* sp., *Lycoperdon* sp. (Giant Mine), and *Lycoperdon* sp. (Con Mine), from Yellowknife [120]. The mushroom *Lycoperdon* sp. from the Giant Mine tailings pond also contained a proportionally higher amount of arsenate, although the major arsenic species in both specimens of *Lycoperdon* sp. was arsenobetaine (**54**). Arsenobetaine (**54**) was also observed to be the major arsenic species extracted from *L. echinatum* (78%), *L. perlatum* (88%), and *L. pyriforme* (62%); minor components included As (III), As (V), **47** and **50** [121]. The arsenobetaine (88% of extracted arsenic) was determined as the water-soluble species in the mushroom *Coprinus comatus*. Minor components included **51**, arsenocholine (**55**) and an unknown arsenic compound [121]. Arsenic content of *Paxillus involutus* was unusual because a major proportion of extracted arsenic (36%) was in an unidentified form (unknown compound Y). An unknown peak possessing a retention time similar to that for unknown Y, in an almost identical chromatographic system, was observed in mussels [122].

Arsenic compounds were determined in methanol extracts from 50 mushrooms. The main arsenic compound found in many mushrooms (Agaricales and Aphyllophorales) was **54**. Dimethylarsinic and methylarsonic acids were present in many mushrooms, but generally as minor components. *Collybia butyracea* contained mainly **54** (8.8 mg/kg) and dimethylarsinic acid (**47**) (1.9 mg/kg). In *Laccaria laccata*,

Leucocoprinus badhamii and *Volvariella volvacea*, **47** was the major metabolite. Arsenocholine (**55**) and the tetramethylarsonium ion (**53**) were present in a few species, generally in low concentrations. Arsenobetaine (**54**) was the main compound in *Sparassis crispa* [123].

Arsenic species such as arsenic (III), arsenic (V), **47**, and **50** have also been found in many cultivated vegetables and fruits: garlic, onion, potato, carrot, beetroot, spinach, asparagus, cabbage, rice, radish, chard, corn, tomato, and beans, and the concentration of these arsenic species can be varying depending on the locations where the vegetables and fruits were collected [124-126].

Aqueous extracts of the marine brown alga *Hizikia fusiforme* (phylum Sargassaceae) contained methylarsonic acid **50** and dimethylarsinic acid **47** in addition to inorganic arsenic and other unknown organic compounds [127-129]. Further investigation of the *Hizikia* species showed that these algae contain also arsenosugars such as **56-60**. Studies of similar type directed at *Laminaria japonica* [130] and *Laminaria digitata* [131] indicated that arsenic species were present as **47**, **50**, and **54-59**, as well as some novel arsenolipids **61-63**.



No. R

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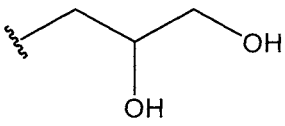

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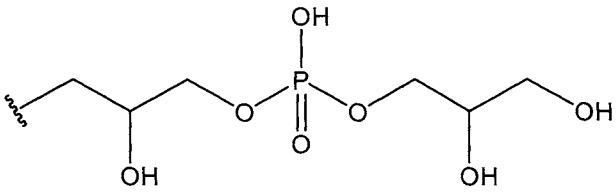

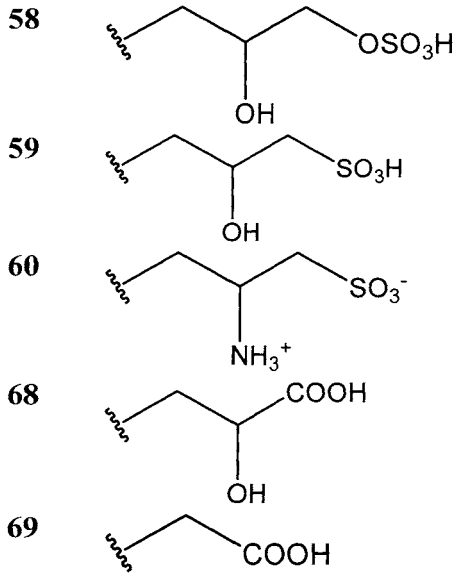

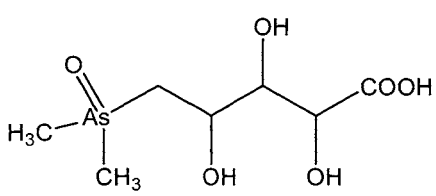

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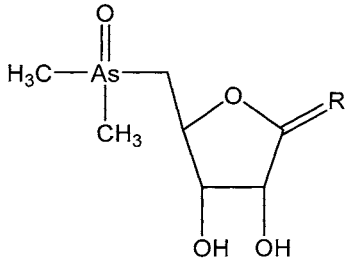
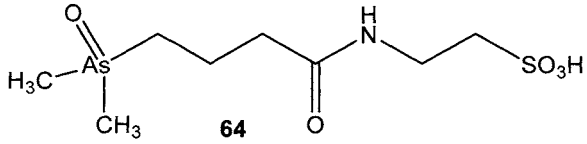
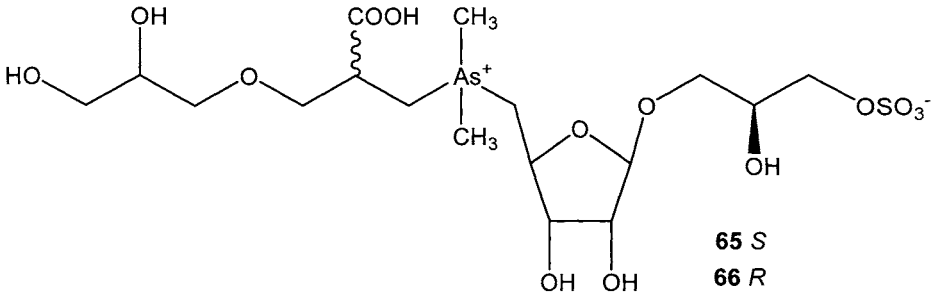


63 R = O
70 R = H_2



64

Four organoarsenic species **56-59** have been isolated from *Laminaria* sp. [132]. Compound **64** and two diastereoisomers **65** and **66** were found as major compounds [133]. Arsenic-containing ribofuranosides **56-59** as well as inorganic compounds have been isolated from freshly collected samples of the Australian brown kelp *Ecklonia radiana* [134].



The distribution of arsenoribosides and inorganic arsenic compounds has been studied in three classes of marine algae: Phaeophyta (brown), *Echlonia radiata, Padina fraseri, Lobophora* sp., *Hormosira bankii, Sargassum* sp.; Rhodophyta (red), *Corallina officinalis, Amphiroa anceps, Laurencia* sp.; and Chlorophyta (green), *Cladophora subsimplex, Codium lucasii,* and *Ulva lactuca* collected from the coastline near Sydney (Australia) [135]. The authors isolated three major arsenoribosides **57-59** from all examined samples. Biotransformation of arsenic by the brown alga *Fucus serratus* was monitored by HPLC analysis, and arsenite, arsenate, **47**, **50**, and arsenosugars **56-59** were found [136].

Some studies attempted further characterization of the arsenic compounds in algae [137], which involved the growth of microalgae, *Tetraselmis chuii* [138], *Chaetoceros concavicornis* [139] in media containing radiolabeled arsenate.

Unicellular microalgae, *Chladomydomonas reihardtii* [140] and *Polyphysa peniculus* [141] as well as green algae *Chlorella* sp. [142], *Chlorella vulgaris* [143], and *Phormidium* sp. [144] can accumulate and biotransform inorganic arsenic to methylated species. Other marine microalgae, such as *Dunaliella salina, Chattonella antiqua, Heterosigma akashimo, Skeletonema costatum, Chaetoceros debile*, and *Thalassiosira weissflogii* [145], are tolerant to arsenate and accumulate As(III) and As(V) in high concentrations, and can transform arsenic (III) and arsenic (V) acids to other arsenic species such as **47**, **50**, **51**.

Thirty different types of Chinese edible seafood, including brown algae, red algae, fish, crab, shrimp, mussels, oysters, and clams, which are very popular foodstuffs in the Chinese cuisine, were examined for their total content of arsenic as well as its different compounds [146].

Arsenolipids such as **56-59** were detected in all of the extracted algae samples (1.5-33.8 μg/g dry wt) and fish samples (0.018-0.78 μg/g wet wt). Arsenobetaine **54** was detected in all of the extracted fish and shellfish samples (0.025-6.604 μg/g wet wt). A major share of arsenic components in seafood was organic arsenic with a low toxicity [146].

The total arsenic concentrations of *Porphyra* collected from the China Sea ranged from 2.1 to 21.6 mg/kg [147]. The analysis report also showed that arsenosugars were the only arsenic species that could be detected in all of the extracts of samples. Arsenosugar PO_4 (**57**) was the major compound in most samples (up to 13.9 mg/kg of dry weight), followed by arsenosugar OH (**56**) (up to 6.2 mg/kg of dry weight). The arsenosugars were stable during a short-term heating at 100 °C. A substantial increase of dimethylarsinic acid (**47**) was detected in urine samples collected from six people. The results obtained indicated that arsenosugars had been metabolized to **47**, which is more toxic than arsenosugars.

The authors [148] describe arsenic binding to *Fucus vesiculosus* metallothionein (which is a metalloprotein having low molecular weight and high cysteine content, which binds a range of metals), characterized by electrospray ionization mass spectrometry. Five arsenic metallothioneins were detected with increasing As to protein ratios. These results provide important information about the metal-chelation behavior of this novel algal metallothionein, which is a putative model for arsenic binding to *F. vesiculosus in vivo*.

A study [149] examined arsenic compounds in raw and cooked edible seaweed and the bioaccessibility of arsenosugars (**56-59**). An *in vitro* digestion (pepsin, pH 2; pancreatin-bile extract, pH 7) was used to estimate arsenosugar bioaccessibility. Cooking of *Undaria pinnatifida* and *Porphyra* sp. did not alter the arsenic species present in the methanol-water extract, but it produced a substantial increase (2 and 5 times) in the As(V) extracted from *Hizikia fusiforme*. In all of the seaweeds analyzed, arsenosugar bioaccessibility was high (> 80%) and did not vary as a result of cooking. Arsenosugar degradation as a result of *in vitro* digestion was not observed.

Water-soluble arseno-compounds were detected in freeze-dried samples of the marine sponges *Halichondria permolis* (13 μg/g dry wt), *H. japonica* (3.42 μg/g dry wt), *H. okadai* (5.5 μg/g dry wt) and *Haliclona* sp. (0.81 μg/g dry wt), which were collected near Japan. Arsenobetaine **54**

and the two arsenosugars **56** and **57** were identified in all targeted samples, and these three compounds accounted for 74-96% of the water-soluble arsenic in all four sponge species. The most abundant arseno-compound in all four species of sponge was arsenosugar **57**, while arsenosugar **56** was only a minor component. The arsenobetaine **54** and arsenosugar **57** concentrations were considerably higher in the three sponges from Demospongiae (*Halichondria*) compared with the single species from Calcispongiae (*Haliclona*). According to [150], arsenosugar **57** has also been found in some marine sponges from Philippines.

Water-soluble fractions containing **54** were isolated from two species of jellyfish, *Aurelia aurita* and *Carybdea rastonii*. Besides arsenobetaine **54** (the major arsenic compound), the tetramethylarsonium ion **53** and arsenocholine **55** were also detected [151, 152]. Nine species of jellyfish, including the two species treated in the previous paper [151], were classified into arsenocholine-rich and arsenocholine-poor species.

The hydrothermal vent shrimp, *Rimicuris exoculata*, which is known to be a primary consumer of the chemolithoautotrophic bacteria, contained 13 μg/g of arsenic almost exclusively as arsenobetaine **54**. The major parts of the extractable arsenic species in the adductor muscle/mantle tissues and in the gill were present as riboside derivatives **56** and **57**, while **54** was present at 16% and 3.6%, respectively, in these tissues [153]. In spite of the absence of biosynthetically active algae, the pattern of arsenic species found in the shrimp species in the deep sea is similar to that found in their counterparts from the ocean surface.

The commercially important western rock lobster *Panulirus longipes cygnus* also contains arsenobetaine **55** as the main arsenic compound [154]. The arsenosugars **56-59** were present in a freshwater crayfish *Procambarus clarkii* collected in the area affected by a toxic mine-tailing spill at Seville, Spain [155]. Different concentrations of the arsenosugars **56** (0.18 μg/g dry mass), **57** (0.077 μg/g), **58** (0.08 μg/g), and **59** (0.14 μg/g) were determined. No significant differences were seen with respect to the total arsenic contents between the sexes but they were seen between the area affected by the spill and the area not affected.

Arsenobetaine **54** was present in the extracts of the earthworm, *Lumbricus terrestris,* as a minor constituent and traces of dimethylarsinic acid **47,** ribosides **56,** and **57** were also detected [156]. The major arsenic compound in the earthworm extracts was an arsenic-containing

dimethylarsinic acid **47**, methylarsonic acid **50**; carbohydrate **54** (55%), and glycerol arsenosugar **56** were also present as minor constituents [157]. Two species of earthworm, *Lumbricus rubellus* and *Dendrodrilus rubidus*, collected from an arsenic-contaminated mine spoil site and an uncontaminated site, were investigated for arsenic compounds [158]. Arsenobetaine **54** was the only organic arsenic species detected in both species of earthworms, with the remainder of the extractable arsenic being arsenate and arsenite.

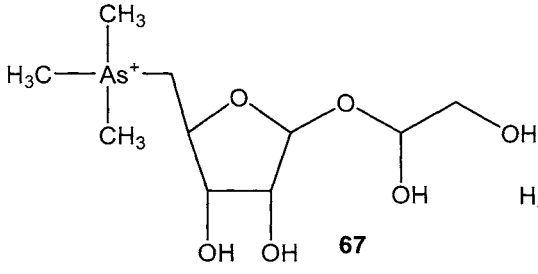
Arsenic compounds were determined in the marine lungworm *Arenicola marina* collected from Odensee Fjord, Denmark [159]. In contrast to most other marine animals, *A. marina* contained most water-soluble arsenic in inorganic forms, and arsenobetaine **54** was present as a minor constituent (6% only). Other arsenic compounds detected in *A. marina* were dimethylarsinate **47** (4%), tetramethylarsonium ion **53** (1.5%), arsenocholine **55** (<1%), and two arsenosugars (**56**, **57**, 1% and 3%, respectively). A new arsenobetaine, i.e. trimethylarsoniopropionate (**62**), previously only reported in fish, was also present in trace amounts (<1%).

Arsenic compounds were examined in three marine gastropods from Thailand, *Thais bitubercularis*, *T. distinguenda*, and *Manila musiva* [160], and the presence of arsenobetaine **54** (93-95% of total extractable arsenic), arsenocholine **55** (3.1-4.6%), tetramethylarsonium ion **53** (0.21-2.2%), a new natural product (**67**), one unknown arsenic compound (each approx. 0.1%), and an unresolved mixture of arsenic compounds (up to 1%) were observed.

The arsenobetaine **54** and riboside **56** as the major water-soluble arsenic compounds were characterized in the sea scallops (*Placlopecten magellanicus*) and Icelandic scallops (*Chlamys islandica*) collected near Newfoundland [161]. This result indicated that consumption of the scallops poses little human risk.

The tetramethylarsonium ion **53,** arsenobetain **54**, and arsenocholine **55** were detected in the soft tissues of both the pearl-free and the pearl-containing pearl oysters *Pincdata fucata* [162]. More than 20 years ago, arsenic-containing sugars **56** and **58** have been isolated from the kidney of the giant clam, *Tridacna maxima* [163]. Water-soluble arsenic species were determined in the kidney of the *Tridacna derasa* [164]. A total of fifteen organoarsenic species were identified, and 13 of these possessed

the dimethylarsinoyl group, i.e. ribofuranosides **56-59**, **68**, **69**, acyclic compounds **61-62**, and dihydroxyfuran **70**. Arsenobetaine and dimethylarsinic acid were also detected. The major species (up to 50% water-soluble arsenic) was 5-dimethylarsinoyl-2,3,4-trihydroxypentanoic acid (**71**). Of the organoarsenic compounds found in *Tridacna derasa*, **56-59** are well known to be elaborated by marine algae [127-129], are presumably products of the zooxanthellae in the clam mantle, and have not been further metabolized by the clam. On the other hand, as has been recently observed, **68** has been previously isolated from the kidney of the related *T. maxima*, has not been found in algae, and might well be the result of metabolism by the clam of algal products [165]. The major arsenic compounds in the seaweed, *Hormosira banksii,* were arsenosugar **56** and an unidentified compound. Arsenobetaine **54** accounted for 95% of arsenic in the carnivorous gastropod *Morula marginalba*, the concentration of arsenocholine was higher and the concentrations of the minor arsenic compounds lower than in the herbivorous gastropod *Austrococchlea constricta* [166].



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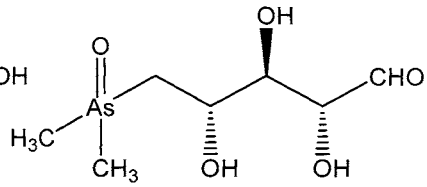

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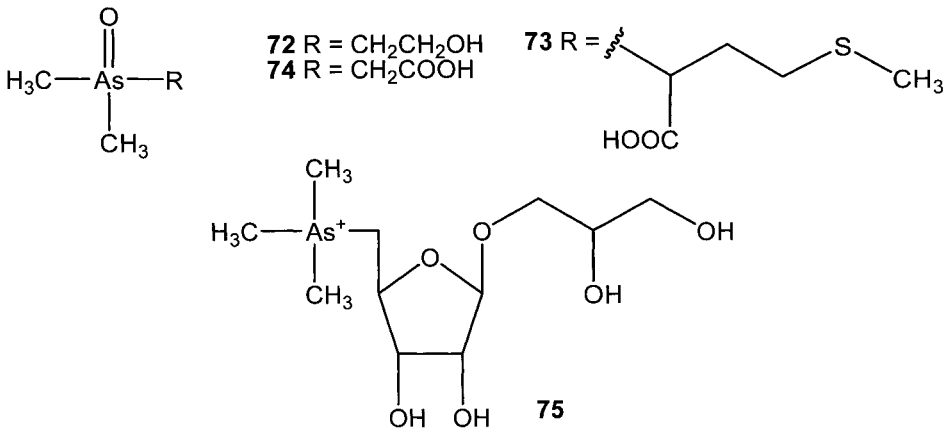
The presence of dimethylarsinate **47**, arsenobetaine **54**, arsenocholine **55**, and trimethylarsoniopropionate **62** was detected in kidney, liver, and lung of the sperm whale *Physeter catodon* cropped in the Andaman Sea [167].

A study [168] evaluated the intake, uptake, and excretion (urine and feces) of arsenic by sheep that live on the Orkney Islands and naturally consume large amounts of arsenosugars through their major food source - seaweed. The sheep eat a broad variety of seaweed species, *Laminaria digitata* and *L. hyperborea* having the highest arsenic content (~74 mg/kg dry mass). In a feeding trial, the average daily intake of arsenic by 12 ewes was ~35 mg (97% of water-extractable arsenic was present as

arsenosugars) gained from feeding on the two brown algae. To test the possible influence of microflora on the metabolism of arsenosugars, six of the sheep were adapted to feeding on grass for 5 months before the start of the trial, and the remaining six sheep were kept on their normal seaweed diet. No significant difference in seaweed/arsenic intake and arsenic excretion was found between the two groups of sheep. The arsenic excreted in the feces represented 13% of the total consumed, and the urinary excretion was estimated at about 86%. The main arsenic metabolite excreted in urine was dimethylarsinic acid (**47**) (60%), minor amounts being found of methylarsonic acid (**50**), tetramethylarsonium ion (**53**), dimethylarsinoylethanol (**72**), and arsenate (V) together with seven unknown arsenic compounds.

In a set of experiments, the alga *Laminaria* was ingested by healthy volunteers [169]. Total arsenic and speciation analysis revealed no marked increase in arsenic blood, serum, and packed cells levels up to 7 h after ingestion. Dimethylarsinic acid (**47**), methylarsonic acid (**50**), and dimethylarsinoylethanol (**72**) have been positively identified in urine sampled after algae intake. Another 5 compounds remained unknown. In simulated gastric fluid incubated with algae, the larger share of the arsenosugars degraded within a short time span into a compound with a mass of 254 Da.

The concentrations of the three arsenicals (**75-77**) were determined in 37 marine organisms comprising algae, crustaceans, bivalves, fish and mammals by high-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICPMS) [170]. All three organoarsenics, which occurred at μg/kg concentrations, were detected in 25, 23 and 17 of the 37 samples analyzed, respectively. The limits of detection were 2-3 μg/kg dry mass. The data illustrate that all three compounds are common minor constituents in practically all marine samples.



Dimethylarsinic acid (**47**), arsenobetaine (**54**), two arsenosugars (**56**, **59**), and several unidentified arsenic species were detected in extracts of the haemolymph of the Dungeness crab, *Cancer magister*, by using HPLC-ICPMS [171]. The results demonstrate that arsenic compounds present in the diet of crabs are not fully metabolized in the gut. They are, at least partly, taken up into the haemolymph. The occurrence of arsenobetaine (**54**) and arsenosugars suggests that the use of repeated haemolymph sampling in crustaceans could facilitate investigations into the kinetics of the biotransformation pathways of arsenic compounds.

Analysis of 10 crude fish oils from various regions of the world for arsenolipids was performed by normal phase HPLC-ICPMS with various mixtures of organic solvents as mobile phases [172]. All ten fish oils appeared to contain the same 4 - 6 major arsenolipids, but in varying amounts depending on the origin of the fish. Further chromatography of some of the oils under both normal phase and reversed-phase conditions indicated the presence of many more minor arsenolipids. Unfortunately, the authors did not provide any data on the structures of the arsenolipids they described.

The pathway of formation of organoarsenics was examined in marine animals, e.g. fish (*Odax cyanomelas*), abalone (*Haliotis rubra*), and sea urchins (*Heliocidaris erythrogramma* and *Centrostephanus rodgersii*) that are directly exposed through their diets to dimethyl arsenoriboses in macroalgae (*Phyllospora comosa* and *Halopteris platycena*) [173]. The identification of dimethyl arsenoriboses (**56**, **57**, **59**) in both dominant

macroalgae species suggest that these arsenic species are to some degree accumulated directly from their diets without degradation or conversion. An unknown arsenic species in *H. rubra* intestinal tissue was identified as 2',3'-dihydroxypropyl 5-deoxy-5-trimethyl arsonioriboside (**75**) (5.0 μg/g dry mass) using tandem mass spectrometry. The presence of **75** in marine animal tissues may be due to microbial-mediated processes that promote the reduction and methylation of dimethyl arsenoriboses released during the breakdown of macroalgae in their diets. The identification of a large amount of trimethyl glycerol arsenoribose in *H. rubra* intestinal tissue suggests that this species is a main constituent in the pathway for arsenic metabolism in this marine animal.

CONCLUSION

Arsenic is a virtually ubiquitous element and arsenic compounds are present in a wide variety of environment components including living organisms, both terrestrial and aquatic. Because of the chemical properties of arsenic, it can be found as part of many types of organic compounds - arsenolipids, arsenosugars and many other types of compounds. Their known toxicity notwithstanding, arsenic compounds, especially organic ones, are integral components of the food chains of many organisms including humans and may play potentially important biological roles in these organisms. Their deeper and more detailed knowledge may contribute to their resurrection as medically or environmentally useful or beneficial agents.

Selenium

The relative proportion of selenium in the environment is very low. In the Earth's crust, selenium is present in a concentration of 0.05 – 0.09 mg/kg. Its level in sea water is usually 0.45 μg/kg, in stream water 0.2 μg/kg. In compounds, selenium is present as Se^{2-}, Se^{2+}, Se^{4+}, and Se^{6+}. In general, it is present in the environment in elemental form or in the form of selenide (Se^{2-}), selenate (SeO_4^{2-}), or selenite (SeO_3^{2-}). In soils, the identity and amounts of the various oxidation-state species depend enormously on the redox-potential conditions, with the lower oxidation states predominating in anaerobic conditions and acidic soils, while the higher oxidation states are favored in alkaline and aerobic conditions. Both selenites and selenates are taken up by plants and converted to protein-bound selenocysteine and selenomethionine, soluble inorganic forms, several free amino acids, and volatile organoselenium compounds. The elemental form of selenium, selenium dioxide, and volatile organoselenium compounds produced by industries and plants are incorporated in the environment. Selenium occurs naturally in water in trace amounts - see above - as a result of geochemical processes, such as weathering of rocks and erosion of soils, and is usually present there as selenate or selenite.

Although most selenium compounds are markedly toxic, considerable attention in the last years has been devoted to the effects of selenium shortage in the daily food intake. A permanently low selenium intake in foods has been found to have an adverse effect on the cardiovascular system and to increase the risk of myocardial infarction and vascular afflictions. The lack of selenium in the nutrition of pregnant women can negatively affect the development of the embryo. In the organism, selenium functions as an antioxidant that destroys free radicals and reduces thereby the risk of cancer. On the other hand, the daily intake of selenium should not exceed a certain limit. An optimum daily dose is currently thought to be 70 μg for men and 55 μg for women. On exceeding the physiological requirements of selenium for an adult man (70 μg/day), the threshold for toxicity may be as low as 700 μg/day.

One biological function for selenium is incorporation into the amino acid selenocysteine that is found in some proteins [174]. Microbial dissimilatory reduction of selenate to selenite and then to elemental

selenium has been described [175]. Selenide undergoes biomethylation to produce methylselenide and dimethylselenide [176].

Practically all small organic selenium compounds in plants, yeast, bacteria or animals are the isologues of corresponding sulfur compounds (Tables 14 and 15).

Table 14. Distribution of selenium compounds in plants, fungi, algae, etc

Compounds	Species
Selenocystathionine (**76**)	*Aspergillus fumigatus*
	Aspergillus terreus
	Astragalus pectinatus
	Astragalus praleongus
	Brassica oleracea capitata
	Lecythis ollaria
	Morinda reticulata
	Neptunia amplexicaulis
	Penicillium chrysogenum
	Stanleya pinnata
Se-Methylselenocysteine (**77**)	*Allium cepa*
	Allium sativum
	Allium tricoccum
	Astragalus bisulcatus
	Astragalus crotalariae
	Astragalus praleongus
	Brassica oleracea botrytis
	Brassica oleracea capitata
	Dunaliella primolecta
	Melilotus indica
	Oonopsis condensata
	Phaseolus lunatus
γ-Glutamyl-*Se*-methylselenocysteine (**78**)	*Allium cepa*
	Allium sativum
	Aspergillus terreus
	Astragalus bisulcatus

	Penicillium chrysogenum
	Phaseolus lunatus
Selenomethionine (**79**)	*Allium tricoccum*
	Aspergillus fumigatus
	Aspergillus terreus
	Brassica juncea
	Brassica oleracea capitata
	Melilotus india
Se-Methylselenocysteine *Se*-oxide (**80**)	*Brassica oleracea capitata*
Selenobiotin (**82**)	*Phycomyces blakesleeanus*
Selenocysteine (**83**)	*Fusarium* sp.
	Vigna radiata
Dimethyl selenide (**84**)	*Penicillium* sp.
γ-Glutamylselenocystathionine (**858**)	*Astragalus pectinatus*
γ-Glutamylselenomethionine (**86**)	*Allium sativum*
Se-Adenosylselenohomocysteine (87)	*Saccharomyces cerevisiae*
Selenocysteic acid (**88**)	*Dunaliella primolecta*
	Fusarium sp.
Se-Methylselenomethionine (**89**)	*Aspergillus fumigatus*
	Dunaliella primolecta
Selenolanthionine (**90**)	*Saccharomyces cerevisiae*
4-Selenouridine (**91**)	*Escherichia coli*
3-Butenyl isoselenocyanate (**92**)	*Stanleya pinnata*
Selenosinigrins (**93**)	*Armoracia lapathifolia*
	Stanleya pinnata
Selenosugars (**94-96**)	*Astragalus racemosus*

With few exceptions, they are isologues of sulfur amino acids or their derivatives. As a rule, their abundance in nature roughly parallels that of selenium in the environment or available feeds, respectively. This is not surprising since, in most cases, neither the initial steps of selenium assimilation nor the enzymes of the trans-sulfuration pathway discriminate between sulfur and selenium [177]. Usually the Michaelis constants (K_m) of these enzymes are slightly lower for the seleno

isologues, and this is balanced by smaller maximum velocities (V_{max}). There are, however, exceptions to this rule. A highly specific selenate reductase has recently been purified from the proteobacterium *Thauera selenatis* [178]. Some cystathionine lyases reportedly prefer selenocystathionine (**76**) over cystathionine [179]. Also the key enzyme leading to *Se*-methylated selenocysteine (**77**) and derivatives thereof, the *S*-adenosyl methionine-dependent selenocysteine methyltransferase, works much less efficiently with, and has a lower affinity for, cysteine. Interestingly, this enzyme was discovered in *Astragalus bisulcatus*, a plant that can be considered as the prototype of the selenium accumulators (see below). The closely related species, chick-pea milkvetch *Astragalus cicer*, which is not a selenium accumulator, lacks selenocysteine methyltransferase under conventional culture conditions. *A. cicer* can, however, be slowly adapted to a selenium-rich substratum. The selenium tolerance thus achieved is accompanied by the appearance of selenocysteine methyltransferase. Evidently, the ability of specialized plants to methylate selenocysteine more efficiently and to accumulate selenium in the better tolerated *Se*-methylated forms is one of the prerequisites for surviving on seleniferous soils. In accordance with this assumption, **77** and derivatives such as γ-glutamyl-*Se*-methylselenocysteine (**78**) were predominantly detected in typical selenium accumulators of the genus *Astragalus* and other selenium-tolerant plants like *Brassica* and *Allium* species.

Table 15. Distribution of selenocompounds in various biological materials

Biological Materials	Selenate	Selenite	Percentage Distribution[1]			Other[2]	References
			77, 78	**79**	**83**		
Rats injected with selenite				6-10	64-70	20-34	[180-182]
Rats injected with **79** (1 day)				63	22	15	[181, 182]
-"- (5-35 days)				14-25	46-57	18-40	[181, 182]
17 Fish	15-36						[183]
20 Vegetables	1-50						[184]
Wheat grain	12-19[3]		1-4	56-83	4-12	4-26	[185, 186]
Wheat straw	97[4]					3	[185]
Corn				61-64	15-16	20-24	[187]
Rice	1-3	5-13		68-81	6-10	19-31	[187]
Soybeans				>80			[188]
Grassland legume			10-13	51-70	19-39[5]		[189]
Phytoplankton[6] (15%)	1.0 (0.15)	83 (12)	12.8 (1.9)	3.2 (0.5)			[190]
Astragalus praelongus (95%)	1.4	9	52	37			[190]
Commercial preparation (95%)	0.6	98.7		0.7			[190]
Se-enriched yeast[6] (8.5%)		27 (2.3)	13 (1.1)	59 (5.0)		5 (0.4)	[190]
Se-enriched garlic	2-5	8	47-87	1-6	1-13	4-36	[191-193]
Se-enriched yeast	4		6-20	23-63	13-21	13-51	[190, 194-196]
Se-enriched onions			42-55	1-4	7-38	21-35	[192, 193]
Se-enriched broccoli florets			63	5	11	21	[192]
Se-enriched broccoli sprouts	20		45	12		3	[197]
Se-enriched wild leeks (bulbs)	12-25		35-50			1-3	[198]

[1] In most cases the percentage distributions were calculated from the areas under the curves of the chromatograms.

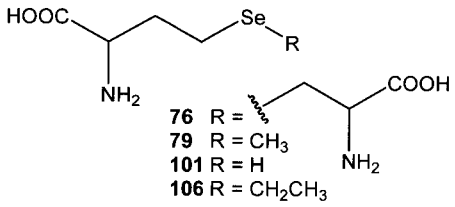
[2] Includes **76, 87, 90, 97, 98, 100**, and unknown.

[3] Combination of selenate, selenite and **88**.

[4] Mixture of selenate and **88**.

[5] **83** and **100**.

[6] Numbers in parentheses represent the percentage of selenium extracted by aqueous solution.



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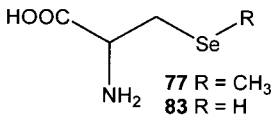
79 R = CH_3
101 R = H
106 R = CH_2CH_3



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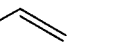
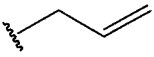
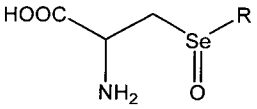


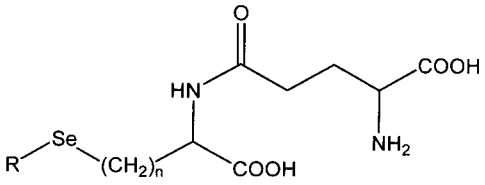
98 R =



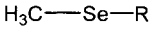
99 R =



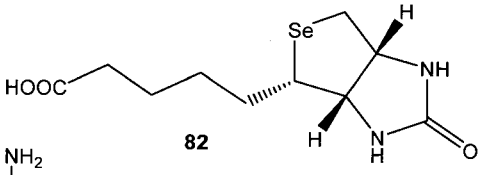
80 R = CH_3
88 R = OH



78 n = 1, R = CH_3
86 n = 2, R = CH_3
97 n = 2, R = CH_2CH_3



81 R = H
84 R = CH_3



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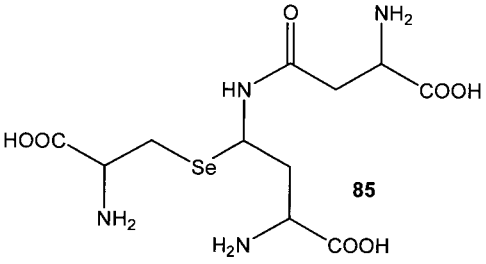

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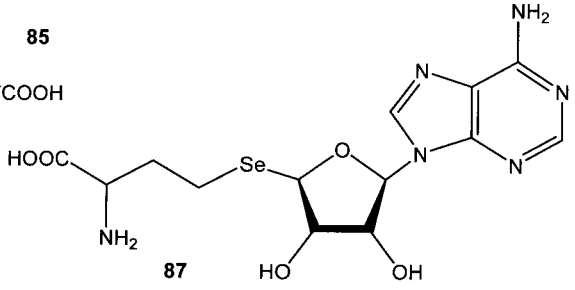

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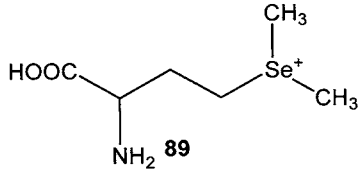

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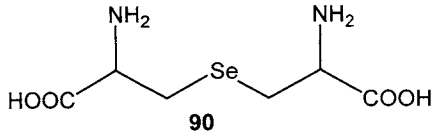

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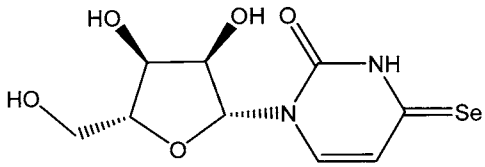

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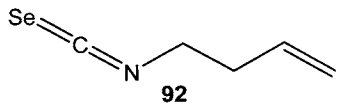

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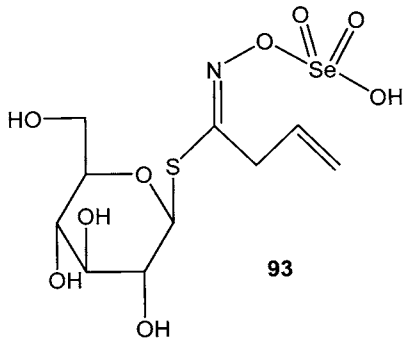

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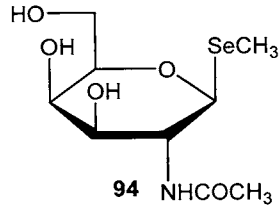

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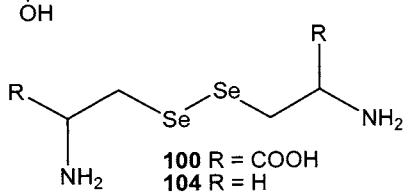
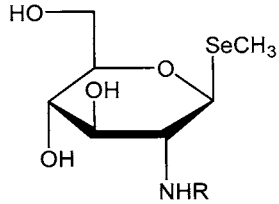


100 R = COOH
104 R = H



95 R = H
96 R = $COCH_3$

Amino acid **78** and its γ-glutamyl derivative are other components of the major pool of seleno compounds in accumulator plants, while selenomethionine (**79**) is the major compound in microorganisms like yeast, which is not specialized in selenium utilization. *Se*-Methylselenocysteine *Se*-oxide (**80**), which is found in marine algae, tends to spontaneously decompose with the formation of pyruvate and ammonia *via* aminoacrylic acid and methaneselenic acid (**81**). The latter reacts with sulfhydryls or selenols to selenodisulfides and diselenides,

respectively. Another group of seleno metabolites characteristic of some accumulator plants are the isoselenocyanates and their precursors, the selenosinigrins. Like their sulfur isologues they appear to be common in *Crucifera*. Their biosynthesis has been studied in horseradish and *Stanleya pinnata*. Selenobiotin (**82**) was detected in *Phycomyces* (*Zygomycetae*). Its biological role remains obscure. The inability of the trans-sulfuration pathway to discriminate between sulfur and selenium gives rise to the remaining selection of low molecular weight selenium compounds so far identified in nature.

Plants absorb Se from soil primarily as selenate and translocate it to the chloroplast, where it follows the sulfur assimilation pathway. Se is reduced (enzymatically and non-enzymatically) to selenide, which reacts with serine to form selenocysteine (**76**). It can be further metabolized to selenomethionine (**79**) and methylated to form products such as *Se*-methyl selenomethionine (**89**). Alternatively, selenocysteine-specific methyl transferase may form *Se*-methyl selenocysteine (**83**), allowing the plant to accumulate extraordinarily large amounts of Se.

Se-enriched plants may be divided into two broad groups [199], i.e. selenium accumulators and/or selenium non-accumulators. Selenium-accumulating plants can be divided into three subgroups: selenite-accumulators (broccoli and cucumber), selenomethionine accumulators (grains such as wheat, and mushrooms) and *Se*-methyl selenomethionine accumulators (garlic and onion) [200].

Most plants, which are considered to be non-accumulators, contain only moderate foliar Se concentrations, that rarely exceed 100 μg/g dry weight when growing on seleniferous soils [201]. Yet, a small number of plants, growing on naturally occurring soils containing only 2-10 ppm Se, can accumulate well over 1 mg/g dry weight Se, and these plants have been classified as Se hyperaccumulators [202]. The largest group of Se-hyperaccumulating plants belongs to the genus *Astragalus* (Fabaceae) [203]. Twenty-five species of *Astragalus* have been characterized as Se hyperaccumulators [204]. Some species can accumulate up to 0.6% of shoot dry weight as Se from soils with 2-10 μg/g dry weight. This is 100-1000 times more Se than found in adjacent non-accumulating plants including other *Astragalus* species [204-206].

There are limited reports about the chemical forms of Se in these plants, but the available information suggests that the Se is often in

methylated forms such as selenocystathione (**76**), *Se*-methyl selenocysteine (**77**), γ-glutamyl-*Se*-methyl selenocysteine (**78**), methyl selenol (**81**), γ-glutamyl selenocystathione (**85**), and selenohomocysteine (**101**). These forms of Se may be safely stored in membrane-bound structures within the plant; Se hyperaccumulators have a relatively small percentage of their total Se sequestered in the protein fractions of the plant. Certain species of *Astragalus* may accumulate in excess of 2 mg Se/g plant tissue, and that Se often is in forms such as **76-78**, **81**, **85**, and **101**. Selenocysteine-specific methyltransferase is the enzyme needed for production of many of these compounds, and insertion of the gene for this enzyme into an *Astragalus* species that does not accumulate Se converts the plant to a Se hyperaccumulator. Recently, this gene has been inserted into *Arabidopsis*, allowing accumulation of **77** and **85**.

Se-enriched garlic contains Se primarily as γ-glutamyl-*Se*-methyl selenocysteine (**78**). Se from garlic does not accumulate in tissues to the same extent as Se from foods enriched in selenomethionine. Species of *Brassica* that are reported to accumulate Se include: broccoli (*Brassica oleracea*), Indian mustard (*Brassica juncea*), Brussels sprouts (*Brassica oleracea* L.), and canola (*Brassica napus*). *Brassica* spp., especially broccoli and canola, also have been used for phytoremediation, a process that uses Se hyperaccumulators to remove high (potentially toxic) concentrations of Se from soil and/or irrigation water. In a single growing season, broccoli may extract up to 20% of soluble Se from Se-laden drainage water.

Although not true plants, mushrooms are generally grouped with vegetables for dietary purposes. Some but not all mushrooms accumulate Se. A survey of 83 species of wild mushrooms reported Se concentrations ranging from 0.01 to 20 ppm. *Agaricus bisporus* can accumulate very high concentrations of Se. Other mushrooms that may accumulate Se include *Boletus edulis* and *B. macrolepiota*. A limited number of studies have reported Se from mushrooms to have low bioavailability, and it has also been reported to be present in low-molecular weight compounds that are not selenocysteine, selenomethionine, or selenite (Table 16).

A few investigators have studied the blue-green alga *Spirulina platensis* as a possible source of Se/iodide pharmaceuticals. Se in *Spirulina* has been reported to be in a high-molecular-weight form. Overall bioavailability of Se from *Spirulina* was reported to be low, but

an ultrafiltrable soluble fraction was highly bioavailable. Se-enriched kelp is commercially available as a dietary supplement.

Se-enriched yeast is the most abundant commercially available source of supplemental Se, with at least a dozen products. Selenomethionine is reported to be the primary chemical form of Se in yeast. Because manufacturers of Se-enriched yeast do not adhere to a single production and/or quality standard, the form of Se probably varies considerably from product to product.

Mutations in ATPS (sulfate adenylyl transferase, EC 2.7.7.4) in the yeast *Schizosaccharomyces pombe* resulted in increased selenate tolerance [207]. The selenate resistant phenotype of these mutants was correlated with low sulfate uptake capacity and low ATPS activity.

Table 16. Examples of plants that are Se accumulators or hyperaccumulators and are used as food

Plant	Concentration (mg/kg)	Compound	Reference
Accumulators			
Wheat	0.1–15	**79**	[208]
Brazil nuts	2.0–35 and more	**79**	[209]
Mushrooms	0.1–20	Unknown[1]	[210, 211]
Brussels sprouts	0.03–7.0	Unknown	[212]
Hyperaccumulators			
Garlic	>1200	**77**	[213]
	<300	**78**	[213]
Broccoli	~1000	**77**	[214]
Ramp	>500	**79**+inorganic Se	[215]

[1] not seleno AA or selenite

Selenium-containing supplements are thought to be more effective when the selenium is ingested in an "organic" form, and many suppliers provide the selenium as selenized yeast, see above, which contains largely selenomethionine (**79**) bound in proteins in addition to many other unknown selenium species. Consumers of such products intent on improved health should be aware, however, of the toxicity of selenium and the possible toxic consequences of overindulgence. Because urine is a major excretory route for selenium, metabolic changes delineating the boundary between essential and toxic concentrations are likely to be

reflected in urinary selenium species. Indeed, all evidence indicates that selenium is methylated in biological systems after reduction of selenite/selenate to hydrogen selenide (excreted by respiration) and trimethylselenonium ion (**102**) (excreted in the urine). The general feeling that methylation processes are a form of detoxification seems to hold for selenium because dimethylselenide (**84**) has much lower acute toxicity (at least 200-fold) than do the inorganic selenium species selenite/selenate and selenoamino acids (Table 17). The rapid excretion of **84**, 71-79% by the rat in 6 h, is also suggestive of a metabolically inert detoxification product. Surprisingly, **102** is at least 20-fold more toxic than dimethylselenide, and thus the further methylation step to give **102** does not appear to benefit the organism in terms of detoxification.

HPLC/ICPMS is a powerful analytical technique and is now the most commonly used method for determining selenium urinary metabolites [215]. Over the last 10 years, most of the reports of selenium species in urine have used HPLC/ICPMS, sometimes together with molecular MS techniques. In this period, a total of 16 selenium species (see below) have been reported in urine, most of them novel human metabolites and some of them completely new compounds.

These 16 compounds identified in urine mostly by HPLC/ICPMS include the compound **102** plus 15 other selenium metabolites (Table 18). For many of these, the assignments have been made on very little evidence and require confirmation before the compounds can be accepted as typical urine metabolites [216, 217]. Some, such as methylselenol, have already been retracted by their discoverers. Selenosugar **95** is now firmly established as a major urinary metabolite when selenium is administered, and it is also a constituent of natural urine. There have also been reports of selenosugar **94** and selenosugar **96** as minor constituents. Selenite appears to be a common minor metabolite in normal urine.

Table 17. Acute toxicity of some selenium species to the rat

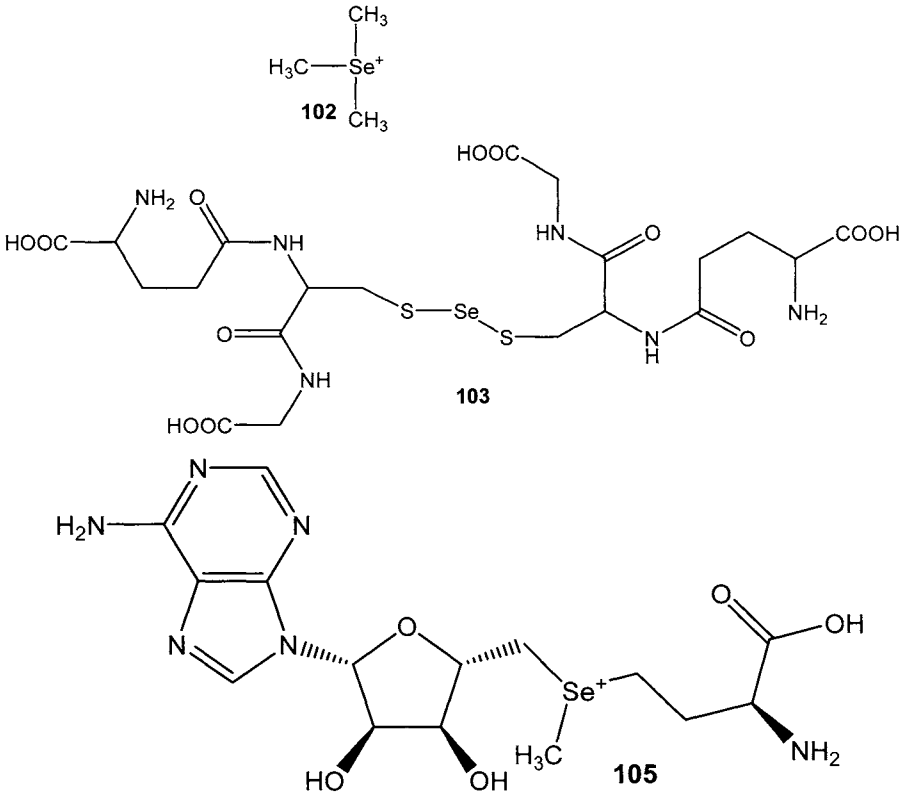
Compound	Se toxicity to rat
Sodium selenite	Oral; 10-day LD_{50}[a] = 3.2 mg/kg Se
	Intraperitoneal; 48-h LD_{75} = 3.5 mg/kg
Sodium selenate	Intraperitoneal; 48-h LD_{75} = 5.5 mg/kg
Selenocystine (**100**)	Intraperitoneal; 48-h LD_{75} = 4.0 mg/kg
Selenomethionine (**79**)	Intraperitoneal; 48-h LD_{75} = 4.25 mg/kg
Dimethylselenide (**84**)	Intraperitoneal; 24-h LD_{50} = 1600 mg/kg
Trimethylselenonium (**102**)	Intraperitoneal; 4-h LD_{50} = 49.4 mg/kg

[a] LD_{50} and LD_{75}, concentrations at which the dose of selenium is lethal in 50% and 75% of animals, respectively.

Table 18. Summary of reported selenium urinary metabolites: confirmed, unconfirmed, and retracted

Compound	No of reports	Accept as a normal urinary metabolite?
Trimethylselenonium ion (**102**)	46	Yes for now, but the early work urgently needs confirming with modern analytical methods
Selenite	16	Yes, but a minor one
Selenate	5	No
Selenodiglutathione (**103**)	1	No
Methylselenol (**81**)	3	No; the original discoverers have retracted this assignment
Selenocystine (**100**)	2	No
Selenocysteine (**83**)	1	No
Selenoethionine (**106**)	1	No
Se-methyl selenomethionine (**89**)	1	No; reported in the abstract but appears to have been retracted in the text of the same report
Selenomethionine (**79**)	4	Not just yet; several studies report it after ingestion of selenized yeast
Selenocystamine (**104**)	1	Not yet; the HPLC/ICPMS data do not match the MS/MS data
Se-adenosylmethionine (**105**)	1	Not yet; the species is reported as being very unstable
Selenosugar (**94**)	2	Yes, a minor constituent
Selenosugar (**96**)	4	Yes; firmly established as a major selenium metabolite after supplementation with selenite or selenized yeast; also data showing it is present in

		urine without selenium supplementation
Selenosugar (**95**)	1	Yes, a minor constituent
Methylselenite (**106**)	1	Thought to be formed in urine from oxidation of selenosugar **96**



Recently, some organoselenium compounds have found pharmacological applications as therapeutic agents in the treatment of several diseases.

The first compounds demonstrating chemopreventive potential were found in plants such as broccoli and green tea. Today, chemoprevention refers to the use of both natural and synthetic compounds to inhibit carcinogenesis.

The basic idea that selenium is a cancer-protecting trace element began in the 1950s, when researchers tried to find substances that could prevent

tumor formation. The epidemiological literature on selenium and cancer has been reviewed [218-220], and it has been suggested that an increased risk for certain human diseases including cancer is related to insufficient intake of selenium; however, some inconsistency still remains [218].

It is clear that mammalian selenoproteins play critical roles in many vital cellular functions and are, therefore, essential for disease prevention [221]. There is an extensive amount of research into the use of organoselenium compounds for cancer prevention [222-224].

Different authors [225-229] found that the CH_3SeH-precursor and *Se*-methylselenocysteine (**77**) are anti-carcinogenic and somewhat more efficacious than the inorganic compound, selenite. Later work has shown that the CH_3SeH precursors methyl-selenocyanate (CH_3SeCN) and **77** each inhibit mammary cell growth, arresting cells in the G1 or early S phase and inducing apoptosis [230-232]. Various synthetic organoselenium compounds have been tested and found to be anti-carcinogenic.

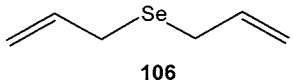
The same group showed that *Se*-allylselenocysteine (**98**), which is expected to yield allylselenol, a fairly hydrophobic metabolite, has more anti-carcinogenic power than **77** and propylselenocysteine (**99**) in rat methylnitrosourea model [233]. The diallyl selenide (**106**) has significant anti-carcinogenic activity using DMB-induced mammary tumor model and is 300 times more active than the diallyl sulfide, a flavor component of garlic [234].

Aromatic selenium compounds in cancer prevention were the first to be synthesized in the 1980s. The applicability of compounds **107-109** in tumor control has been demonstrated in some organs using animal models; they are effective in both the initiation and post initiation phase. These organoselenium compounds are less toxic and chemopreventive than inorganoseleniums and natural organoseleniums, and can be the candidates for excellent chemopreventive agents for human cancers.

γ-Glutamyl-*Se*-methyl selenocysteine **86** is the major selenium compound in natural and selenized garlic, and is an effective carcinogenic agent against mammary gland cancer in rats.

Novel organoselenium compounds like those shown below have been synthesized and used [235]. Oxaselenins, compounds **110-112,** have proliferation-inhibiting effects against human uterine cervical cancer

cells. Compound **112** showed the greatest growth inhibition with IC_{50} of 45.1. Compounds **110** and **111** exhibited only weak inhibitory activities, with IC_{50} of > 100 μM.



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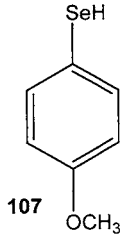

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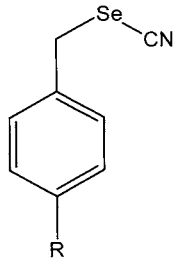
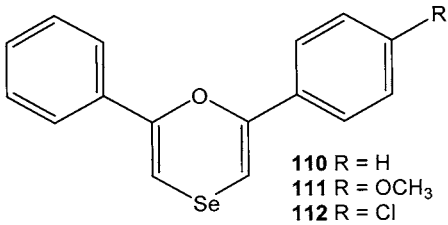
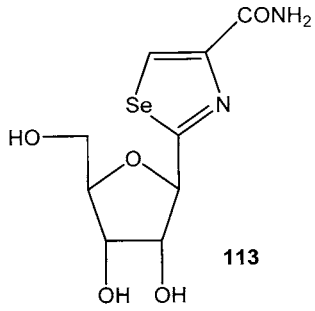


108 R = H
109 R = CH_2SeCN



110 R = H
111 R = OCH_3
112 R = Cl



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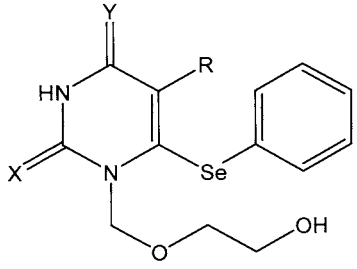
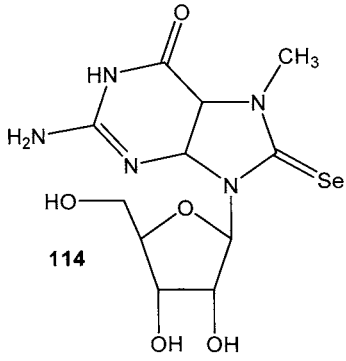




114

	R	X	Y
115	H	O	O

116	CH_3	O	O
117	F	O	O
118	Cl	O	O
119	Br	O	O
120	CH_3	S	O
121	H	O	H_2
122	CH_3	O	H_2
123	F	O	H_2
124	CH_2CH_3	O	H_2

Organoselenium compounds can be used as antiviral and antibacterial agents and this has been reviewed many times [222, 236-238].

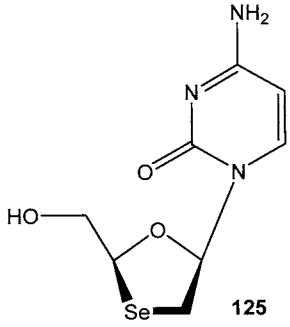
Antivirals

Several interesting organoselenium compounds exhibit antiviral activities. One such is selenazofurin **113**, effective against different types of viruses (herpes simplex, parainfluenza and rhinovirus). The purine analogue, 7-methyl-8-selenoguanosine **114**, has been tested *in vivo* for antiviral activity against Semliki Forest virus infection in a mouse model

Several selenium-substituted acyclouridine derivatives **115-120** were studied and their antiviral activity was evaluated in human peripheral blood mononuclear cells infected with HIV-1. Compounds **115** and **116** are the least and more effective compounds against HIV virus, respectively. Chemical modifications of the acyclic side chain produced compounds **121-124**. Compound **124** is the most potent antiviral against HIV-1. The pharmacokinetics and toxicity studies on compound **124** clearly show that this compound can act as an effective antiviral agent at low concentrations without exhibiting toxicity.

Some 3'-*Se*-substituted dideoxynucleosides in racemic forms, as well as the α- and β-anomers of oxaselenolane nucleosides have been synthesized and exhibited potent anti-HIV and anti-HBV activities. The synthesized racemic nucleosides (**125, 126**) were evaluated for antiviral activity against HIV virus type-1 and hepatitis B virus in peripheral blood

mononuclear cells and 2.2.15 cells. The racemic (±)-β-cytosine (**125, 126**) compounds exhibit the most potent anti-HIV activity (EC_{50} 2.69 and 5.55 μM respectively) and anti-HBV activity (EC_{50} 1.2 μM for both racemic compounds). The anti-HIV activity of resolved α- and β-enantiomers of (**125, 126**) has been evaluated and the (-) enantiomers are more potent than their (+)-counterparts.



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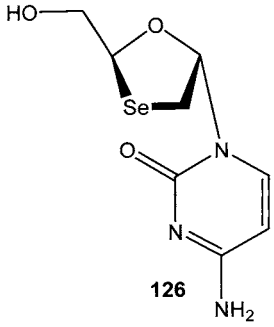
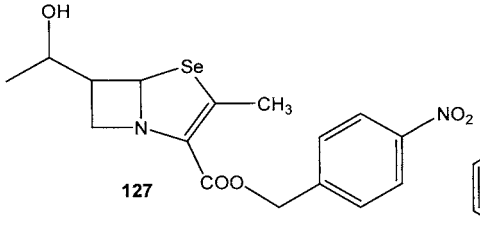


126

Antibacterials and Antifungals

Because of the comparability of organoselenium compounds and organosulfur compounds, many organoselenium compounds were synthesized and their antimicrobial activity was studied [238]. Early studies were centered at the skeletal modification of the naturally occurring β-lactam antibiotics. The optically active **127** has been synthesized and has antibacterial activity [239].



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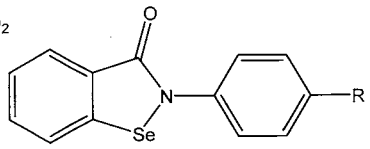


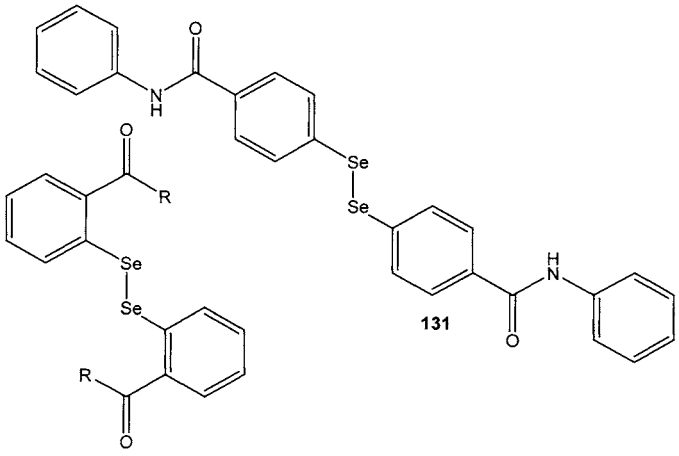
128 R = H
129 R = Cl

Ebselen (**128**) is a heterocyclic compound that possesses antiinflammatory, anti-atherosclerotic, cytoprotective, and antimicrobial activity against *Staphylococcus aureus*. Compound **128** and the *p*-chloro analogue **129** exhibited strong inhibitory activity against the growth of

Saccharomyces cerevisiae Σ127-8b strain. The latter compound also inhibited the growth of *Candida albicans* 258 strains.

On the other hand, diaryl diselenides, compounds (**130-133**), had no inhibitory effect on fungal growth. However, the benzisoselenazolones show antibacterial activities against Gram-negative *E. coli* K-12 Row and Gram-positive *S. aureus* 209P bacterial strains [240].

Since morpholine compounds are typically bactericidal and fungicidal, different selenomorpholines **134-136** were synthesized and their antimicrobial activity was tested against *Staphylococcus aureus*.



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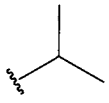

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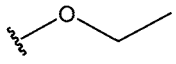

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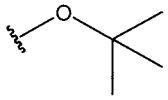

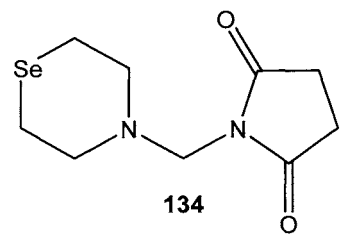

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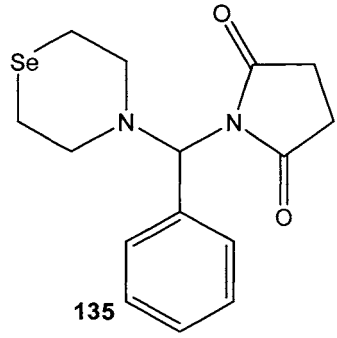

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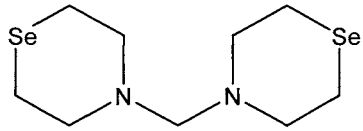
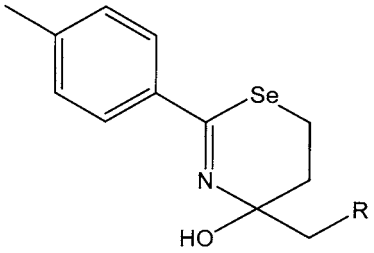


136

Organoselenium Compounds in Regulation of Apoptosis

Selenium compounds such as **77**, ebselen (**128**) or selenodiglutathione (**103**), are chemopreventive in animal models, inhibit cell growth and induce apoptosis *in vitro*. Compound **77** has been the most extensively investigated. It inhibits cell proliferation and induces apoptosis in several tumor cells. Ebselen (**128**) provides an efficient protection against mustard-induced cell death in normal and tumoral lymphocytes and might prove useful as an antidote against alkylating agents. Compound **103** is associated with induction of Fas ligand, a well-known mediator of apoptosis, and activation of so-called stress kinase signaling pathways, particularly in Jun NH_2-terminal kinase.

Compounds **137** and **138** inhibited HT-1080 proliferation through the induction of DNA fragmentation and were investigated for their inhibitory effect on the growth of eight human tumor cell lines, including stomach, lung, prostate and colon cancer cell lines *in vitro*. Both compounds exhibited the strongest cytotoxicity against a gastric adenocarcinoma and their IC_{50} were 2.38 μM and 2.78 μM respectively. Furthermore, both compounds produce induction of apoptosis in human gastric adenocarcinoma cells.



137 R = CH_3
138 R = H

CONCLUSION

Selenium, an essential micronutrient for plants, animals and microorganisms, has a direct impact on human health and environmental safety. Thus, Se supplementation of populations with low or deficient Se intakes may improve health, and supplementation of Se to populations with adequate intakes may reduce the risk of cancer. Selenium levels in the body are relevant for protection against chronic degenerative, neurological or neoplastic diseases.

The anticarcinogenic and chemopreventive properties of selenium compounds have been demonstrated in mammary, colon and prostate cancer prevention. Compounds **77**, **79**, and **83**, which participate in redox reactions, for instance in antioxidant enzymes, are important components of selenium-enriched yeast used as food supplement and therapeutic agent. Organoselenium compounds are promising also as agents reducing viral expression, combating antibacterial and antifungal infections and preventing heart disease and other cardiovascular and muscle disorders. They also exhibit considerable antioxidant activity.

Ongoing investigations into selenium metabolism include state-of-the-art methods such as HPLC/ICPMS in combination with MS/MS. Data on the profile of selenium metabolites will elucidate the element's essential and toxic roles and relate individual Se species with observed health effects.

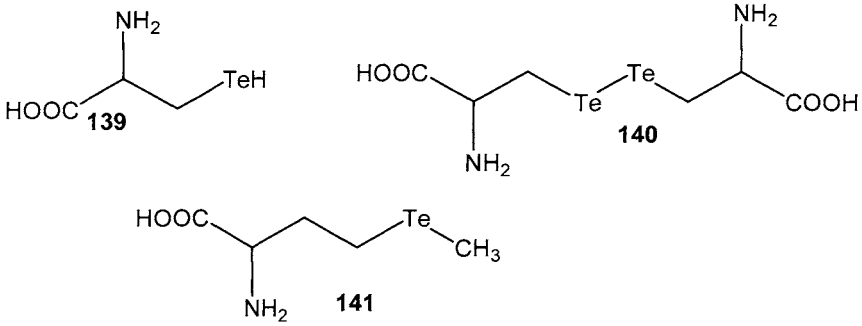
Tellurium

Tellurium is occasionally found as the free element, but is more often found as gold telluride (calaverite), and combined with other metals in the USA, Canada, Peru, and Japan. The principal source of tellurium is the anode sludges produced during the electrolytic refining of blister copper. It is also a component of dusts from blast furnace refining of lead. Tellurium is a relatively rare element, in the same chemical family as oxygen, sulfur, selenium, and polonium (the chalcogens). The relative proportion of tellurium in the Earth's crust is very low, and the content of this element is estimated to be in the range of 1 – 5 μg/kg. Its concentration in seawater is so low that it cannot be reliably determined by current analytical techniques.

Tellurium has no significant biological role. Tellurium and tellurium compounds should be considered to be toxic and need to be handled with care. Organic tellurides have been employed as initiators for living radical polymerization, and electron-rich mono- and di-tellurides possess antioxidant activity. Humans exposed to as little as 0.01 mg/m^3 or less in air develop "tellurium breath", which has a garlic-like odor. This is due to formation of ethyl telluride within the body.

Tellurium, unlike the elements mentioned above, is not thought to be required by biological systems. However, tellurium-resistant fungi grown in the absence of sulfur and in the presence of tellurite ions incorporate tellurium into amino acids and proteins [241]. Tellurium is considered to be toxic to most organisms. Genes correlated with tellurium resistance have been identified, but the mechanisms of tellurium resistance are not completely understood [242]. Reduction of tellurite to insoluble elemental tellurium has been described in Gram-positive and Gram-negative bacteria and archaea, and is one of the mechanisms of microbial tellurium resistance [243]. *Aspergillus fumigatus*, *A. terreus*, and *Penicillium chrysogenum*, the tellurium tolerant fungi, are able to grow on sulfur free medium amended with 0.2% (w/v) tellurite. Tellurium was incorporated into several types of low and high molecular weight proteins. The newly detected telluro-proteins contained an extraordinary high level of tellurium, as well as telluro-cysteine (**139**), telluro-cystine (**140**), telluro-methionine (**141**), and serine [241]. Nitrate reductases [244] and terminal oxidases in the respiratory electron transport chain [245] have been implicated in tellurium reduction. Biomethylation of tellurite, tellurate,

and elemental tellurium to volatile dimethyltellurium has been observed in several species of bacteria and fungi, and may represent another mechanism of tellurium resistance [243]. Genes associated with tellurite resistance (TeR) are found in many pathogenic bacteria. Tellurite can be detoxified through interactions with cellular thiols, such as glutathione, or a methyltransferase-catalyzed reaction, although neither process appears to be involved in plasmid-mediated TeR [242].



Escherichia coli JM109 cells, expressing the genes localized in a 3.8-kb chromosomal DNA fragment from *Geobacillus stearothermophilus* V, produced volatile organotellurium compounds, which were released into the headspace in GC analysis [246]. In addition to dimethyl telluride and dimethyl ditelluride, two new organometalloidal compounds, methanetellurol (CH_3TeH) and dimethyl tellurenyl sulfide (CH_3STeCH_3), were detected.

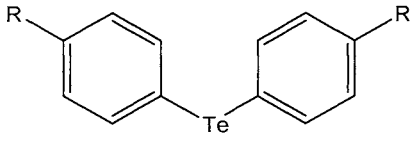
Inorganic and organic tellurium compounds are highly toxic to the central nervous system of mammals. Organotellurium compounds have been reported as potent inhibitors of squalene monooxygenase, causing a dramatic reduction in the rate of cholesterol biosynthesis and leading to degradation of the myelin sheath. The IC_{50} value for these methyltellurium compounds was 100 nM and was 100-fold lower than that of sodium tellurite, indicating a role for hydrophobicity in the enzyme-inhibitor interaction. This study [235] also provides evidence that organotellurium compounds react with vicinal cysteine sulfhydryl groups on squalene monooxygenase. Dimethyltellurium dichloride was the most potent of these compounds, and its neuropathy resembled that caused by elemental tellurium. The data mentioned above are consistent with the hypothesis that demyelination induced by organotellurium compounds is a result of squalene monooxygenase inhibition, and suggest that a

dimethyltelluronium dichloride compound may be the neurotoxic species presented to Schwann cells *in vivo*.

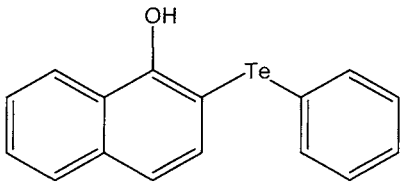
The interaction of organoselenium and organotellurium compounds with δ-aminolevulinate dehydratase (*δ*-ALA-D) will be discussed together here, in view of their similar effects on enzyme activity. Organotellurium compounds can interact directly with low molecular thiols, oxidizing them to disulfides. Reduced cysteinyl residues from proteins can also react with these compounds, which may cause, in the case of enzymes, the loss of their catalytic activity. For instance, δ-ALA-D is a sulfhydryl-containing enzyme that is extremely sensitive to oxidizing agents. This enzyme catalyzes the asymmetrical condensation of two molecules of 5-aminolevulinic acid to form porphibilinogen, an intermediate in tetrapyrol biosynthesis. Hence, this enzyme plays a fundamental role in most living aerobic and photosynthesizing organisms by participating in heme and chlorophyll biosynthesis. The mechanism of porphibilinogen synthesis is similar in animals and plants; however, the enzyme obtained from these sources exhibits subtle structural diversity.

Although the tellurium atom is generally regarded as a toxic metalloid, its role in biological systems has been reviewed [247]. Concerning organotellurium compounds little is known about their biological and pharmacological effects. As pointed out above, glutathione plays a central role in the endogenous antioxidant defense as a reducing agent and nucleophile as well as a substrate for glutathione peroxidases and transferases. Organotellurium compounds are readily oxidized from the divalent to the tetravalent state. Consequently, this property makes tellurides attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals. These novel and potent antioxidants can play an important role in targeting therapeutic interventions in several diseases [235]. A number of diorganoyl tellurides were able to catalyze the reaction of hydrogen peroxide with thiols. The most active compound, **142** (IC_{50} = 30 nM), is among the most potent antioxidants in the microsomal system ever reported. This compound also caused a concentration-dependent delay of the onset of the linear phase of microsomal peroxidation stimulated by iron/ADP/ascorbate. Contrary to the conventional antioxidants examined, diaryl tellurides were found to inhibit peroxidation for a long time, seemingly with an autocatalytic mechanism. 2-Substituted-1-naphthols are the most potent 5-lipoxygenase inhibitors known. Based on this information, a series of 2-substituted-1-

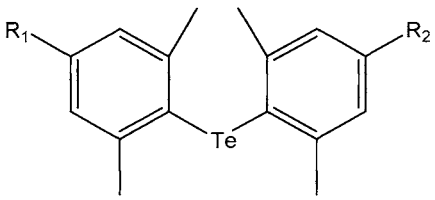
naphthol analogues with the benzylic group replaced by sulfur, selenium, and tellurium were prepared. Data obtained with 2-phenyl tellurenyl-1-naphthol, **143**, indicated that this compound inhibits stimulated leukotriene B4 biosynthesis in human neutrophils. Besides, compounds **144** and **145** prevented leukocyte-mediated cell damage in Caco-2 cells and protected rat kidney tissue against oxidative damage caused by anoxia and reoxygenation.



142 R = $N(CH_3)_2$
144 R = OH
151 R = NH_2
153 R = OCH_3
154 R = CH_3
162 R = NO_2



143

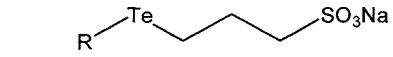


145 $R_1 = R_2 = OH$
152 $R_1 = OH$, $R_2 = OCH_2COOH$

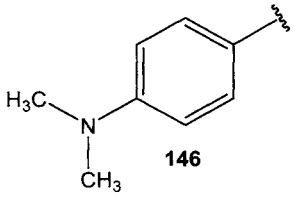
In addition, a cyclodextrinyl ditelluride compound has been pointed out as an excellent glutathione peroxidase-mimic, as demonstrated by its high catalytic efficiency. Moreover, water-soluble organotellurium compounds **146-150** were screened as protectors against peroxynitrite. As a result, the compound **146** has been demonstrated to efficiently protect against peroxynitrite-induced oxidation in solution. Thus, it has been suggested that this molecule acts as a catalyst in scavenging ONOO- in the presence of glutathione. Besides, peroxynitrites can cause tyrosine nitration of proteins and inactivate a variety of enzymes. Among the tested *para*-substituted diaryl tellurides, e.g. **151** offered the most efficient protection against peroxynitrite-mediated oxidation. This compound was 11 times more effective than the methylselenocysteine and approximately 3 times more active than **144** and **147** in this capacity. Compounds **152-154** showed activity similar to that of selenomethionine. Conversely, the heterocyclic organotellurium compounds **155** and **156**

showed no significant activity. Diaryl tellurides act as scavengers of peroxynitrite by an oxygen transfer mechanism similar to that observed with hydrogen peroxide and hydroperoxides.

Organotellurium compounds have been investigated as chemopreventive agents. In 1987, the compound **157**, coded AS101, was demonstrated for the first time to present immunomodulating properties and, when administered to mice, to mediate antitumor effects. This compound has also been described to stimulate human lymphoid cells to proliferate and produce lymphokines. In fact, AS101 stimulates the production of IL-1, IL-2, colony-stimulating factor, tumor necrosis factor, and other cytokines *in vitro*.



R =



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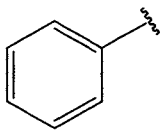

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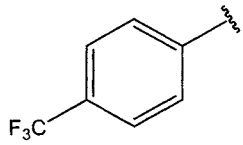

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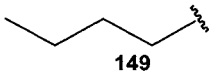

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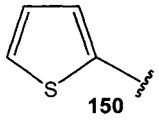

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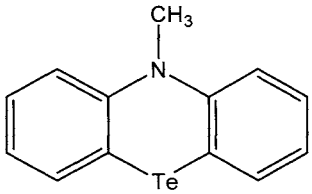

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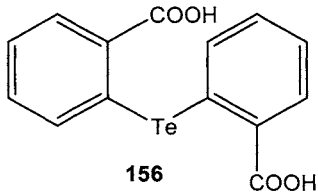
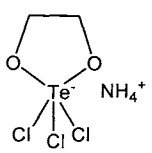


156

Telluranthrene **158** and compound **159** demonstrated bacterial cytotoxicity and a capacity to induce apoptotic cell death in eukaryotic HL-60 cells. The EC_{50} values for telluranthrene **158** and diphenyl ditelluride **160** were 196 and 33 μM, respectively. The series of organotellurium compounds **142**, **144**, **151**, and **161** function as selective inhibitors of thioredoxin reductase and have potential antitumor effects. They were good noncompetitive inhibitors of thioredoxin reductase,

having IC_{50} values less than 10 μM. Compounds **144** and **150** were tested for antitumor activity against MCF-7 breast cancer; however, the antitumor activity was observed only at doses that produced lethality.



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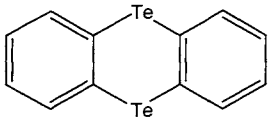

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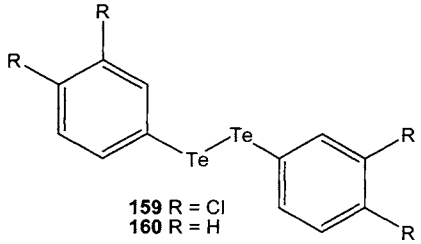
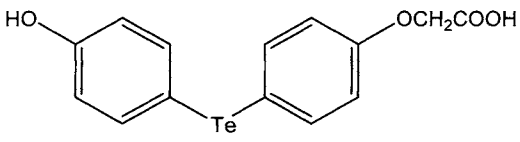


159 R = Cl
160 R = H

The diaryl telluride, alkyl aryl telluride, and dialkyl telluride carrying sulfopropyl groups were prepared and were found to be the most efficient tellurium based inhibitors of thioredoxin reductase ever tested. The results clearly showed that of the four cyclic aryl alkyl chalcogenides **162-165**, all primitive analogues of vitamin E, only the tellurium compound, **165**, showed interesting inhibition characteristics.



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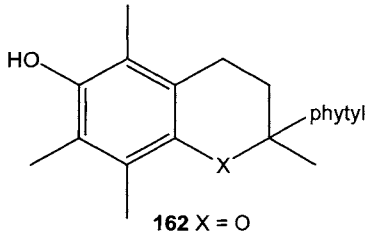
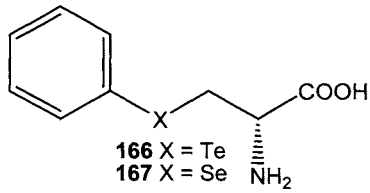


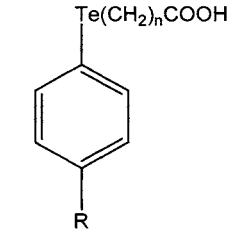
162 X = O
163 X = S
164 X = Se
165 X = Te

Further results demonstrated that phenyltelluriocysteine, **166**, is bioactivated to its corresponding tellurol analogously to what has been shown previously for phenylselenocysteine, **167**. Microsomal activation studies show that the three conjugates seem to show the trend Te > Se > S; phenyltellurocysteine was the strongest inhibitor of several human cytochrome P450 isoenzymes followed by phenylselenocysteine, while phenylthiocysteine was the weakest inhibitor. The results suggest that phenyltellurocysteine might be a significant novel class of prodrugs to generate biologically active tellurols.

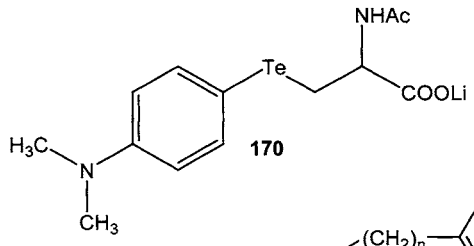


166 X = Te
167 X = Se

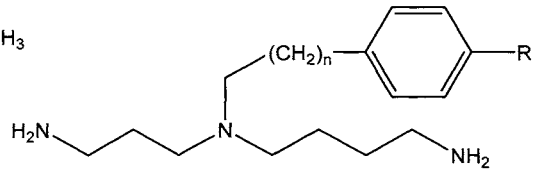
The thioredoxins are small ubiquitous redox proteins with the conserved redox catalytic sequence Trp-Cys-Gly-Pro-Cys-Lys, where the Cys residues undergo reversible NADPH dependent reduction by selenocysteine containing flavoprotein thioredoxin reductases [248]. Thioredoxin expression is increased in several human primary cancers including lung, colon, cervix, liver, pancreatic, colorectal and squamous cell cancer. The thioredoxin/thioredoxin reductase pathway therefore provides an attractive target for cancer drug development. Organotellurium steroid, lipid, amino acid, nucleic base, and polyamine inhibitors were synthesized on the basis that they might be selectively or differentially incorporated into tumor cells. Some of the newly prepared classes of tellurium-based inhibitors (lipid-like compounds **168** and **169**, amino acid derivative **170**, nucleic base derivative **171**, and polyamine derivatives **172** and **173**) inhibited TrxR/Trx and cancer cell growth in culture with IC_{50} values in the low micromolar range.



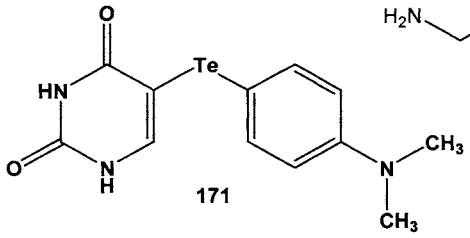
168 n = 4, R = H
169 n = 11, R = $N(CH_3)_2$



170

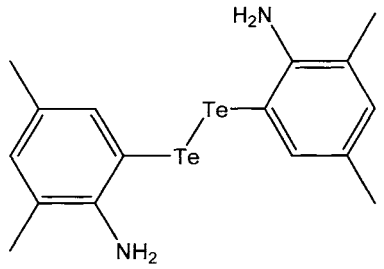


172 R = H, n = 3
173 R = $N(CH_3)_2$, n = 4

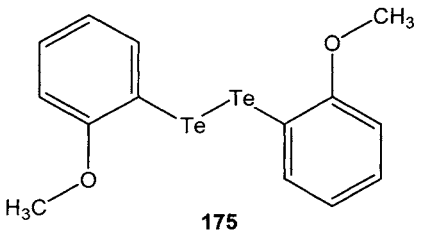


171

Two substituted aryl organotellurium compounds and a tellurium-free analogue of one of these were evaluated for *in vitro* cytotoxicity using human promyelocytic (HL-60) cells as an experimental system [249]. Both tellurium-containing ditellurides (**174, 175**) were cytotoxic at concentrations as low as 5 μM. Data indicate that both compounds induce apoptosis in both a time and dose dependent manner; however, 2,2'-dimethoxybiphenyl, structurally comparable to the first of these but without the tellurium bridge, did not produce apoptosis under the concentrations and time course studied. Therefore, the telluride moiety was apparently an important factor in the apoptotic effect.



174

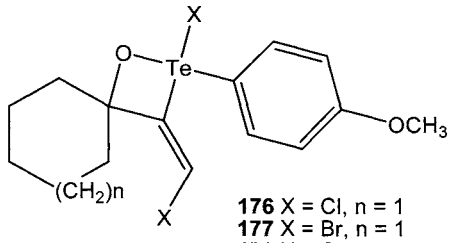


175

The effect of diphenyl ditelluride (**160**) on rat thymocytes was examined under *in vitro* conditions using a flow cytometer with fluorescent probes [250]. Incubation of thymocytes with **160** at 300 nM or more for 24 h significantly increased the populations of shrunken cells and of cells with hypodiploidal DNA. Benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethylketone, a paninhibitor of caspases, greatly suppressed the increase in the hypodiploidal cell population, suggesting the involvement of caspase activation in **160** toxicity. Hence, it is possible that **160** would increase the population of thymocytes undergoing apoptosis if its blood concentration in mammals reached at least 300 nM or more.

New organotellurium compounds, e.g. **176-178**, with specific cysteine protease inhibitory activity were synthesized [251]. All compounds tested exhibited high specific second-order constant for cathepsin B inactivation. Compound **176** was the best inhibitor of the series, showing a second-order constant of 36,000 $M^{-1}s^{-1}$. This value is about 100-fold higher than the second-order rate for cysteine protease inactivation shown by the historic Te compound **157**. The inhibition was irreversible and time and

concentration dependent; no saturation kinetics were observed, suggesting a direct bimolecular reaction. The results described in this paper show that the new organotellurium compounds are powerful inhibitors of cathepsin B, constituting promising potential anti-metastatic agents.



176 X = Cl, n = 1
177 X = Br, n = 1
178 X = Cl, n = 2

A study [252] of the *in vitro* effect of diphenyl ditelluride (**160**), and ebselen (**128**) on Na^+, K^+-ATPase activity of rat brain demonstrated that all these compounds significantly inhibited (in the μM range) Na^+, K^+-ATPase activity. Diphenyl ditelluride, at low concentrations, provoked an increase in Na^+, K^+-ATPase activity. Dithiothreitol, at 3 mM, protected the enzyme against the inhibition caused by diphenyl ditelluride, diphenyl diselenide and ebselen. Post-incubation of diphenyl diselenide-treated homogenate with dithiothreitol completely recovered enzyme activity. Dithiothreitol was also able to abolish the enzyme inhibition induced by 20 μM diphenyl ditelluride and was partially able to alleviate the inhibition induced by high concentrations of this organotellurium compound; however, it did not alleviate ebselen-induced Na^+, K^+-ATPase inhibition. Cerebral Na^+, K^+-ATPase is a potential molecular target for the toxic effect of organochalcogens and the inhibition may occur through a change in the crucial thiol groups of this enzyme.

It was demonstrated that solutions of $(t\text{-}Bu_2SnO)_3$ and $(p\text{-}MeOC_6H_4)_2TeO$ (Sn/Te ratio=1:1) rapidly absorb CO_2 to form an air-stable molecular tellurastannoxane $[\{(p\text{-}MeOC_6H_4)_2TeOSn(t\text{-}Bu_2)CO_3\}_2]$ showing significant intramolecular Te···O interactions in both solution and the solid state [253]. The desorption of CO_2 occurs at rather low temperatures, which suggests applications of the complex for instance as a phase-transfer catalyst or as a precursor for the preparation of organic carbonates.

CONCLUSION

Tellurium is so rare in nature that it has no real significance for living organisms and is not thought to be required by biological systems. The highly toxic organotellurium compounds damage cells, e.g. by oxidizing sulfhydryl groups and depleting endogenous reduced glutathione in a variety of tissues. They cause damage to the central nervous system and block a number of other vital functions of the organism.

Astatine

The last element of the group of metalloids is astatine. It has been estimated that the whole Earth's crust contains less than 44 mg astatine and this element with the atomic number 85 can thus be considered one of the rarest naturally occurring elements on Earth. All isotopes of this radioactive element have short half-lives and are products of several radioactive decay series. ^{219}At ($t_{1/2}$ = 54 s) occurs in one rare side branch of the ^{235}U decay series while ^{215}At, one of the products of a side branch of the ^{215}Po decay series, undergoes a very fast β decay ($t_{1/2}$ = 1x10^{-4} s). ^{218}At ($t_{1/2}$ 2 s) is one of the products of the ^{238}U decay series, which was also recorded to contain trace amounts of ^{217}At ($t_{1/2}$ = 0.0323 s) and ^{216}At ($t_{1/2}$ = 3x10^{-4} s). Isotopes with the longest half-lives are ^{210}At and ^{211}At ($t_{1/2}$ approximately 1/3 days).

It has not as yet been possible to isolate macroscopic amounts of the element. Astatine chemistry can only be studied by trace methods in highly dilute solutions, and this study is understandably fraught with a high risk of experimental errors and faulty conclusions. Despite all these obstacles, increasingly more information is gradually being gathered about the nature of the element. The most suitable for these studies is the isotope ^{211}At ($t_{1/2}$ 7.21 hrs). Recently, the first data have appeared about the organic compounds of At; still, the obstacles in the study of inorganic At compounds including separation of the products, identification of their trace amounts and the danger of radioactive radiation make the acquisition of any information on organic At compounds very difficult. It has so far been possible to synthesize some compounds of the RAt, $RAtCl_2$, R_2AtCl a $RAtO_2$ types (R is phenyl or p-tolyl); all these syntheses were performed with iodine containing trace amounts of At.

Compared with radioactive iodine, astatine is a better tool for destroying anomalous thyroid gland tissues because it emits radiation with shorter range in tissues (70 μm) and higher energy (5.9 MeV), and

consequently has a better local affect than iodine, which emits a lower-energy β radiation with a range of up to 2 mm. However, the very low availability and high price of astatine-containing materials severely limit any practical applications [254, 255].

CONCLUSION

Because of the very low half-life of its isotopes, the radioactive astatine has no significance for living systems.

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