8.07
Marine Natural Products and Marine Chemical Ecology

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8.07.1 INTRODUCTION

The oceans cover nearly 70% of the whole surface area of the earth and more than 30 phylums and 500 000 species of marine organisms live in them. In the oceans the circumstances are quite different from those on the land. The undersea environment is a closed system with high salinity, high pressure, and relatively constant temperature. Animals, plants, and microorganisms living in the ocean therefore are expected to produce quite different secondary metabolites from those produced by terrestrial organisms. Since the 1970s, a great number of new marine natural products have been isolated from various marine organisms. Most of these marine natural products possess a variety of unique chemical structures that have never been encountered among natural products of terrestrial origins, whereas these marine natural products frequently exhibit interesting biological activity which may be of great importance in many fields of biological sciences. The aims of research projects concerned with marine natural products may be (i) to find novel compounds that are useful as leads for drug development, (ii) to provide good tools for basic studies of life science, and (iii) to study the roles and biological functions of secondary metabolites in the life of marine organisms. The third subject, which is called ''marine chemical ecology,'' has, since the mid 1980s, become a field of study.

This chapter describes studies on marine natural products, particularly those of interest from the viewpoint of marine chemical ecology, and consists mainly of two parts. The first part deals with marine natural products related to marine chemical ecology. Classification of the phenomena associated with marine chemical ecology is arbitrary, and here we describe them in seven sections (Sections 8.07.2–8). In the latter part of this chapter (Section 8.07.9), topics in marine natural products chemistry are described from various viewpoints irrespective of the relationships to ecological subjects. As the dividing line between the sections may sometimes be obscure, the selection of compounds and topics is arbitrary and not necessarily comprehensive.

A series of excellent reviews on marine natural products chemistry, published by Faulkner,[1–12] cover all the literature describing marine natural products, organized phylogenetically. A special issue of *Chemical Reviews* appeared in 1993,[13] providing broad aspects of contributions on marine natural products chemistry. The present authors wrote a review in 1992[14] covering the nitrogen-containing secondary metabolites isolated from marine organisms, mainly reported in the late 1980s.

Several good books or reviews dealing with general or specialized subjects in marine natural products research have been published,[15–21] in particular, those describing the role of marine natural products in chemical ecology.[22–25]

8.07.2 FEEDING ATTRACTANTS AND STIMULANTS

Feeding is one of the most fundamental behaviors of all living organisms. It was suggested by many biological studies that chemical substances or chemical changes in the environment may initiate the feeding behavior of marine organisms and promote the ingestion of foods, and those chemicals may be designated as "attractants" and "stimulants," respectively. This sections deals with studies of feeding attractants and stimulants of marine animals. Excellent reviews have been published on this subject by Sakata.[26–28] This section contains descriptions of feeding attractants of stimulants of particularly fish and mollusks.[26–29]

8.07.2.1 Fish

It is well known that chemical substances participate in feeding behaviors of fish and this phenomenon is called "chemoreception." Fish possess a sense of smell and taste and their sensitivity is much higher than that of man (Table 1).[28] The gustatory organs (taste buds) of fish are distributed not only in the mouth but also on the palp, lip, and skin. The senses of taste and smell cannot be clearly categorized for fish because signal communications by chemical substances are mediated with water. It has been suggested that the gustatory organs of fish may play a role in receiving signals of chemical substances over long distances.

Table 1 Comparison of taste sensitivity of man and fish.[28,30]

	Lowest concentration of taste (mol l^{-1})		
Substance	*Man*	*Fish*[a]	*Ratio*
Raffinose		1/245 760	
Sucrose	1/91	1/81 921	900:1
Lactose	1/16	1/2560	160:1
Glucose	1/13	1/20 480	1575:1
Galactose	1/9	1/5120	569:1
Fructose	1/24	1/61 440	2560:1
Arabinose	1/13	1/15 360	1182:1
Saccharin	1/9091	1/1 536 000	169:1
Quinine hydrochloride	1/1 030 928	1/24 576 000	24:1
Sodium chloride	1/100	1/20 480	205:1
Acetic acid	1/1250	1/204 800	164:1

[a] Cypriniformes family.

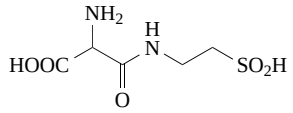
Hashimoto *et al.*[31] studied feeding attractants and stimulants of the eel *Anguilla japonica* in extracts of mussels *Tapes japonicus* by watching the behavior of eels using samples mixed with gelatin. Seven amino acids were isolated in the active fraction, and among them the effective concentrations of glycine (Gly), L-alanine (Ala), and L-arginine (Arg) were revealed to be 2×10^{-5} mM, 1×10^{-7} mM, and 5×10^{-8} mM, respectively. Mixtures of these amino acids were more effective than each pure amino acid, suggesting that the synergetic effect of amino acids may be important. Quaternary ammonium salts, nucleic acid derivatives, and organic acids proved to be inactive.

Amino acids were generally identified as feeding attractants and stimulants of fish of other kinds (Table 2). They were mostly normal amino acids such as Gly, Ala, and Arg, while unusual amino acids were also reported such as arcamine (**1**) and strombine (**2**).[32] In addition to amino acids, inosine, inosine-5′-monophosphate (5′-IMP (**3**)), adenosine-5′-monophosphate (5′-AMP (**4**)), betaine [Bet, $(CH_3)_3N^+CH_2CO_2^-$], and trimethylamine *N*-oxide [TMAO, $(CH_3)_3N^+O^-$] were also reported as active substances of turbot, plaice, or Dover sole (Table 2).

Table 2 Feeding attractants and stimulants of fish.

Predator fish	*Prey animals*	*Active substances*	*Ref.*
Bathystoma rimator	*Arca zevra*	arcamine (**1**)	32
Bathystoma rimator	*Strombus gigas*	strombine (**2**)	32
Merlangius merlangus	*Arenicola marina*	Gly, Ala, Ser, Thr, Leu, Glu, Val	27, 33
Chrysophrys major	*Perinereis vancaurica tetradentata*	Gly, Ala, Val, amphoteric fluorescent substance	34
Lagodon rhomboides	*Penacus duorarum*	Gly, Asp, Ile, Phe, Bet	27
Salmo gairdnerii		Gly, Ala, α-Aba,[a] Val	35
Scophthalmus maximus	squid extract	5′-IMP, inosine	36
Pleuronectes platessa	squid extract	amino acids, AMP, TMAO	37
Limanda limanda	squid extract	amino acids	37
Soleo solea	*Mytilus edulis*	Bet	27

[a] α-Aba: α-aminobutyric acid.



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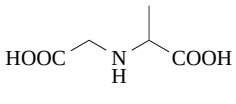

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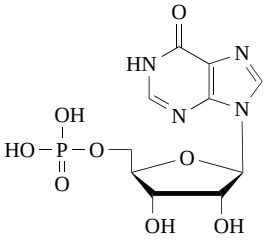

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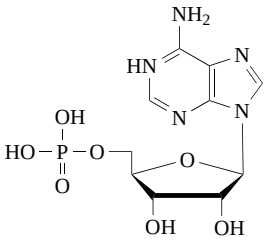


(4)

Ina and Matsui[34] studied the relationship between the feeding-stimulating activity and concentration of three amino acids, Gly, L-Ala, and L-Val; the results are shown in Figure 1, in which biting counts represented how many times the fish (sea bream *Chrysophyrys major*) bit the sample kneaded with starch and corresponded to the feeding-stimulating activity. Ina and co-workers[38] also examined the structure-activity relationships of neutral amino acids as feeding stimulants for sea bream *C. major* and revealed the following important structural factors. (i) It was essential for feeding-stimulating activity that amino acids contain α-hydrogen, and free α-amino and α-carboxyl groups. (ii) Active amino acids possess a carbon chain with less than five carbons. More than six carbons reduced the effectiveness. Those with an aromatic ring (e.g., Phe) were inactive. (iii) The methyl group of the terminal position of the aliphatic chain was necessary; the activity was almost lost for those whose terminal methyl was substituted with other functionalities (e.g., Cys, Ser, Orn, and cysteic acid). (iv) Di- and tripeptides were inactive, even those consisting of active amino acids. (v) Only L-amino acids have activity. (vi) L-Cys, L-Pro, and L-Trp exhibited strong synergistic responses to active amino acids such as Gly, L-Ala, and L-Val, whereas the responses of α-Aba, Ser, Thr, Asp, Glu, Asn, and Met were weak.

8.07.2.2 Mollusks

The phylum Mollusca is represented by classes of Gastropoda and Bibalvia, and the former contains commercially important seafood products such as abalones and turbots. It is known[26–29] that gastropods search about for foods and "chemoreception" may be participating in their feeding behaviors, and some amino acids, propionic acid, and trimethylamine were shown to play important roles in the feeding preferences or chemoreception of particular species of gastropods. Abalones *Haliotis* sp. were reported to feed on most algae, and their feeding preference on brown algae were

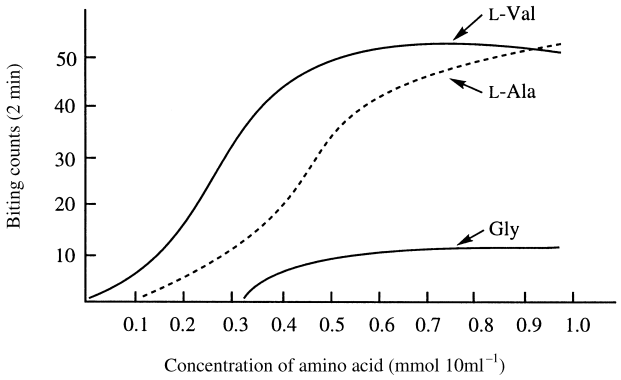
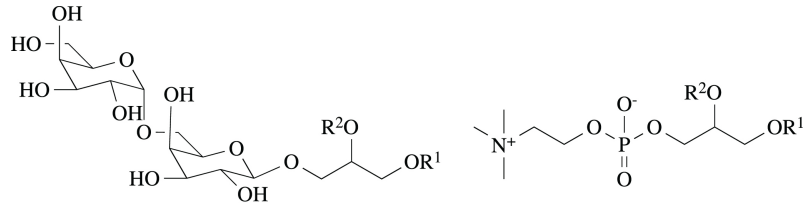


Figure 1 Relationship between the feeding-stimulating activity and concentration of Gly, L-Ala, and L-Val.

shown by examination of the stomach contents. Very few studies, however, have been described on the chemical investigation of the feeding behaviors of abalones.

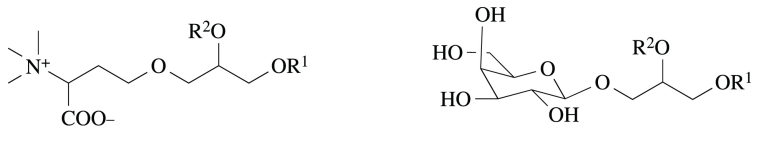
Sakata *et al.*[39] studied the chemical substances involved in feeding behaviors of the herbivorous gastropod *Haliotis* sp. and developed an excellent biological test of feeding preference of the young abalone *Haliotis discus* using Avicel plates. Abalones, about one year old and starved for at least a day before the test, moved around the test plate, and when they found a spot where their phagostimulant was absorbed, they bit off the Avicel from the sample zone. This test therefore showed not the feeding-attracting activity but the feeding-stimulating activity. Using this method, the methanol extracts of several algae were examined to reveal that extracts of brown alga *Undaria pinnatifida* were the most active. Isolation and identification of the feeding stimulant of the young abalones contained in the extracts of *U. pinnatifida* were studied using the Avicel plate tests to obtain two kinds of active substances, digalactosyldiacylglycerols (DGDG (**5**)) and phosphatidylcholines (PC (**6**)).[40]



(5) **(6)**

$R^1 = R^2 =$ acyl (C_{14}–C_{22})

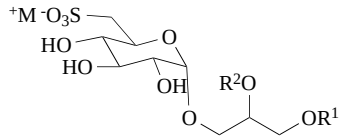
Feeding stimulants of a seahare *Aplysia juliana* were investigated from extracts of a green alga *Ulva pertusa* to isolate two kinds of genetic glycerolipids, DGDG (**5**) and 1,2-diacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (DGTH (**7**)).[41] The composition of fatty acid methyl esters (C_{14}–C_{22}) liberated from DGTH (**7**) was analyzed by GLC after hydrolysis with methanolic KOH, and FD and FAB mass spectral data provided evidence for identification of DGTH (**7**). From the less polar fractions of the silica gel column chromatography of the $CHCl_3$/MeOH (1:1) extract of *U. pertusa*, monogalactosyldiacylglycerols (MGDG (**8**)) was obtained and revealed to be less active. The *Aplysia* clearly responded to filter paper on which only 100 μg of DGTH (**7**) was absorbed while DGDG (**5**) was as active as DGTH (**7**).



(7) **(8)**

$R^1 = R^2 =$ acyl (C_{14}–C_{22})

Sakata and co-workers also reported that the Avicel plate method was applicable to investigation of the feeding stimulants for other gastropods such as the turban shell *Turbo cornutus* and the top shell *Omphalius pfeifferi*.[28–30] The turban shell *T. cornutus* moved around for food at night and often fed on a brown alga *Eisenia bicyclis*. Feeding stimulants of *T. cornutus* isolated from the methanol extract of *E. bicyclis* were revealed to be DGDG (**5**), PC (**6**), and 6-sulfoquinovosyldiacylglycerol (SQDG (**9**)). The methanol extract of the green alga *Ulva pertusa* was also studied to isolate feeding-stimulating substances of the top shell *O. pfeifferi* to identify the active glycerolipids such as DGDG (**5**), DGTH (**7**), and SQDG (**9**).[42] Comparisons of the feeding-stimulating activity of glycerolipids isolated from brown and green algae are summarized in Table 3. It is interesting that SQDG (**9**) was very active for the turban shell *T. cornutus* and the top shell *O. pfeifferi* but inactive for the abalone *Haliotis discus*. DGDG (**5**), PC (**6**), and DGTH (**7**) were active for all four gastropods, and among them, PC (**6**) was the most active substance. In addition, the glycerolipids, DGDG (**5**), PC (**6**), and SQDG (**9**), were revealed to be feeding stimulants not only of mollusks but also of echinoderms such as the sea urchin *Strongylocentrotus intermedius*.[27,28]



(9)

$R^1 = R^2 = acyl$

Table 3 Feeding-stimulating activity of glycerolipids for gastropods.[a]

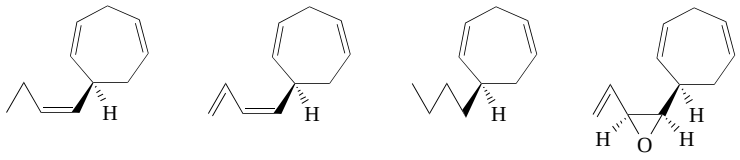
	Haliotis discus	*Turbo cornutus*	*Omphalius pfeifferi*	*Aplysia juliana*
DGDG (**5**)	14–28	15–25	18–23	800
PC (**6**)	10–20	15	< 15	< 50
DGTH (**7**)	10–20	24	< 10	100
SQDG (**9**)	> 300	20–40	< 20	> 1000

[a] Minimum amount of sample for each sample zone of the Avicel plate test or for each filter paper of the test for seahare (μg).

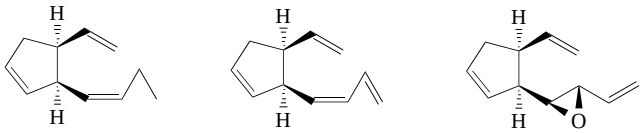
8.07.3 PHEROMONES

8.07.3.1 Sex Attractants of Algae

Brown algae or seaweeds are widely distributed throughout the oceans. Female gametes of marine brown algae release and attract males by chemical signals. In the early 1970s, Müller *et al*.[43] studied the sex attractant of a cosmopolitan brown alga *Ectocarpus siliculosus* and observed that culture dishes of the mature gynogametes of *Ectocarpus* release a faint aromatic fragrance, which was not perceived with the male culture. The scented substance was lipophilic and can be adsorbed on charcoal. When a cotton yarn or filter paper which assimilated the scented substance released from the mature gynogametes of *Ectocarpus* was presented to androgametes, the male gametes congregated around the allurement. By using this phenomenon as a biological test, the attractant substance of *Ectocarpus* was investigated from as many as 14 900 culture dishes of mature gynogametes (ca. 1 kg) to isolate 92 mg of the active substance, named as ectocarpene, which was identified to be (+)-(6*S*)-(1*Z*-butenyl)-1,4-cycloheptadiene (**10**) from mass and NMR spectroscopic data as well as degradation experiments. Diimide reduction of the side-chain of (**10**) followed by ozonolysis of the resulting 6-butyl-1,4-cycloheptadiene afforded (*R*)-2-butylsuccinic acid. Ectocarpene (**10**) was the first sex-attractant of algae whose chemical structure was established. In following years, a series of 11 hydrocarbons (**10**)–(**20**) was isolated and identified as pheromones that were involved as chemical signals in the sexual reproduction of more than 40 species of brown algae (Table 4).[44–47]



(10) **(11)** **(12)** **(13)**



(14) **(15)** **(16)**

(17) **(18)** **(19)** **(20)**

Table 4 Male gametes-attracting substances of brown algae.

Pheromone	*Order*	*Algal species*
Ectocarpene (**10**)	Ectocarpales	*Ectocarpus siliculosus*
	Sphacelariales	*Sphacelaria rigidula*
	Dictyosiphonales	*Adenocystis utricularis*
Desmarestene (**11**)	Desmarestiales	*Desmarestia aculeata*
Dictyotene (**12**)	Dictyotales	*Dictyota dichotoma*
Lamoxirene (**13**)	Laminariales	*Laminaria digita*
Multifidene (**14**)	Cutleriales	*Cutleria multifida*
	Laminariales	*Chorda tomemtosa*
Viridiene (**15**)	Syringodermatales	*Syringoderma phinneyi*
Caudoxirene (**16**)		*Perithalia caudata*
Hormosirene (**17**)	Fucales	*Hormosira banksii*
	Fucales	*Xiphophora chondrophylla*
	Durvillaeales	*Durvillaea potatorum*
	Scytosiphonales	*Scytosiphon lomentaria*
Finavarrene (**18**)	Fucales	*Ascophyllum nodosum*
	Dictyosiphonales	*Dictyosiphon foeniculaceus*
Cystophorene (**19**)	Fucales	*Cystophora siliquosa*
Fucoserratene (**20**)	Fucales	*Fucus serratus*

These sex pheromones were all lipophilic and volatile compounds consisting of C_8 or C_{11} linear or monocyclic hydrocarbons or their epoxides; monocyclic ones contained a cyclopropane, cyclopentene, or cycloheptadiene structure. Identification of these compounds was based on a combination of GC and MS, and comparison with synthetic authentic compounds. For instance, the presence of spermatozoid-releasing and -attracting substances released from the eggs was first suggested for the *Fucus* species in the first half of the twentieth century,[48] while the structure of the active compound, fucoserratene (**20**),[49] was only disclosed two years later than that of ectocarpene (**10**) because of the difficulty of cultivation of algae of the *Fucus* species. The structural elucidation of fucoserratene (**20**) was established by means of UV, mass, and NMR spectroscopic studies of (**20**) and its perhydrogenation product, viz., *n*-octane. The positions and geometrics of alkenes were revealed by comparison of gas chromatographic behaviors with all isomeric conjugated 1,3,5- and 2,4,6-octatrienes.

As shown in Table 4, the relationships between chemical structures of pheromones and taxonomical classifications of algae appeared unclear. Mature female gametophytes of brown algae of the order Laminariales, which includes the large kelps used as food such as tangle, secrete a highly volatile material which induces an explosive discharge of antheridia and spermatozoids. The active

substance was investigated by mass cultures of female gametophytes of *Laminaria digitata*. Suspensions of cultures of mature female gametophytes with developed oogonia and eggs were extracted by circulating a stream of air in a closed-loop system,[50] and volatile compounds were collected on a filter of activated carbon. After desorption with dichloromethane, the volatile substances were analyzed by glass capillary gas chromatography. When a SiO_2 particle adsorbing the active substance was added to mature male gametophytes, mass spermatozoids were released and large haloes of spermatozoids were successively formed around the particle.[51] After six years of research, the active substance was identified to be lamoxirene (**13**), as only minute quantities could be produced and isolated in the laboratory. Lamoxirene (**13**) induced the mass release of male gametes of *Laminaria digitata* within 8–12 s at a threshold of ca. 50 pmol. Lamoxirene (**13**) also induces the spermatozoid releasing and attracting of five species of brown algae of the Laminariales order.[52] Kajiwara observed that each of the culture suspensions of mature female gametophytes of eight species of Japanese brown algae (*Laminaria japonica*, *L. angustata*, *L. angustata f. longissima*, *L. coriacea*, *L. diabolica*, *Cymathaese japonica*, *Kjellmaniella crassifolia*, and *Undaria pinnatifida*) of the Laminariales order induced release of spermatozoids from antheridia of all these eight species of brown algae. Among the 64 combinations, no differences were observed in the spermatozoid-releasing activity.[44] Thus, lamoxirene (**13**) proved to be a common spermatozoid-releasing and -attracting substance to brown algae belonging to the Laminariales order.

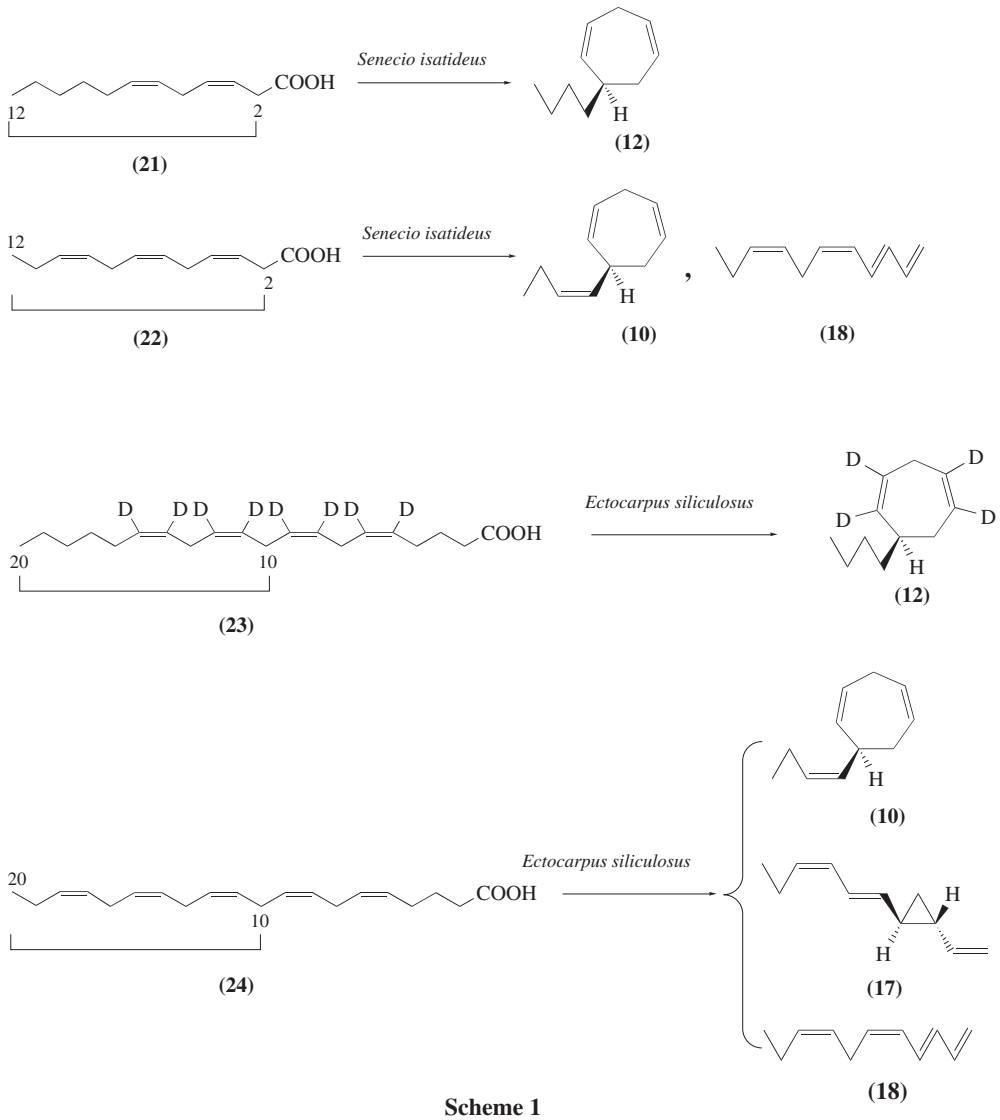
The sexual reproduction of brown algae of orders Scytosiphonales and Chordariales is an example of isogamy. A pleasant fragrance emanated from the motile female gametes of brown algae of these orders when settled on a surface to attract androgametes. The secretions from the female algal cells of *Colpomenia bullosa* (Scytosiphonales) were collected on charcoal. The volatile constituents were desorbed with dichloromethane and analyzed by GC, GC–MS, and HPLC to compare with those of the secretions from the male cells. A characteristic peak of the female secretions was collected by preparative HPLC (Zorbax ODS) to obtain ca. 100 μg of active substance, which was revealed to be homosirene (**17**) on the basis of GC–MS and ^{13}C NMR spectral data.[53] The absolute configuration of (**17**) obtained from the female secretions of *C. bullosa* was established as (1*R*,2*R*) by chiral HPLC analysis. The female secretions of *Colpomenia bullosa*, *Scytosiphon lomentaria* (Scytosiphonales), and *Analipus japonicus* (Chordariales) were examined by GC and GC–MS under low-temperature conditions to avoid thermal isomerization of homosirene (**17**) into ectocarpene (**10**). It was revealed that the secretions of *C. bullosa* and *S. lomentaria* contained 5% of ectocarpene (**10**) in addition to homosirene (**17**) (95%). The female secretions of *A. japonicus* was shown to consist of 88% of ectocarpene (**10**) and 12% of homosirene (**17**). The optical purity of homosirene (**17**) from gynogametes of thalli of brown algae of different geographic origins was analyzed to reveal that the ratio of enantiomers, (−)-(1*R*,2*R*)-(**17**) and (+)-(1*S*,2*S*)-(**17**), varied with the species and sometimes depended on the locality (Table 5).[47] Brown algae appeared to produce characteristic enantiomeric mixtures of pheromones, which has been presumed, though without any experimental substantiation, to be a simple means for identification of the chemical signals of their own species. The separation of the enantiomers of the alkenic hydrocarbons from the pheromone blend of seaweeds was achieved by GC on modified cyclodextrins as chiral stationary phases.[54]

Table 5 Enantiomer compositions of hormosirene (**17**) from secretions of female gametes or thalli of brown algae.

Genus and species	*Origin*	*Major enantiomer*	*ee* (%)
Dictyopteris acrostichoides	Sorrento, Australia	(−)-(1*R*, 2*R*)	74.2
Dictyopteris membranaceae	Villefranche, France	(−)-(1*R*, 2*R*)	71.2
Dictyopteris prolifera	Hikoshima, Japan	(−)-(1*R*, 2*R*)	90.0
Dictyopteris undulata	Hikoshima, Japan	(−)-(1*R*, 2*R*)	92.0
Analipus japonicus	Muroran, Japan	(+)-(1*S*, 2*S*)	66.0
Analipus japonicus	Akkeshi, Japan	(+)-(1*S*, 2*S*)	90.0
Durvillaea potatorum	Sorrento, Australia	(−)-(1*R*, 2*R*)	51.7
Haplospora globosa	Halifax, Nova Scotia	(+)-(1*S*, 2*S*)	83.3
Hormosira banksii	Flinders, Australia	(−)-(1*R*, 2*R*)	82.8
Xiphophora gladiata	Hobart, Tasmania	(−)-(1*R*, 2*R*)	72.3
Xiphophora chondrophylla	Flinders, Australia	(−)-(1*R*, 2*R*)	82.0

The biosynthesis of these marine algal pheromones is of great interest, and extensive investigations have been carried out.[47] The C_{11} hydrocarbons such as (*S*)-ectocarpene (**10**), dictyotene (**12**), and finavarrene (**18**) have also been isolated from terrestrial higher plants. Model experiments using

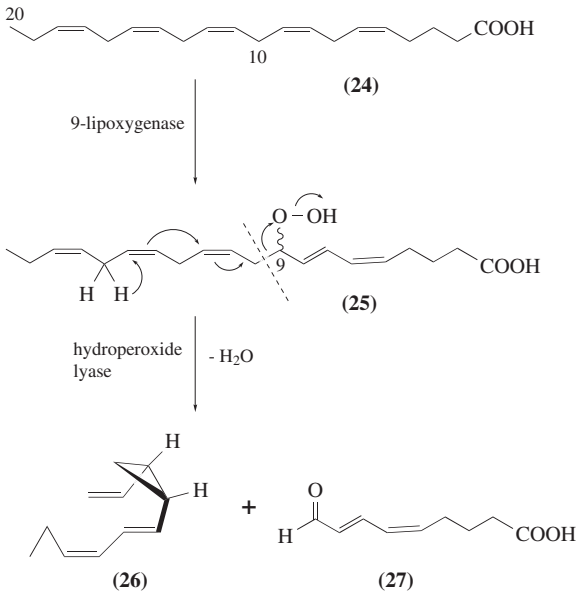
Senecio isatideus (Asteraceae) showed that these C_{11} hydrocarbons were derived from unsaturated C_{12} carboxylic acids (**21**) or (**22**) through oxidative decarboxylation, as shown in Scheme 1. In marine algae, however, the C_{11} hydrocarbons were revealed not to arise from the unsaturated C_{12} carboxylic acids precursors (**21**) or (**22**), but were derived from the polyunsaturated C_{20} fatty acids.[55] Externally supplied [$^2H_8$]-arachidonic acid (**23**) was very effectively converted into labeled dictyotene (**12**) in high yield by female gametes of *Ectocarpus siliculosus* (Scheme 1). The deuteration pattern of [$^2H_4$]-dictyotene (**12**) indicated that the $C_{11}H_{18}$ hydrocarbon (**12**) was formed from the C-10–C-20 positions of the arachidonic acid ([$^2H_8$]-(**23**)). From this result, the $C_{11}H_{16}$ hydrocarbons with one more degree of unsaturation such as (*S*)-ectocarpene (**10**), homosirene (**17**), and finavarrene (**18**) were implied to be generated from the aliphatic terminus of the more highly unsaturated eicosapentaenoic acid (**24**).[56]



Scheme 1

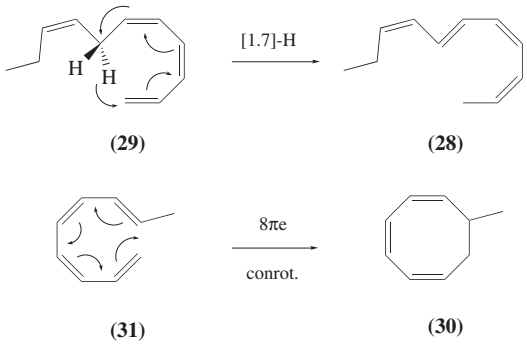
The first functionalization of the eicosanoid (**24**) was assumed to involve 9-lipoxygenase to yield 9-hydroperoxyicosa-(5*Z*,7*E*,11*Z*,14*Z*,17*Z*)-pentaenoic acid (9-HPEPE (**25**), Scheme 2), which was postulated to release the *cis*-disubstituted cyclopropane (**26**) with (1*R*,2*S*)-configuration and the C_9-dicarbonyl fragment (**27**) by a homolytic cleavage with hydroperoxide lyase.[56]

An uncommon C_{11}-hydrocarbon, 2*Z*,4*Z*,6*E*,8*Z*-undecatriene (giffordene (**28**)) is the major product of the fertile gametophytes of a brown alga *Giffordia mitchellae*.[57] The unusual stereochemistry of giffordene (**28**) may be accounted for by involvement of a spontaneous antarafacial [1.7]-hydrogen shift of a thermolabile intermediate (**29**) with a terminally unsaturated (1,3*Z*,5*Z*,8*Z*)-tetraene



Scheme 2

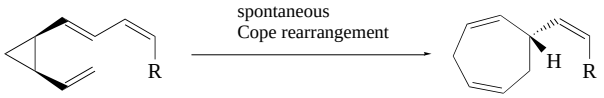
structure (Scheme 3), which could be supposed to be generated from the 9-HPEPE precursor (**25**) with an appropriate cisoid conformation. The thermolabile precursor (**29**) was synthesized under low-temperature conditions ($< -30\,^{\circ}C$),[58] and kinetic data showed that a half-life of compound (**29**) under the natural environment (18 °C) was ~2.5 h. The activation energies of the [1.7]-hydrogen shift ($E_a = 67.4$ kJ mol^{-1}; $\Delta S_{298} = -91.9$ J mol^{-1} K^{-1}) was considerably lower than that of the well-known [1.7]-hydrogen shift in the conversion of previtamin D_3 into vitamin D_3. The biosynthesis of 7-methylcyclooctatriene (**30**), isolated from the Mediterranean brown alga *Cutleria multifida*,[59] may be accounted for by another pericyclic reaction, viz., an 8πe electrocyclization of (1,3*Z*,5*Z*,7*E*)-nonatriene (**31**), as shown in Scheme 3. This thermolabile precursor (**31**) was also prepared under low-temperature conditions ($< -30\,^{\circ}C$) to determine kinetic data.[58] The half-life of acyclic precursor (**31**) was limited to a few minutes at ambient temperature, and the activation energies of the 8πe electrocyclization reaction ($E_a = 59.4$ kJ mol^{-1}; $\Delta S_{298} = -89.7$ J mol^{-1} K^{-1}) were the lowest values known for natural pericyclic reactions.



Scheme 3

The generation of (*S*)-ectocarpene (**10**) and other 6-substituted cyclohepta-1,4-dienes such as desmarestene (**11**), dictyotene (**12**), and lamoxirene (**13**) may be assumed to proceed by rearrangement of the thermally labile divinylcyclopropane (**26**) (Scheme 2) through a spontaneous [3,3] sigmatropic reaction (Cope rearrangement) as shown in Equation (1). To verify this hypothesis, the thermally labile divinylcyclopropane (**26**) and its analogues (**32**) and (**33**) were prepared and the activation energies of the Cope rearrangements yielding (**10**), (**11**), and bisnor-derivative (**34**),

respectively, were determined, as shown in Table 6.[60] The two temperatures in Table 6 were typical for the surroundings of marine algae in spring in Arctic and Mediterranean regions, and the half-lives for the transformation proved to be relatively long (18–77 min).



(1)

(26) R = C_2H_5
(32) R = CH=CH_2
(33) R = H

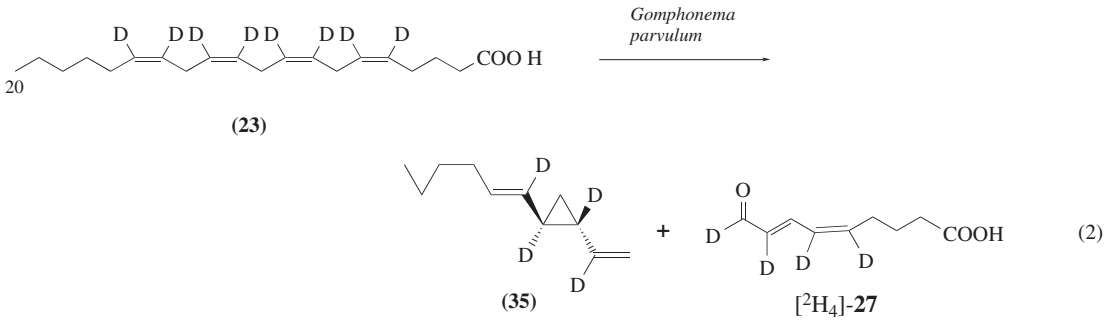
(10) R = C_2H_5
(11) R = CH=CH_2
(34) R = H

Table 6 Activation parameters and half-lives for the cope rearrangement of cyclopropanes.

Cyclo-propane	*Cyclo-heptadiene*	E_a (kJ mol^{-1})	ln *A*	$t_{1/2}$ (8 °C) (min)	$t_{1/2}$ (18 °C) (min)
(**26**)	(**10**)	63.8 ± 1.2	18.9 ± 0.4	56	21
(**32**)	(**11**)	62.6 ± 1.5	18.5 ± 0.5	45	18
(**33**)	(**34**)	64.9 ± 1.2	19.0 ± 0.4	77	30

The cyclohepta-1,4-dienes (**10**)–(**13**) were considered as sex pheromones of brown algae, but a question arose as to whether thermolabile cyclopropane precursors like (**26**) were actually the active pheromones. Comparative biological tests of (**10**) and (**26**) using male gametes of *Ectocarpus siliculosus* were carried out and revealed that the unstable cyclopropane (**26**) was much more active than the stable cycloheptadiene (**10**). A threshold concentration of (**10**) was determined as approximately 10 nmol L^{-1} in seawater, while that of (**26**) was found to be significantly lower, around 5 pmol L^{-1}. The release of the pheromone by female gametes, chemotactic orientation of male gametes, and fusion of sexual cells all occurs within only a few minutes. The half-life for the rearrangement of (**26**) was significantly longer than the time required for a sexual encounter in algae. Thus, it may be suggested that the degradation of the cyclopropanes by Cope rearrangement inactivates algal pheromones. Marine brown algae have developed the fastest and most effective signal system for spontaneous deactivation of attractant and/or release factors. These results suggested that systems in which cycloheptadienes were identified as active factors should be reexamined since thermally labile cyclopropyl precursors may be the actual pheromones and have even lower threshold concentrations.

The biosynthetic sequence involving the functionalization at C-9 of unsaturated C_{20} fatty acid, as depicted in Scheme 2, was further supported by a study on the algal pheromones produced by a freshwater diatom *Gomphonema parvulum*,[61] which was revealed to produce significant amounts of C_{11} hydrocarbons; its major volatile component was identified as homosirene (**17**). Approximately 10^7–10^8 cells of *G. parvulum* were sonicated, centrifugated, and the supernatant crude preparation was treated with deuterium-labeled arachidonic acid (**23**) to give deuterium-labeled dihydrohomosirene (**35**) together with 9-oxo-nona-5*Z*,7*E*-dienoic acid (**27**), as shown in Equation (2). The polar fragment (**27**) was identified by comparison with a synthetic authentic specimen. When the experiment was conducted in the presence of $^{18}O_2$ gas, the oxygen isotope was incorporated into the aldehyde function of (**27**). These observations further supported the current concept of the algal pheromone biosynthesis (Scheme 2).



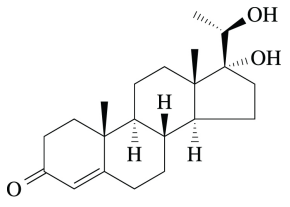
(**23**)

(**35**) + [$^2H_4$]-**27**

(2)

8.07.3.2 Others

Some secondary metabolites such as steroids and prostaglandins are identified as pheromonal attractants of teleosts.[62] 17α,20β-Dihydroxy-4-pregnen-3-one (**36**) was revealed to be a potent female sex pheromone of goldfish *Carassius auratus*,[63] Ovulating goldfish release (**36**), and milt (sperm and seminal fluid) volume was increased by exposure to (**36**). The goldfish olfactory epithelium was extremely sensitive to (**36**), and the increase in milt volume induced by (**36**) was abolished by sectioning the medial olfactory tracts, which was implicated in the control of sex behavior in male goldfish.



(36)

8.07.4 SYMBIOSIS

8.07.4.1 Invertebrates and Microalgae

Symbiotic association (living together; the wide sense of ''symbiosis'') of animals, plants, or microorganisms with different species is frequently found and there are three styles of symbiosis, (i) parasitism, (ii) commensalism, and (iii) mutualism. The first case implies that by living together only one of a pair (the symbiont) is advantaged but the partner (the host) is damaged. In the second case, only the symbiont is advantaged while the host is neither advantaged nor damaged. The third one is the narrow sense of ''symbiosis,'' in which both the symbiont and host are advantaged by living together.

Symbiotic association between marine invertebrates and microalgae is a well-known phenomenon classified as mutualism (the third case), and Table 7 shows a list of symbiotic marine microalgae and their host invertebrates.[64] These symbiotic microalgae live inside the cells of host invertebrates. Flatworms take microalgae from their food and the algae go into the parenchyma under the epidermis of the hosts. Symbionts of bivalves live in the edges of the mantle, the hematocoel, or the renal tissues. Sometimes symbionts of bivalves are found inside the gill cells or in the ameboid hemocytes. Nudibranchs have their symbiotic algae reside in the branched hepatopancreas on their back processes or in the alimentary canals.

The morphology and life history of some species of symbiotic microalgae are almost the same as those of free-living species, but those for other species are quite different. Dinoflagellates of the genus *Symbiodinium* living inside invertebrates are always found as vegetative cells which have very thin and faint cell walls and no flagella, while those of free-swimming ones have very bold cell walls. The symbiotic dinoflagellates *Amphidinium* sp. and *Prorocentrum* sp. found inside flatworms have two flagella and cell walls, but the cell walls of *Amphidinium* sp. appear relatively thin. Prasinophytes of free-swimming species are found in the periods of vegetable cells or moving spores; the latter have four flagella with equal length. In both periods, free-swimming prasinophytes possess cell walls and stigma, while those symbiotically living inside invertebrates lose both cell walls and stigma and their cells are wrapped by plasma-membrane.

The way in which the symbiotic algae are handed down to the next generation of the host invertebrates also depends on the combinations of algae and invertebrates. Protozoans give their symbiont algal cells almost equally to the divided daughter cells during cell fission. For flatworms, bivalves, and nudibranchs, the symbiotic associations with microalgae are reconstructed by the next generation. On their oviposition, however, it is frequently found that microalgae of the parent host cells are adhered to the egg capsule, and hatched larvae of the host invertebrates can easily take the algae into their own cells as symbionts.

These symbiotic associations of microalgae and invertebrates are maintained by quite dexterous mechanisms and succeed for generations. Sponges recognize cyanophytes by their choanocyte or digestive cells as symbiotic algae and have a mechanism not to digest algae. It is now recognized

Table 7 Symbiotic marine microalgae and their host invertebrates.[63]

Host invertebrates	*Genus*	*Symbiotic microalgae*	*Genus*
Sponges	*Cliona*	dinoflagellate	*Symbiodinium*
	Aplysina	cyanophyte	
	Jaspis	cyanophyte	
Coelenterates	*Clavularia*	dinoflagellate	*Symbiodinium*
(Octocorallia)	*Heliopora*	dinoflagellate	*Symbiodinium*
	Lobophylum	dinoflagellate	*Symbiodinium*
	Jubipora	dinoflagellate	*Symbiodinium*
	Xenia	dinoflagellate	*Symbiodinium*
	Zoantles	dinoflagellate	*Symbiodinium*
Tunicates	*Trididemnum*	prochlorophyte	*Prochloron*
	Didemnum	prochlorophyte	*Prochloron*
	Diplosoma	prochlorophyte	*Prochloron*
	Lissoclinum	prochlorophyte	*Prochloron*
Flatworms	*Convoluta*	prasinophyte	*Tetraselmis*
	Convoluta	diatom	*Lichmorphora*
	Convoluta	dinoflagellate	*Symbiodinium*
	Amphiscolops	prasinophyte	*Tetraselmis*
	Amphiscolops	dinoflagellate	*Symbiodinium*
	Amphiscolops	dinoflagellate	*Amphidinium*
	Amphiscolops	dinoflagellate	*Prorocentrum*
	Pseudaphanostoma	dinoflagellate	*Symbiodinium*
	Haplodiscus	dinoflagellate	*Amphidinium*
Bivalves	*Hippopus*	dinoflagellate	*Symbiodinium*
	Corculum	dinoflagellate	*Symbiodinium*
	Fragum	dinoflagellate	*Symbiodinium*
	Tridaena	dinoflagellate	*Symbiodinium*
Nudibranchs	*Aeolidiella*	dinoflagellate	*Symbiodinium*
	Melibe	dinoflagellate	*Symbiodinium*
	Elysia	chloroplast of Codiales	
	Plakobrunchus	chloroplast of Codiales	

that the microalgae are fundamental to the biology of their hosts, and their carbon and nitrogen metabolisms are linked in important ways. Table 8 shows substances given to host invertebrates by symbiotic algae. Glycerol is the main chemical substance released by symbiotic dinoflagellate *Symbiodinium* sp. For coelenterates and bivalves, more than 20–40% of the carbon metabolites produced by photosynthesis of *Symbiodinium* sp. was released into the host cells as glycerol. Galactose, glycolic acid, alanine, glutamic acid, and lipid particles are also reported as released substances from symbiotic algae to the host invertebrates.

Table 8 Substances given by symbiotic algae to host invertebrates.[64]

Symbiont	*Host*	*Carbon metabolites*
Dinoflagellate	coelenterates	
Symbiodinium sp.	*Pocillopora damicornis*	glycerol
Symbiodinium sp.	*Porites poriles*	glycerol
Symbiodinium sp.	*Condylactis gigantea*	glycerol, lipid particles
	bivalve	
Symbiodinium sp.	*Tridaena crocea*	glycerol
	nudibranch	
Symbiodinium sp.	*Melibe pilosa*	lipid particles?
Symbiodinium sp.	*Pteraeolidia ianthina*	glycerol
Cyanophyte	*Placobranchus ianthobapsus*	galactose
Chloroplast of Codiales	*Tridachia crispata*	galactose
Prochlorophyte	tunicate	
	Diplosoma virens	glycolic acid
Prasinophyte	flatworm	
Tetraselmis convolutae	*Convoluta roscoffensis*	alanine, glutamic acid

Nitrogen fixation in coral reef sponges with symbiotic cyanobacteria has been reported.[65] The presence of unicellular cyanobacteria in sponges was confirmed by electron microscopy. Cyanobacteria were observed in large vacuolated cells (termed bacteriocytes) throughout the tissue of a sponge *Siphonochalina tabernacula*. The cyanobacteria observed in the thin brown pigmented ectosome of a sponge *Theonella swinhoei* occupied 10–15% of the ectosome volume. Cyanobacteria numbers decrease away from the ectosome and were not evident in the deeper part of the endosome, while symbiotic bacteria were rare in the ectosome and particularly numerous in the endosome. Nitrogenase activity was determined using the acetylene reduction technique immediately after collection of the sponges on reef-based platforms. Nitrogenase activity was detected in the two sponges (*S. tabernacula* and *T. swinhoei*) which harbor symbiotic cyanobacteria, and was absent in the third sponge *Inodes erecta* which lacked cyanobacteria and a symbiont. The activity of the ectosome of *T. swinhoei* was higher than the endosome; the former contained cyanobacteria while the latter was almost free of cyanobacteria. In addition, when sponge tissues were incubated in light or dark considerably more ethylene was produced in the illuminated sample. The nitrogenase activity was therefore concluded to be due to symbiotic cyanobacteria.

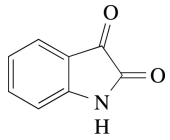
What is the purpose of symbiotic association with invertebrates for algae? One family of colonial didemnid ascidians (Urodhordata) harbor the symbiotic unicellular prokaryotic alga *Prochloron* sp., which synthesizes chlorophylls a and b but no phycobilin pigments. During investigation of conditions for laboratory cultivation of this unusual alga, it was found that *Prochloron* sp. is a naturally occurring tryptophan auxotroph that survives in nature by close association with the host.[66] Cell division in *Prochloron* cultures, freed from the host ascidian *Diplosoma similis*, occurred under acidic conditions (pH 5.5) in the presence of tryptophan. Among 21 common levorotatory amino acids tested as a supplement to the basal medium, only L-tryptophan affected *Prochloron* growth. The metabolic dysfunction that rendered *Prochloron* auxotrophic may involve only the initial step of the tryptophan biosynthetic pathway from chorismic acid (the final intermediate common to both tyrosine and tryptophan synthesis) since shikimic acid alone did not support the algal growth but anthranilic acid did. The growth rates of *Prochloron* culture were slow compared with those of many other algal cultures. Although the growth rate of *Prochloron* in the acidian *D. similis* was not known, it may be imagined that symbiotic association with didemnid ascidians is advantageous to the alga. It was also reported[64] that recycling of nitrogen metabolites such as uric acid or ammonia was carried out by symbiotic microalgae and was also beneficial to algal physiology. Thus, it may be profitable for algae to live in the host invertebrates as symbionts, compared with free living in the oligotrophic environments of tropical or subtropical oceans lacking in inorganic or other nitrogenous nutrients.

Investigations by Muscatine and co-workers on the "host factor" that participates in symbiotic association between dinoflagellates and reef-building corals or other tropical anthozoans have revealed that free amino acids exhibited "host-factor" activity and induced the release of photosynthate from symbiotic dinoflagellate *in vitro*.[67] Symbiotic dinoflagellates incubated with a crude homogenate of their own host tissue fixed $^{14}CO_2$ in the light and released a substantial fraction of the fixed carbon to the incubation medium, principally as [^{14}C]glycerol, [^{14}C]alanine, and [^{14}C]glucose. In contrast, the release of labeled organic carbon compounds by dinoflagellates incubated without homogenate of the host tissue was significantly less. Thus, it was shown that a factor (a chemical substance, i.e., a "host factor") present in the homogenate of the host tissue evoked the release of fixed carbon as low molecular weight compounds from symbiotic dinoflagellates. The host factor of the Hawaiian reef coral *Pocillopora damicornis* was investigated from the crude aqueous extract of this host using size-exclusion chromatography and HPLC. The host-factor active fraction showed absorbance features characteristic of the hydrophilic mycosporine-like amino acids abundant in marine cnidarians. Further investigation on isolation of individual mycosporine-like amino acids from lyophilized methanolic extracts of *P. damicornis* tissues led to identification of the host factor as a set of free amino acids. Synthetic amino acid mixtures, based on the measured free amino acid pools of *P. damicornis* tissues, not only induced the selective release of ^{14}C-labeled photosynthetic products from isolated symbiotic dinoflagellates but also enhanced total $^{14}CO_2$ fixation. Comparison of the radiochromatograms of the ^{14}C-labeled photosynthetic products released and fixed by symbiotic dinoflagellates of *P. damicornis* incubated with a synthetic free amino acid pool showed that the release of ^{14}C-labeled products was selective and not the result of dinoflagellate lysis. In addition, the released products were qualitatively identical to those released by dinoflagellates incubated in crude aqueous extracts of their own host. The products released by dinoflagellates isolated from *P. damicornis* differed from those released by dinoflagellates isolated from the sea anemone *Aiptasia pulchella*. These differences may be a reflection of the diversity of dinoflagellate species found in symbiosis with cnidarians, rather than host-factor specificity.

Symbiotic dinoflagellates of flatworms produce a variety of secondary metabolites with unique bioactivity such as strong cytotoxicity or vasoconstrictive activity, which is described in Section 8.07.9.4 The biological significance of these unique metabolites in the lives of dinoflagellates and/or flatworms is unknown.[68]

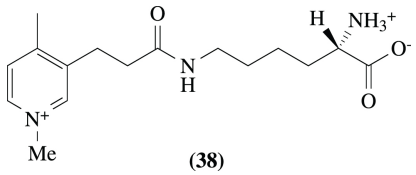
8.07.4.2 Others

Embryos of the shrimp *Palaemon macrodactylus* are known to resist infection by the pathogenic fungus *Lagenidium callinectes*. Fenical and co-workers found that a penicillin-sensitive bacterial strain (*Alteromonas* sp.) that was consistently isolated from healthy embryos effectively inhibited the growth of the fungus *L. callinectes in vitro*.[69] This bacterial strain was shown to produce and release an antifungal compound, which was identified as 2,3-indolinedione (**37**) (known as isatin). Bacteria-free embryos which were exposed to the fungus quickly died, whereas embryos reinoculated with the bacteria or treated only with 2,3-indolinedione (**37**) thrived. Thus it was clearly revealed that the symbiotic *Alteromonas* sp. bacteria protect shrimp embryos from fungal infection by producing and liberating the antifungal metabolite (**37**).



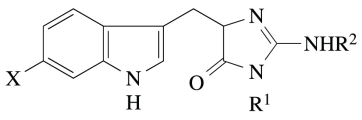
(37)

The species-specific partnership between anemone fish and the giant sea anemone is a well-known phenomenon of symbiosis in marine environments. The anemone fish recognize their specific partner anemone on the basis of chemical substances secreted by the anemone. Naya and co-workers studied the chemical substances secreted by the sea anemone which elicit symbiotic behavior of the fish and identified several nitrogenous compounds that induce characteristic symbiotic movements.[70,71] The sea anemone *Radianthus kuekenthali*, which was a specific host of the fish *Amphiprion perideraion*, was homogenized, and fractionation of the aqueous layer of the extract yielded 48 μg of a cationic compound as the sole active factor, which was named amphikuemin (**38**). The structure of (**38**) was established by hydrolysis, spectroscopic data, and synthesis. The synthetic L-enantiomer of (**38**) was identical with natural material in all respects including biological activity. Amphikuemin (**38**) was active in the attracted swimming assay using *A. perideraion* at a concentration of 10^{-10} M. Analogues of amphikuemin (**38**) were also prepared; *N*-demethyl-L-amphikuemin was totally devoid of activity, while the activity of the synthetic quaternary salt 4′-demethyl-L-amphikuemin was greatly reduced to 2.0×10^{-8} M.

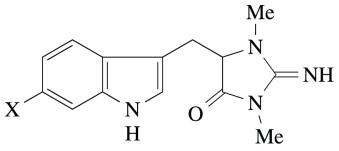


(38)

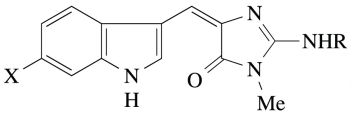
The organic layer of the extract of *R. kuekenthali* was also examined to give 16 tryptophan-derived alkaloids, dihydroaplysinopsins (**39**)–(**44**) and aplysinopsins (**45**)–(**54**), which are known as sponge-metabolites.[72] Dihydroaplysinopsins (**39**)–(**44**) induced attracted swimming of *A. perideraion* at the effective dose of 10^{-6} M, more weakly than amphikuemin (**38**). Aplysinopsins (**45**)–(**54**) with structural variations in ring C, double-bond geometry at C-8 and C-1′, and the presence and absence of bromine caused the fish *A. perideraion* to perform an up-and-down movement of the head, like a seesaw. *A. clarkii*, another guest fish of the host animal *R. kuekenthali*, did not respond to either amphikuemin (**38**) or the aplysinopsins and dihydroaplysinopsins. It is thus likely that these two different species of symbiotic fish recognize their common host through different chemicals.



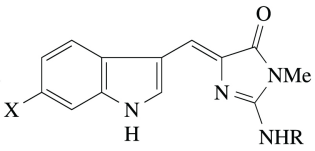
(39) X = Br, R^1 = Me, R^2 = H
(40) X = H, R^1 = Me, R^2 = H
(41) X = Br, R^1 = H, R^2 = Me
(42) X = H, R^1 = H, R^2 = Me



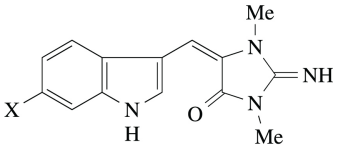
(43) X = Br
(44) X = H



(45) X = Br, R = Me
(46) X = H, R = Me
(47) X = Br, R = H
(48) X = H, R = H

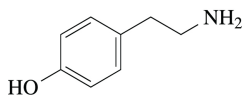


(49) X = Br, R = Me
(50) X = H, R = Me
(51) X = Br, R = H
(52) X = H, R = H



(53) X = Br
(54) X = H

Chemical substances involved in the symbiotic movements of another host/guest pair, *Stoichactis kenti* (sea anemone) and *A. ocellaris* (anemone fish) were also studied, and tyramine (**55**) and tryptamine (**56**) were identified as active substances that induced attracted swimming with tail wagging and active searching behavior, respectively, both at a dose of 10^{-6} M. It was observed that addition of tyramine to a partially purified fraction of the secretion of *S. kenti* increased the activity while further purification sometimes led to reduction in the activity. The symbiosis-inducing activity in the pair of *S. kenti–N. ocellaris*, therefore, may depend on the synergistic effect of multiple unidentified chemicals.



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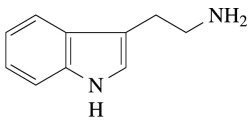

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8.07.5 BIOFOULING

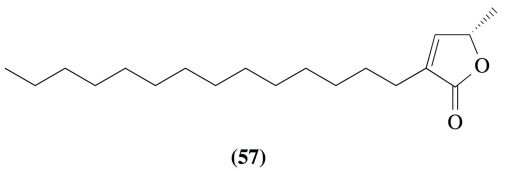
Many species of sessile organisms live in the sea. Barnacles, mussels, hydroids, bryozoans, and tunicates are representative. In their life cycles, the larvae of sessile organisms swim and float until they find a suitable place to settle and grow. The search for an appropriate place where they undergo metamorphosis and grow into adults is quite important for them, because it influences their entire life and the conservation of the species. It is believed that they have elaborate chemical control mechanisms to induce the larval settlement at the most satisfactory location for the particular species. They also possess chemical defense systems against larval settlement of other competing sessile organisms.[73–76]

8.07.5.1 Microorganisms

A biofilm formation by marine bacteria is observed in a few hours when a plastic plate is put into the sea. This biofilm formation is followed by attachment of microalgae such as diatoms to give a "slime," which is called "microfouling." The microalgal slime triggers larval settlements and metamorphosis of various marine sessile organisms such as barnacles and blue mussels to lead to "macrofouling." Although it is believed that chemical signals are involved in the attachment of marine bacteria and microalgae, the process is not well understood. Organisms already growing at

some ecological site have chemical control systems (allelopathy) for preventing the settling of other attaching organisms such as diatoms.

Research on chemical substances that regulate biofilm formation has been carried out.[77] ''Macrofouling'' causes serious problems on ship hulls, fishing facilities, and other artificial objects in the sea such as the cooling systems of power plants. Organotin compounds like TBTO [bis-(*n*-tributyltin)-oxide] have been used as antifouling agents, but they exhibit toxic effects on the marine environment including fish and shellfish. Antifouling substances with less toxicity, therefore, are required. On the other hand, substances that induce or promote biofilm formation may be of use in fish farming. A bioassay system was developed using a marine bacterium possessing attaching properties (*Rhodospirillum salexigens* SCRC 113), and evaluation of the biofilm formation was monitored by measuring the quantity of polysaccharide produced by the bacteria as well as the absorbance of the culture medium by the attaching bacteria. As a result, one biofilm formation accelerator, hateramine (**57**), was isolated from a marine sponge, and 10 compounds (**58**)–(**67**) were identified as biofilm formation inhibitors from natural and synthetic compounds. Bisdeacetylsolenolide D (**58**), aeroplysinin-I (**59**), and new nitroalkanes (**60**) and (**61**) were natural products isolated from marine sponges. Ethyl *N*-(2-phenethyl)carbamate (**62**) was isolated from the marine bacterium SCRC3P79 (*Cytophaga* sp.) and its inhibition activity was remarkable. A synthetic analogue (**63**) also exhibited significant inhibition activity of biofilm formation and showed antibacterial activity. Compounds (**64**)–(**67**) were prepared by synthesis and significantly inhibited the biofilm formation not only in the laboratory bioassay but also in the field experiment.



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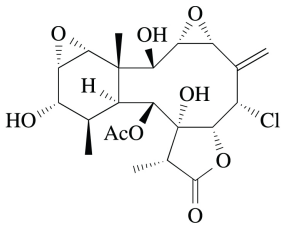

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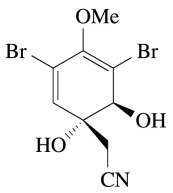

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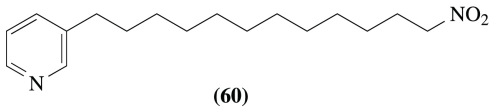

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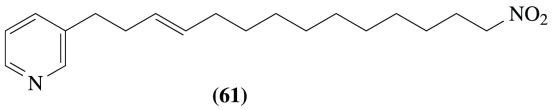

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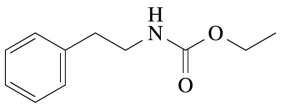

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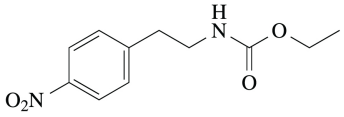

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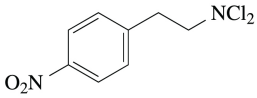

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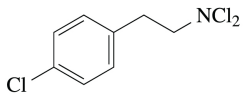

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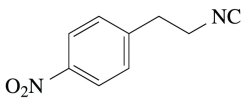

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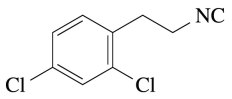
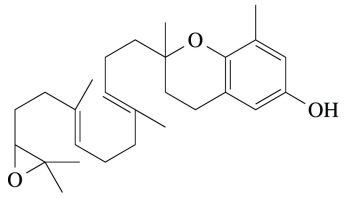


(**67**)

8.07.5.2 Hydrozoa

The larvae (pulanula) of the Japanese hydrozoan *Coryne uchidai* search for their attaching site by swimming and crawling about, and frequently settle on seaweeds. From an extract of the seaweed

Sargassum tortile, δ-tocotrienol epoxides (**68**) and (**69**) were identified as larval settlement/metamorphosis-inducing substances.[73,74] The inducing activity was further confirmed by the use of synthetic (**68**) and (**69**). More interestingly, the epoxide (**68**) also exhibited inhibition of metamorphosis, depending upon the concentration. When the epoxide (**68**) was presented to the larvae for too long, the metamorphosis induced by (**68**) was interrupted at an early stage of polyp formation, but was restarted by diluting with fresh seawater.



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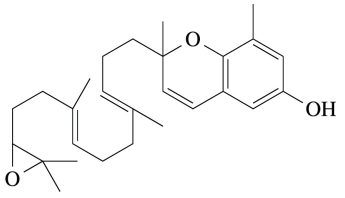


(**69**)

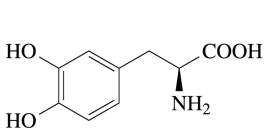
The larval settlement and metamorphosis of a hydroid *Hydractinia echinata* were induced by a biofilm of Gram-negative bacteria. The active substance released from the biofilm is unknown but assumed to be an unstable hydrophobic substance. Metal ions such as Li^+, Rb^+, and Cs^+ are revealed to induce larval metamorphosis, and tumor-promoting phorbol esters are also reported to be active.[78] Bacterial biofilms also induced larval settlement of a hydroid *Halcordyle disticha*. Catecholamines were active in the induction of larval metamorphosis of *H. disticha* but inactive in that of *H. echinata*.[79]

8.07.5.3 Polychaetes

Some lugworms live in colonies of tubes made from sea sand. The larval settlement and metamorphosis of a reef-building tube worm *Phramatopoma californica* were induced by extracts of their tubes, and the active substance was identified as a mixture of fatty acids (C_{14}–C_{22}).[80] Tests using individual fatty acids revealed that $C_{16:1}$, $C_{18:2}$, $C_{20:4}$, and $C_{20:5}$ fatty acids were active with the concentration of 100 μg g^{-1}; only the *Z* isomer of $C_{16:1}$ fatty acid was active, while its *E* isomer was inactive. Too large a dose (1 mg g^{-1}) of $C_{20:4}$ and $C_{20:5}$ fatty acids induced unusual metamorphosis. In addition, the terminal carboxyl group proved to be essential for the metamorphic-inducing activity. Marine bacterial biofilms also induced larval settlement of a polychaetes *Janua brasiliensis*, and the active compound was believed to be biopolymers like glycoproteins.[81]

8.07.5.4 Mollusks

The larvae (veliger) of oysters settle down around their parents as with many other marine invertebrates, and extracts of parent oysters induced the larval settlement. Larval settlements of the eastern oyster *Crassostrea virginica* and the pacific oyster *C. gigas* were induced by the biofilms of Gram-negative bacterium. From the culture broth of this bacterium, L-3,4-dihydroxyphenylalanine (L-DOPA (**70**)) and its polymerized product, a black melanin pigment (molecular weight, 12 000–120 000), were identified as active substances.[82] L-DOPA (**70**) is a biosynthetic precursor of the catecholamines, well-known neurotransmitters, such as (−)-adrenalin (**71**) and (−)-noradrenalin (**72**). L-DOPA (**70**) induced larval settlement and metamorphosis and the most effective concentration was 2.5×10^{-5} M; at this concentration settlement and metamorphosis of approximately 50% of the larvae were induced. However, when (**70**) was presented to the larvae for too long at concentrations higher than 5×10^{-6} M, (**70**) proved to be toxic.[83] (−)-Adrenalin (**71**) and (−)-noradrenalin (**72**) were also strongly active but, interestingly, they induced larval metamorphosis without attaching to suitable substrates. D-DOPA (**73**), dopamine (**74**), acetylcholine (**75**), and GABA (**76**) were revealed to be inactive in larval settlement and metamorphosis of oysters.



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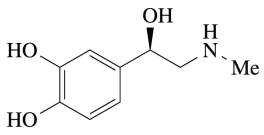

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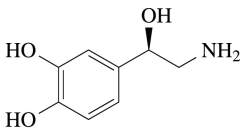

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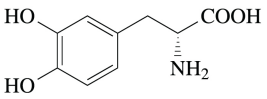

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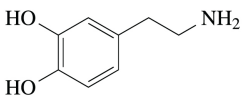

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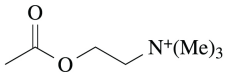

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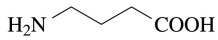
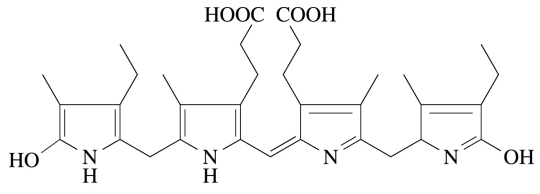


(76)

The planktonic larvae of the gastropod mollusk, *Haliotis rufescens*, the large red abalone of the eastern Pacific, are recruited from the plankton to crustose red algae including species of *Lithothamnium*, *Lithophyllum*, and *Hildenbrandia*.[84] This recruitment to the intact crustose red algae is normally dependent upon larval contact with the recruiting algal surface, and the crustose red algae produce or contain molecules that induce substratum-specific settlement, attachment, and metamorphosis of the *Haliotis* larvae. Such molecules were associated with macromolecules and were demonstrated to be produced by a number of red algae, but were not produced by the green or brown algae. The substratum specificity of larval settlement and metamorphosis was shown to result from the unique availability of these chemical inducers at the surfaces of the crustose red algae. By purification based on size-separation by gel-filtration, followed by ion-exchange chromatography over a diethylaminoethyl (DEAE)-acrylamide matrix, the principal inducer was resolved from the red algal phycobiliprotein.[85] Phycoerythrobilin (**77**) (a linear tetrapyrrole bile pigment) and its specific protein conjugate, phycoerythrin, were the accessory photosynthetic pigments responsible for the coloration of the coralline red algae. Both free and conjugated phycoerythrobilin (**77**) exhibited half maximal induction of settling of the *Haliotis* larvae at $\sim 10^{-6}$ M and were toxic to the larvae at concentrations higher than 10^{-5} M. Other linear and cyclic tetrapyrroles and other proteins including hemoglobin, which contains the cyclic tetrapyrrole heme group, were inactive over comparable concentrations.



(77)

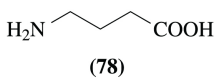
Table 9 summarizes the induction activity of several extracts of algae and a series of amino acids for larval settlement and metamorphosis of *Haliotis rufescens*.

GABA (γ-aminobutyric acid (**78**)), a neurotransmitter in higher animals, was found to be a potent inducer of rapid settling of the *Haliotis* larvae contained in the crustose red algae. GABA (**78**) was active in inducing behavioral metamorphosis, viz., inducing to settle and begin their characteristically snail-like plantigrade attachment, gliding locomotion, and grazing behavior on the clean glass of the test vials, at concentrations as low as $\sim 10^{-7}$ M, with half-maximal effectiveness (50% settling) at $< 10^{-6}$ M. Prolonged exposure of larvae to higher concentrations of the free inducer (**78**) was

Table 9 Induction activity of larval settlement and metamorphosis of *Haliotis rufescens*.[84]

Inducer	*Settled* (%)
None	0
Lithothamnium sp. and *Lithophyllum* sp.	82
Bossiella sp.	4
Diatoms	0
Bacteria and microalgae	0
Macrocystis pyrifera	0
Lithothamnium extract, 2 μg of protein per ml	2
The same, boiled	6
The same, protease digested; boiled	23
GABA (γ-aminobutyric acid)	$\geqslant$ 99
α-Aminobutyric acid (D- and L-)	0
β-Aminobutyric acid (D- and L-)	0
n-Butylamine	0
n-Butyric acid	0
n-Pentanoic acid	0
Succinic acid	0
γ-Guanidinobutyric acid	0
γ-Hydroxybutyric acid	58
δ-Amino-*n*-valeric acid	89
ε-Amino-*n*-caproic acid	74
L-Glutamic acid	12
D-Glutamic acid	0
L-Glutamine	0
L-Aspartic acid	0
Other neurotransmitters and effectors	0

toxic. GABA (**78**) is present in high concentrations ($\sim 10^{-2}$ M) in the crustose coralline red algal extracts. Structural analogues of GABA (**78**) such as γ-hydroxybutyrin acid (**79**), δ-amino-*n*-valeric acid (**80**), and ε-amino-*n*-caproic acid (**81**) showed significant activity in the induction of settling. The absolute requirements for the inducing activity may be the presence of the primary carboxyl group of GABA (**78**) and that of specific substitution at the γ-position. Increasing and decreasing the chain length of GABA homologues progressively decreased their activity. In addition, L-α,ω-diamino acids, such L-α,β-diaminopropionic acid (**82**) and L-lysine (**83**), facilitated, at a concentration of 10^{-5} M, the induction of larval metamorphosis in the presence of GABA (2.5×10^{-7} M).[86] On the other hand, other neurotransmitters and effectors were inactive; tested substances were L-epinephrine, L-norepinephrine, serotonin, histamine, acetylcholine, choline, and indole-3-butyric, -acetic, -propionic, and -acrylic acids.



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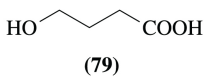

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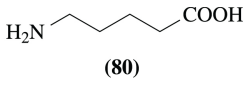

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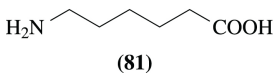

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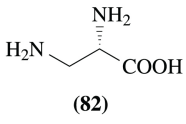

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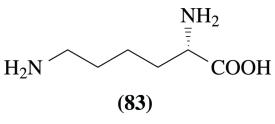
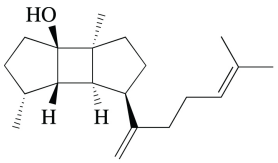


(83)

The larval settlement and metamorphosis of *Haliotis rufescens* as well as those of the tube worm *Phramatopoma californica* and the nudibranch *Phestilla sibogae* were induced by increase in the concentration of potassium ion (K^+) in the seawater. Addition of the K^+-channel blocker, tetraethylammonium chloride, inhibited the larval settlement and metamorphosis of *H. rufescens* but affected no changes for *P. californica* and *P. sibogae*.[87]

The neutral methanol extract of the brown alga *Dilophus okamurai* was found to inhibit the settlement and metamorphosis of the swimming larvae (veliger) of the abalone *Haliotis discus hannai*. Two active components were isolated and identified as spartane-type diterpenes (**84**) and (**85**).[88] There herbivorous abalones can scarcely be seen in the community of the brown alga *D. okamurai* which grows in a depth of water very similar to that of the brown alga *Eisenia bicyclis*. Thus it was suggested that *D. okamurai* contains an antifeeding substance against the abalone. The

two diterpenes (**84**) and (**85**) inhibited the larval settlement and metamorphosis of the abalone *H. discus hannai* at a concentration of 5 ng ml^{-1}. It is unknown whether these diterpenes are true antifeedants.



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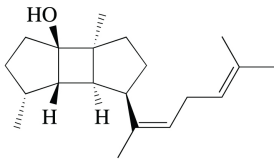
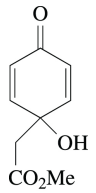


(85)

The larval settlement and metamorphosis of the scallop *Pecten maximus* were induced by jacaranone (**86**), which was isolated from extracts of the alga *Delesseria sanguinea*, at a concentration of 0.5 mg l^{-1}.[89] Among the compounds isolated from *D. sanguinea* only (**86**) displayed this property.



(86)

8.07.5.5 Barnacles

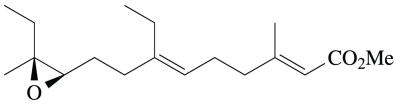
Barnacles are one of the representative marine fouling invertebrates which cause serious problems on ship hulls and other man-made objects. Larvae (cypris) of barnacles settle around their parents. Extracts of parents of the Atlantic barnacle *Balanus balanoides* contained a substance that induced larval settling and metamorphosis. This substance was suggested to be a chemically stable protein bridged with yellow pigments (quinones?), which kept their activity after heating but became inactive by treatment with sodium perchlorate. This protein was named ''arthropodin'' and proved to consist of subunits with molecular weights of 18 000 and 5000–6000.[73,74]

From the adult barnacle *B. amphitrite* a protein with molecular weight 3000–5000 was isolated as a larval-settlement inducing pheromone.[90] This active pheromone was a substance released from the adult barnacle into the water, and the lowest effective concentration was 10^{-10} M. It was revealed that di- or tripeptides also induced larval settlement (Table 10). It seems to be a requirement that active dipeptides have a basic amino acid residue at the carboxyl terminal and the other amino acid is neutral or basic; other combinations of amino acid residues proved to be inactive. The most active peptides were L-His-L-Lys (basic–basic) and L-Leu-Gly-L-Arg (neutral–neutral–basic) and their lowest effective concentration was 2.0×10^{-10} M.

Table 10 Induction activity of dipeptides in larval settlement of the barnacle *B. amphitrite*.[89]

Peptides	*Concentrations at which larval settlement was induced* (M)
Leu–Arg (neutral–basic)	2.0×10^{-8}–2.0×10^{-10}
His–Lys (basic–basic)	2.0×10^{-8}–2.0×10^{-10}
Glu–Lys (acidic–basic)	inactive
Lys–Gly (basic–neutral)	inactive
Glu–Gly (acidic–neutral)	inactive
Gly–Ser (neutral–neutral)	inactive
Arg–Glu (basic–acidic)	inactive
Gly–Asp (neutral–acidic)	inactive
Glu–Glu (acidic–acidic)	inactive

Inducers of larval settlement for barnacles were well discriminated from those of larval metamorphosis. The proteins (''arthropodin'') or peptides described above corresponded to the former, while juvenile hormone (**87**) and its synthetic analogue ZR-512 (**88**) were reported as examples of the latter.[73,74,91] ZR-512 (**88**) caused larval metamorphosis of cypris of *B. galeatus* at concentrations as low as 10 ppb without settling to the surface, and was 1000 times as strongly active as compound (**87**), while another analogue ZR-515 (**89**) with methoxy and isopropyl ester groups was inactive. Compound (**88**), however, was ineffective when juvenile hormone (**87**) (0.5–10 ppb) was introduced to the larvae before addition of (**88**). Thus, many things remain to be studied for a fuller understanding of the mechanism and roles of chemical signals of larval settlement and metamorphosis.



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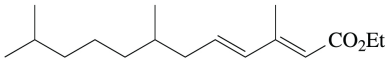

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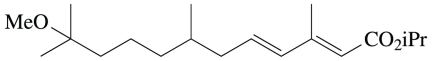
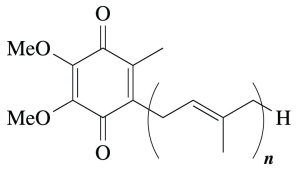
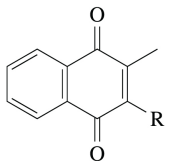


(89)

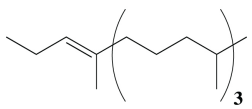
On the other hand, the search for settlement inhibitors has been extensive because of the serious problems caused by marine fouling invertebrates. To clarify the influence of marine bacteria on the settling of fouling invertebrate larvae, marine bacterial products that inhibit settling by cyprids of the barnacle *Balanus amphitrite* were screened. The culture broth of a bacterial strain belonging to *Alteromonas* sp., which was associated with the marine sponge *Halichondria okadai*, effectively inhibited settling of the cyprids.[92] The active substance was isolated from the extract of the mycelium and was identified as ubiquinone-8 (**90**). Compound (**90**) and its related compounds, ubiquinone-0 (**91**), ubiquinone-9 (**92**), ubiquinone-10 (**93**), vitamin K_1 (**94**), and vitamin K_3 (**95**) were examined for inhibitory activities and toxicities. As a result, compound (**91**), which had no polyprenyl group, showed higher inhibitory activity and higher toxicity than other polyprenylated ubiquinones, suggesting that the length of the polyprenyl side-chain was an important factor influencing both inhibitory activity and toxicity of ubiquinones. Similarly, vitamin K_3 (**95**) with no polyprenyl group was more active and more toxic than vitamin K_1 (**94**), which has a polyprenyl group. Bacterial films on solid substrates were reported to play important roles as repellents or attractants for the settlement of marine sessile organism larvae, as discussed in Section 8.07.5.1. As ubiquinones and related compounds are commonly found in bacteria, they may retard the settling of invertebrate larvae.



(90) $n = 8$
(91) $n = 0$
(92) $n = 9$
(93) $n = 10$



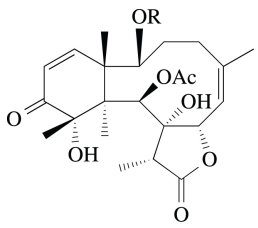
(94) R =



(95) R = H

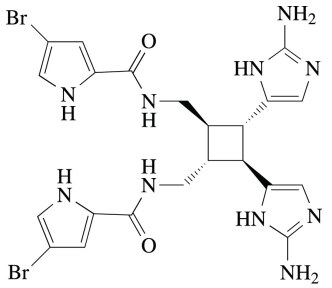
Studies on antifouling substances against barnacle larvae have been reported and some new active secondary metabolites were isolated from marine invertebrates such as the sea pansy, sponges, and nudibranchs. From extracts of the Atlantic sea pansy *Renilla reniformis*, three new briarein-type

diterpenes, renillafoulins A (**96**), B (**97**), and C (**98**) were isolated. All three compounds inhibited larval settlement of the barnacle *B. amphitrite* with EC_{50} (50% effective concentration) values ranging from 0.02 μg ml^{-1} to 0.2 μg ml^{-1}.[93]

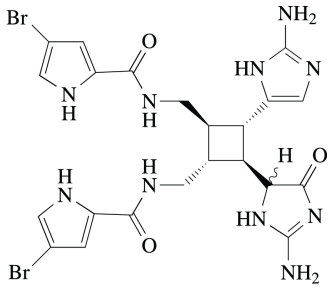


(**96**) R = $COCH_3$
(**97**) R = $COCH_2CH_3$
(**98**) R = $COCH_2CH_2CH_3$

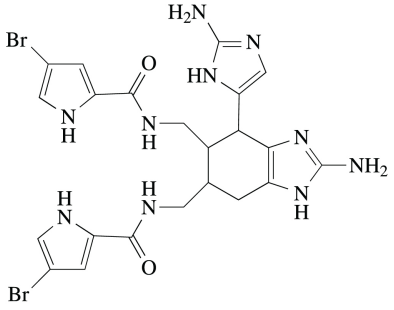
Dimeric bromopyrroles (**99**)–(**101**) isolated from the Caribbean sponge *Agelas conifera* were found to be active in barnacle settlement; the assay using *B. amphitrite* gave settlement-inhibition EC_{50} values of 44 μg ml^{-1} and 21 μg ml^{-1} for compounds (**99**) and (**101**), respectively, and settlement-facilitation EC_{50} values of 29 ng ml^{-1} and 17 ng ml^{-1} for compounds (**99**) and (**100**), respectively.[94]



(**99**)

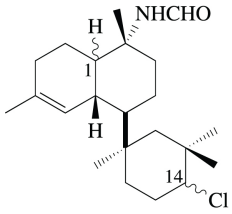


(**100**)

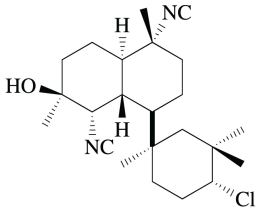


(**101**)

Antifouling substances from Japanese marine invertebrates have been investigated extensively. From the marine sponge *Acanthella carvernosa*, collected off Yakushima Island, 1000 km southwest of Tokyo, three new diterpene formamides, kalihinenes X (**102**), Y (**103**), and Z (**104**) along with known kalihinol A (**105**) and 10-formamidokalihinene (**106**) were isolated.[95] The new kalihinenes (**102**)–(**104**) inhibited attachment and metamorphosis of cyprid larvae of the barnacle *B. amphitrite* with EC_{50} values of 0.49, 0.45, and 1.1 μg ml^{-1}, respectively, while no toxicity was found at these concentrations. Kalihinol A (**105**) (EC_{50} 0.087 μg ml^{-1}) and 10-formamidikalihinene (**106**) (EC_{50} 0.095 μg ml^{-1}) also showed potent antifouling activity, more active than $CuSO_4$ (EC_{50} 0.15 μg ml^{-1}).



(102) 1β-H, 14α-Cl
(103) 1α-H, 14α-Cl
(104) 1β-H, 14β-Cl



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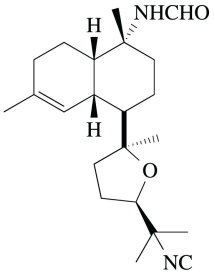


(106)

Antifoulants were also obtained from the sponge of the same species *Acanthella carvernosa*, collected off Hachijo-jima Island. The hexane-soluble fraction of both EtOH and MeOH extracts of this sponge were fractionated with the guidance of antifouling activity assay to afford 14 active terpenoids (**107**)–(**120**), seven of which ((**109**)–(**111**), (**111**), (**116**), (**118**)–(**120**)) were new natural products.[96] Most of them contained either isocyanate, isothiocyanate, isocyanide, or formamide groups. Antifouling activities of these terpenoids against cyprid larvae of the barnacle *B. amphitrite* are shown in Tables 11 and 12. Table 11 summarizes percentages of metamorphosed and dead larvae when the barnacle was exposed to terpenoids (**107**)–(**120**) at a concentration of 5 μg ml^{-1} for 48 h. Larval metamorphosis was completely inhibited by compounds (**115**)–(**120**). Table 12 shows percentages of inhibition at three concentrations for kalihinanes (**116**)–(**120**). Isocyanate and isothiocyanate derivatives (**119**) and (**120**) were highly antifouling (EC_{50} ca. 0.05 μg ml^{-1}).

Table 11 Percentages of metamorphosed and dead larvae of the barnacle *B. amphitrite* exposed to compounds (**107**)–(**120**) at a concentration of 5 μg ml^{-1} for 48 h.

Larvae (%)	(**107**)	(**108**)	(**109**)	(**110**)	(**111**)	(**112**)	(**113**)	(**114**)	(**115**)	(**116**)	(**117**)	(**118**)	(**119**)	(**120**)
Metamorphosed	8	37	29	38	4	8	33	42	0	0	0	0	0	0
Dead	0	13	0	0	0	0	0	0	0	0	0	0	0	0

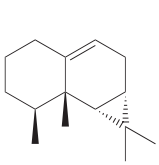
Table 12 Percentages of inhibition of larval settlement and metamorphosis of the barnacle *B. amphitrite*.

Concentration (μg ml^{-1})	(**116**)	(**117**)	(**118**)	(**119**)	(**120**)
5	100	100	100	100	100
0.5	69	61	49	44	67
0.05	24	24	17	50	42

Nudibranchs are well known to sequester secondary metabolites from their sponge diets to protect their soft bodies from predators, and their chemical defense could be used for development of antifouling strategy. In fact, antifouling substances that inhibited larval settlement and metamorphosis of the barnacle *B. amphitrite* were isolated from extracts of four nudibranchs of the family Phyllidiidae from five different collection sites (Table 13). The antifouling substances were

Table 13 Isolation yields and antifouling activity of sesquiterpenes (**121**)–(**130**) from nudibranchs of the family Phyllidiidae (mg/animal).

Species	*Collection site*	(**121**)	(**122**)	(**123**)	(**124**)	(**125**)	(**126**)	(**127**)	(**128**)	(**129**)	(**130**)
Phyllidia pustulosa	Yakushima	1.2	0.4	0.7							
Phyllidia pustulosa	Kuchinoerabu	0.1	0.4		0.2						
Phyllidia pustulosa	Tanegashima		0.3		0.4	0.8	1.6				
Phyllidia pustulosa	Kamikoshiki							13.0			
Phyllidia ocelata	Kamikoshiki								1.0		
Phyllidia varicosa	Shimokoshiki							2.1		2.5	
Phillidiopsis krempfi	Shimokoshiki							1.8	0.8		1.3
Antifouling activity IC_{50} (μg ml^{-1})		10	3.2	7.2	4.6	2.3	0.13	0.14	0.70	0.33	>50



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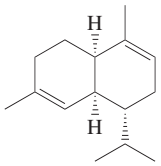

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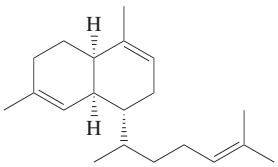

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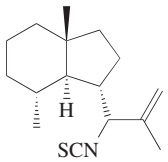

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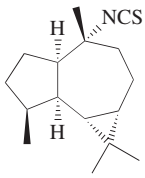

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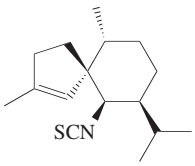

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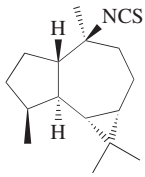

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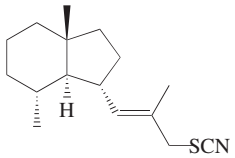

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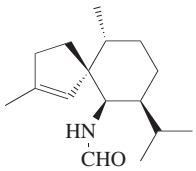

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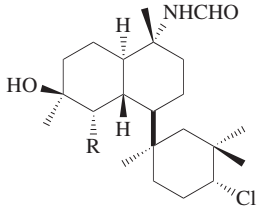

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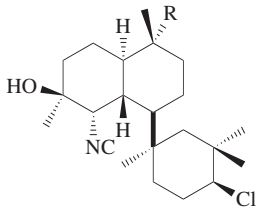
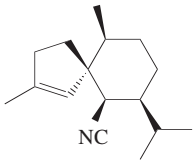
(119) R = NCO
(120) R = NCS



(117) R = NC
(118) R = NHCHO



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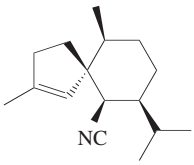

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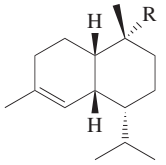
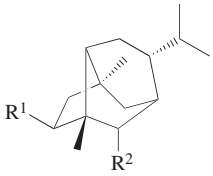
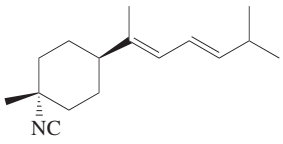


(123) R = NCS
(128) R = NC



(124) R^1 = SCN, R^2 = H
(125) R^1 = H, R^2 = SCN



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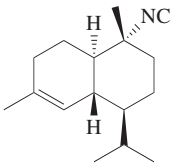

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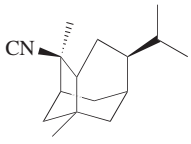

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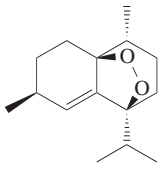


(130)

identified as three new sesquiterpene isocyanides (**121**), (**127**), and (**129**), a new sesquiterpene peroxide (**130**), and six known sesquiterpenes (**122**)–(**126**) and (**128**).[97] These sesquiterpenes had structural similarities to those isolated from the sponge *Acanthella carvernosa* described above. Table 13 shows the isolation yields and antifouling activities of these sesquiterpenes. Compounds (**126**) and (**127**), especially, showed potent antifouling activity with IC_{50} values of 0.13 μg ml^{-1} and

0.14 μg ml^{-1}, respectively, while no toxicity was found at these concentrations. Their activities were comparable to that of $CuSO_4$ (IC_{50} 0.15 μg ml^{-1}). It should be noted that (**130**) showed no activity at 50 μg ml^{-1}, though it contained a peroxide moiety.

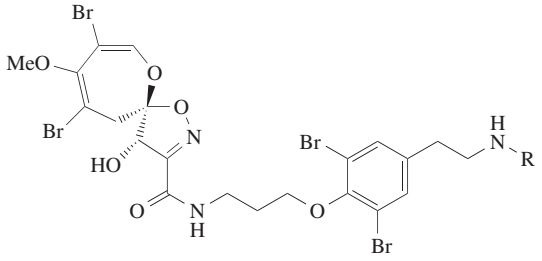
Different classes of natural products containing a bromopyrrole or bromotyramine moiety, isolated from marine sponges, were revealed to have antifouling activity. The MeOH extract of the sponge *Pseudoceratina purpurea* collected off Hachijo-jima Island, 300 km south of Tokyo, was found to be active against cyprids of the barnacle *B. amphitrite*. Bioassay-guided fractionation of the ether-soluble portion of the MeOH extract using silica gel chromatography, gel-filtration on Toyopearl HW-40, and reverse phase (C_{18}) HPLC afforded eight bromopyrrole or bromotyramine derivatives, ceratinamides A (**131**) and B (**132**), psammaplysins A (**133**) and E (**134**), ceratinamine (**135**), moloka'iamine (**136**), pseudoceratidine (**137**), and 4,5-dibromopyrrole-2-carbamide (**138**). Ceratinamides A (**131**) and B (**132**) were new bromotyrosine derivatives with a spiro[4,6]dioxazundecane skeleton.[98] Ceratinamine (**135**) was a new compound containing an unprecedented cyanoformamide functionality.[99] Pseudoceratidine (**137**) was also a new compound consisting of a spermidine moiety and two 4,5-dibromopyrrole-2-carbonyl units.[100] These compounds exhibited antifouling activity (settlement and metamorphosis inhibitory activity) against cyprid larvae of the barnacle *B. amphitrite* with ED_{50} values ranging from 0.10 μg ml^{-1} to 8.0 μg ml^{-1}, as summarized in Table 14. Ceratinamide A (**131**) and psammaplysin A (**133**) were particularly potent. Compounds (**132**), (**133**), (**135**), and (**137**) were lethal to the larvae at a concentration of 30 μg ml^{-1}, while others were not toxic at this concentration. Thus, (**131**) was considered to be a promising antifouling agent. It was interesting that psammaplysin A (**133**) and 4,5-dibromopyrrole-2-carbamide (**138**) induced larval metamorphosis of the ascidian *Halocynthia roretzi*; this subject is discussed further in Section 8.07.5.6. Psammaplysin E (**134**), ceratinamine (**135**), and moloka'iamine (**136**) also exhibited potent cytotoxicity against P388 murine leukemia cells, while psammaplysin A (**133**) and pseudoceratidine (**137**) were antibacterial against *Flavobacterium marinotypicum*. In addition, compounds (**131**)–(**138**) were inactive against *Alteromonus macleodii*, *Pseudomonas nautica*, *Vibrio alginolyticum*, *Bacillus marinus*, *Penicillium chrysogenum*, *Candida albicans*, and *Mortierella ramanniana* at 10 μg/disk, suggesting that antifouling activity was not associated with antibacterial or antifungal activity.

Table 14 Biological activities of compounds (**131**)–(**138**).

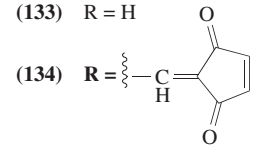
Compounds	*Metamorphosis inducing activity on acidian H. roretzi* ED_{100} (μg ml^{-1})	*Antifouling activity against barnacle B. amphitrite* ED_{50} (μg ml^{-1})	*Antibacterial activity against F. marinotypicum* (mm, 10 μg/disc)	*Cytotoxic activity against P388 cell* IC_{50} (μg ml^{-1})
(**131**)		0.10		> 10
(**132**)		2.4		> 10
(**133**)	1.2	0.27	10	> 10
(**134**)		4.8		2.1
(**135**)		5.0		3.4
(**136**)		4.3		2.1
(**137**)		8.0	15	> 10
(**138**)	25	> 30		> 10

8.07.5.6 Tunicates

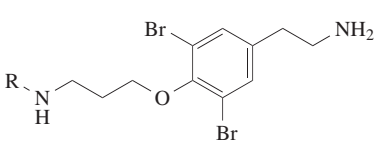
Inducers or promoters of larval settlement and metamorphosis of tunicates have also been investigated. As promoters of settlement and metamorphosis of the tunicate *Ciona savignyi* larvae, two pteridine-containing bromophysostigmine alkaloids, urochordamines A (**139**) and B (**140**) were isolated from the tunic (outer body) of the adult tunicate *C. savignyi*, collected off Asamushi, 600 km northeast of Tokyo, in June 1992.[101] They were stereoisomers at the C-9 position and urochordamine A (**139**) promoted larval settlement and metamorphosis in *C. savignyi* at a concentration of 2 ng ml^{-1}; all larvae treated with (**139**) completed settlement and metamorphosis by the time 50% of the larvae in a control group had settled. Urochordamine B (**140**), however, had no activity at the same concentration, suggesting the importance of the stereochemistry of C-9. It



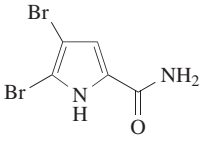
(131) R = CHO
(132) R = $CO(CH_2)_{11}CHMe_2$
(133) R = H



(134)



(135) R = COCN
(136) R = H



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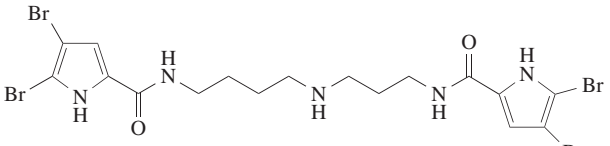
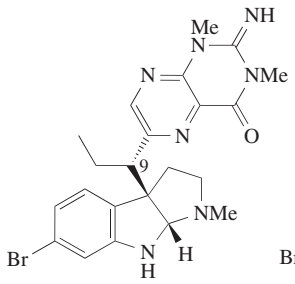


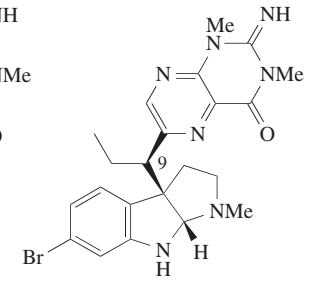
(137)

should be noted that compounds (**139**) and (**140**) were not detected in *C. savignyi* collected at the same place in October. Therefore, compounds (**139**) and (**140**) may be produced only during the spawning season. Moreover, the colonial tunicate *Botrylloides* sp. collected in the Gulf of Sagami in June contained both compounds (**139**) and (**140**). It was found, however, that compounds (**139**) and (**140**) were not obtained from the tunic of *C. savignyi*, collected in 1993 or later, thus raising doubts that compounds (**139**) and (**140**) were produced by the tunicates.[76] Urochordamines A (**139**) and B (**140**) were converted into more polar compounds, urochordamines A′ (**141**) and B′ (**142**), respectively, when left standing in protic solvents.[102] Structure–activity relationships of urochordamines were tested on the promoting activity of larval metamorphosis of *H. rorentzi*. The percentage of metamorphosed larvae at a concentration of 25 μg ml^{-1} were similar whether induced by (**139**) or (**141**). The promoting activity of (**140**) was lower than that of (**139**) or (**141**). Interestingly, (**142**) showed no promoting activity at a concentration of 25 μg ml^{-1}. Compound (**141**) induced larval metamorphosis even at a concentration of 0.25 μg ml^{-1}, while (**139**) was ineffective at a concentration of 2.5 μg ml^{-1}. Therefore, the order of promoting activity was (**141**) > (**139**) > (**140**) > (**142**), indicating that both the structure of the pteridine moiety and the stereochemistry at C-9 were important for larval metamorphosis-promoting activity. The effect of exposure time on metamorphosis was also examined. About 20% of 6 h old larvae metamorphosed when exposed to 25 μg ml^{-1} of (**141**) for 15 min, which was a much lower proportion than at 30 min and 60 min exposures. However, exposure to 25 μg ml^{-1} of (**139**) for 60 min was necessary to induce metamorphosis, while 30 min exposure was ineffective. These results may indicate that urochordamines promote larval metamorphosis in ascidians without a chemoreception pathway. In addition, urochordamines induced metamorphosis of pediveliger larvae of the mussel *Mytilus edulis galloprovincialis*: 87%, 73%, 26%, and 50% of larvae metamorphosed in 6 days after treatment with 2.5 μg ml^{-1} of (**139**)–(**142**), respectively. Urochordamines are the first compounds to be identified that induce metamorphosis of pediveliger larvae, implying again that urochordamines do not act via chemoreception, but via an internal mechanism.

Promoters of larval metamorphosis in two kinds of ascidians, *H. roretzi* and *C. savignyi*, were also isolated from the brown alga *Sargassum thunbergii*, and they were identified as diphlorethol (**143**), a mixture of phlorotannins, and sulfoquinovosyl diacylglycerols (SQDG, (**144**) = (**9**)) composed of $C_{16:0}$ (82%) and $C_{18:1}$ (18%) as analyzed by GC.[103] These compounds induced larval metamorphosis of both ascidians at a concentration of 25 μg ml^{-1} and phlorotannin such as (**143**) appeared to act faster than SQDG (**144**). A large number of phlorotannins from brown algae are



(139)



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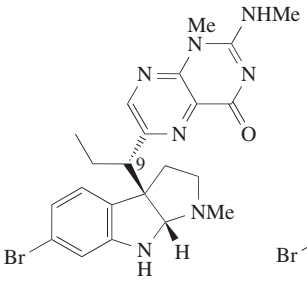
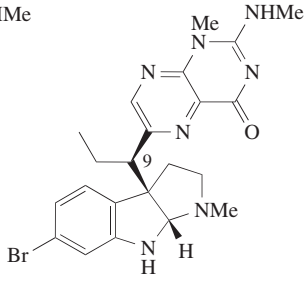
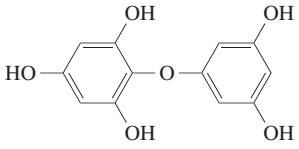


(141)

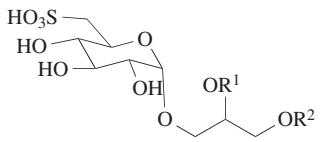


(142)

known to be antibiotics, and SQDG (**144**) is a common lipid of chloroplast membrane, and has been known to exhibit various biological activities such as a feeding-stimulating activity for herbivorous gastropods (Section 8.07.2.2). Furthermore, the fact that δ-tocotrienol epoxides (**68**) and (**69**) isolated from the brown alga *S. tortile* were the promoters of larval settlement/metamorphosis in the hydroid *Coryne uchidai* (Section 8.07.5.2) together with the results above led to the idea that brown alga may play important roles in the larval settlement/metamorphosis of marine animals.



(143)

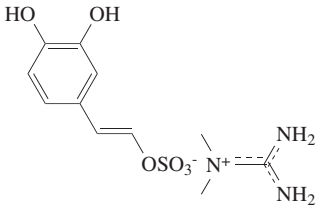


(144)

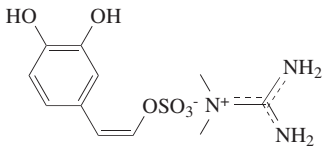
(R^1, R^2: $C_{16:0}$, 82% and $C_{18:1}$, 18%)

N,*N*-Dimethylguanidinium styryl sulfates, (*E*)- and (*Z*)-narains (**145**) and (**146**),[104] 3,4-dihydroxylstyrene dimers (**147**)–(**149**), trimethoxystyrene derivative (**150**), and a hypoxanthin base (**151**) were isolated from the MeOH extract of a marine sponge *Jaspis* sp. as metamorphosis-inducers of ascidian *Halocynthia roretzi* larvae.[105] The EtOH and acetone extracts afforded a triethoxy derivative (**152**) and a naphthyl derivative (**153**), respectively, instead of (**150**). (*Z*)-Narain (**146**), as well as its anion alone, induced larval metamorphosis on *H. roretzi* at a concentration of 5 μM, while *N*,*N*-dimethylguanidine sulfate was inactive at a concentration of 50 μM. (*E*)-Narain (**145**), its anion, (**147**), (**148**), and (**150**) were active at a concentration of 5 μM; (**149**) and (**151**) were much less active, whereas (**153**) did not show any effect. Therefore, the anion moiety, especially the sulfate group, may play an important role as the metamorphosis-inducer. The stereochemistry of the double bond was also important for activity. 4-Carboxy-1,3-dimethylimidazolium ion (**154**), which may be biogenetically related to (**151**), was isolated from the marine sponge *Cacospongia scalaris* as a metamorphosis-inducing compound, showed the same degree of activity as (**151**).

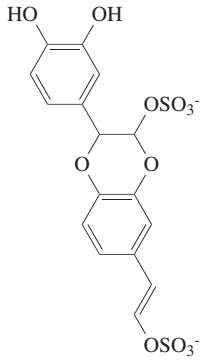
Two new pipecolate derivatives, anthosamines A (**155**) and B (**156**), were isolated from the marine sponge *Anthosigmella* aff. *raromicrosclera* along with a new diketopiperazine (**157**), *cyclo*-(L-Arg-



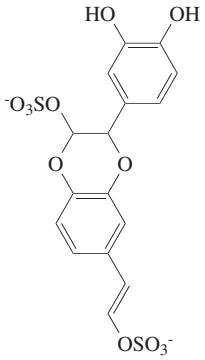
(145)



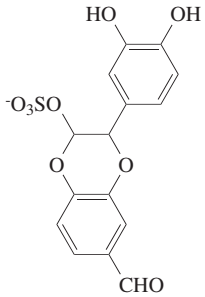
(146)



(147)



(148)



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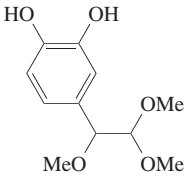
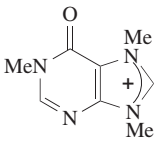


(150)



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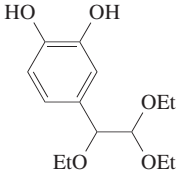

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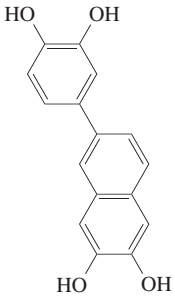
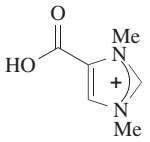
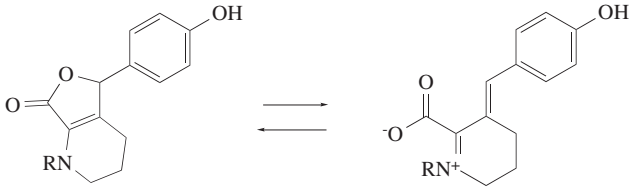


(153)



(154)

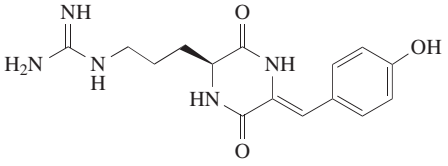
dehydrotyrosine) as metamorphosis-inducers of ascidian *H. roretzi* larvae. Reversible isomerization between amine and iminium ion forms was observed for anthosamines A (**155**) and B (**156**); iminium ion forms (**155b**) and (**156b**) predominate in protic solvent, and amine forms (**155a**) and (**156a**) in aprotic solvent (Equation (3)). Anthosamines A (**155**) and B (**156**) induced larval metamorphosis on the ascidian *H. roretzi* at a concentration of 50 μM completely, while the diketopiperazine (**157**) (50 μM) induced metamorphosis of 50% of larvae. From the Japanese marine sponge *Stelletta* sp., a new bisguanidinium alkaloid, stellettadine A (**158**), containing a norsesquiterpene unit was isolated as a metamorphosis-inducing compound. The absolute configuration of C-6′ was determined as *S* by treatment of (**158**) with $NaIO_4$ in the presence of $RuCl_3$ to afford (*S*)-2-methylglutaric acid. Stelettadine A (**158**) showed metamorphosis-inducing activity with an ED_{100} (100% effective dose) value of 50 μM on ascidian *H. roretzi* larvae.



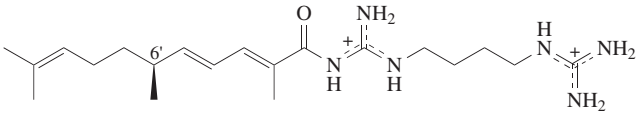
(3)

(155a) R = H
(156a) R = Me

(155b) R = H
(156b) R = Me



(157)



(158)

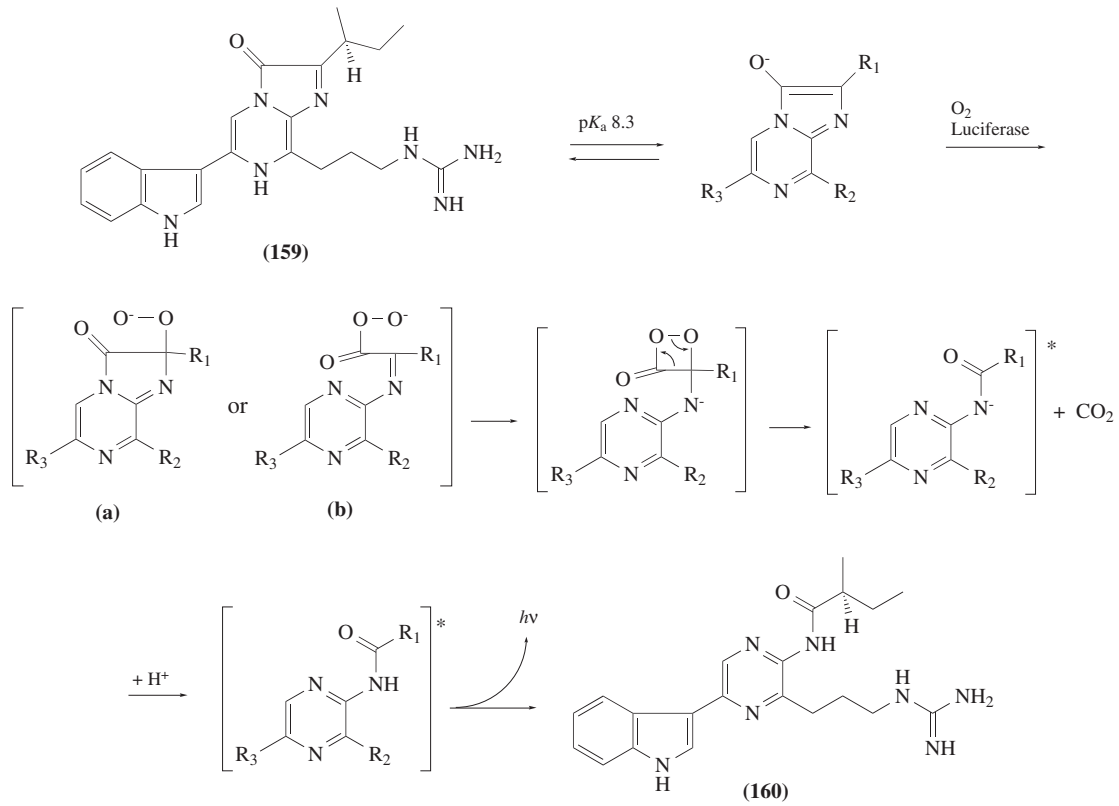
8.07.6 BIOLUMINESCENCE

Bioluminescence of marine organisms is a widely recognized phenomenon, and bioluminescent animals found in the sea are widely distributed over almost all phyla of the animal kingdom from protozoans to vertebrates.[106] Among several bioluminescence mechanisms that have been reported, the luciferin–luciferase (L-L) reaction is probably the most well-known luminous system. In this system, light is emitted as a result of a reaction of a heat-stable substrate (general name: luciferin) with oxygen catalyzed by an enzyme (general name: luciferase), which was initially discovered in the bioluminescence of the luminous beetle *Pyrophorus noctiluca* in 1885. Other luminous systems involve the participation of a luminescent protein (general name: photoprotein) or symbiosis with luminous bacteria.[106]

8.07.6.1 Sea Firefly

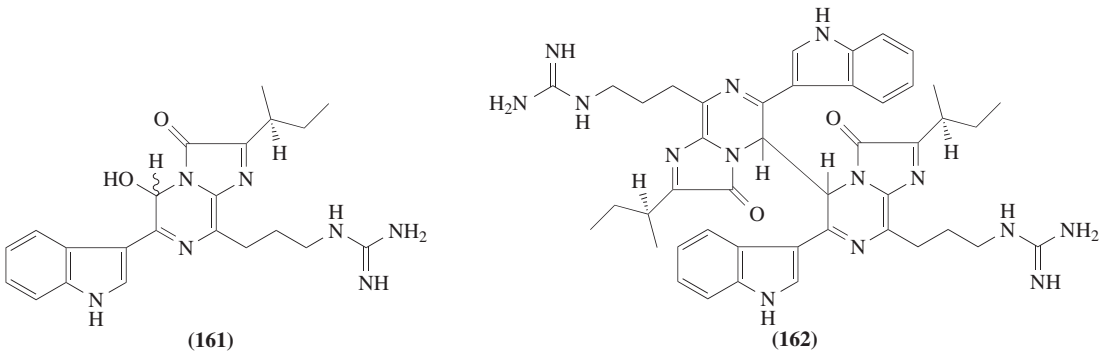
Among many bioluminescent marine organisms, the ostracod crustacean *Vargula hilgendorfii* (formerly *Crypridina hilgendorfii*) has played a central role in contributing to an understanding of the chemical and physical bases of bioluminescence. *V. hilgendorfii* is commonly referred to as "umi botaru" in Japan or the "sea firefly." Until the 1960s this animal occurred in great abundance along the south coastal waters of Japan, but their numbers have since been reduced significantly. *V. hilgendorfii* is a small animal (ca. 3 mm long) which lives in sand at the bottom of shallow waters and becomes an active feeder at night. It emits a strongly luminescent secretion into the seawater when it is disturbed. The secretion consists of luciferin and luciferase, thus producing a bright luminous cloud. The light results from the oxidation of luciferin by molecular oxygen, catalyzed by luciferase, and the mechanism of the reaction has been studied extensively. The structure of the *Vargula* luciferin was determined as an imidazopyrazine compound (**159**) in the 1960s,[106] and the products of the reaction were known to be oxyluciferin (**160**) and carbon dioxide. The mechanism of the *Vargula* bioluminescence can be summarized as shown in Scheme 4.[106] The *Vargula* luciferin (**159**) dissociates to its anion (pK_a 8.3 in water), which is then oxidized by molecular oxygen to give a hydroperoxide anion. The hydroperoxide anion is postulated to be hydroperoxide form (a) or peracid form (b). The anion decomposes through a dioxetane intermediate to form oxyluciferin anion in a singlet excited state. In neutral solutions protonation occurs on the excited anion to give the excited neutral oxyluciferin molecule, which subsequently emits light. This bioluminescence system via decomposition of the dioxetanone intermediate is an example of the CIEEL mechanism[107,108] and the unstable dioxetanone intermediate has not been isolated.[109]

Oxidation of *Vargula* luciferin (**159**) in the presence of luciferase with light production gives oxyluciferin (**160**) which cannot be reduced (Scheme 4). On the other hand, luciferin (**159**) is very susceptible to oxygen even in the absence of the enzyme to form "reversibly oxidized luciferin."[106] Thus, the orange-colored aqueous solution of (**159**) turns red in air, and then becomes colorless owing to further autooxidation. The red substance is also formed by chemical oxidation of (**159**) with ferricyanide, lead dioxide, or diphenylpicrylhydrazyl radical (DPPH). It emits light only very slowly in the presence of the enzyme, but the luminescent activity similar to that of the luciferin (**159**) can be restored by chemical reduction with sodium hydrosulfite ($Na_2S_2O_4$). Oxidation of (**159**) with lead dioxide or DPPH affords a product without light production. This product can be reduced to luciferin (**159**) with sodium hydrosulfite or sodium borohydride ($NaBH_4$). Thus it can be recognized as a reversibly oxidized luciferin. The product, named luciferinol, was isolated and its structure was determined to be (**161**), although it is fairly unstable and difficult to handle.[110] Luciferinol (**161**) is a two-electron oxidation product and has no bioluminescent activity with luciferase. By further HPLC analysis, another oxidation product besides (**161**) was found, particularly when ferricyanide was used as the oxidant. Although it was extremely labile, it was named



Scheme 4

biluciferyl and its structure was determined as (**162**).[111] It was suggested that a one-electron oxidation of (**159**) gives the luciferyl radical, which dimerizes to its dimer (**162**). Biluciferyl (**162**) was oxidized with DPPH into luciferinol (**161**) and reduced with sodium hydrosulfite or acid into luciferin (**159**). Whereas luciferinol (**161**) produces no light with *Vargula* luciferase, very slow bioluminescence was observed with biluciferyl (**162**) in the presence of the enzyme, indicating that biluciferyl (**162**) is an intermediate in the oxidation of (**159**) into (**161**) and that the slow luminescent reaction observed in the reversibly oxidized luciferin could be due to biluciferyl (**162**).



The cloning and nucleotide sequence analysis of the cDNA for the *Vargula* luciferase and the expression of the cDNA in a mammalian cell system has been reported.[112] The *Vargula* luciferase was purified to homogeneity by using tryptamine and *p*-aminobenzamidine affinity column chromatography. Digestion of the luciferase with endopeptides afforded several peptides, and a portion of one peptide fragment was used to design the complementary oligodeoxynucleotide probe. The

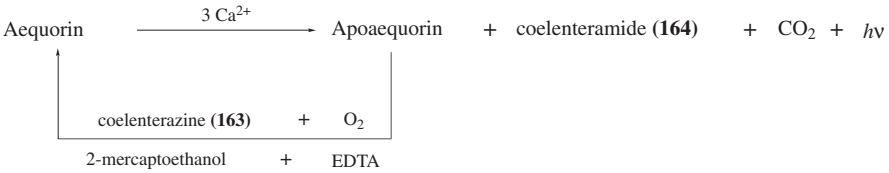
mRNA was extracted from the *V. hilgendorfii* ground to find powder in liquid nitrogen. The cDNA library was constructed and the nucleotide sequence suggested that the primary structure of the *Vargula* luciferase consisted of 555 amino acid residues in a single polypeptide chain with a calculated molecular weight of 62 171. To establish that the cloned cDNA actually encoded *Vargula* luciferase, a mammalian cell system (monkey COS cells) was used to express cDNA. As a result, luciferase activity was clearly detected in the culture medium of transfected COS cells with some luciferase activity also present in the cell extract.

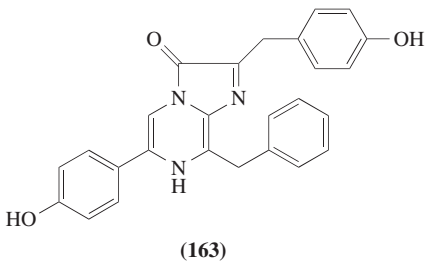
The enzyme, *Vargula* luciferase, showed significant amino acid sequence homologies in two regions with an N-terminal segment of aequorin, a calcium-binding photoprotein which emits light in the bioluminescent reaction of the jellyfish *Aequorea victoria* (Section 8.07.6.2). In spite of the close similarities in substrate structure and mechanisms of the bioluminescent systems in *Vargula* and *Aequorea*, the enzymes and substrates of the two systems showed only slight light-emitting cross-reactions.[112]

8.07.6.2 Jellyfish

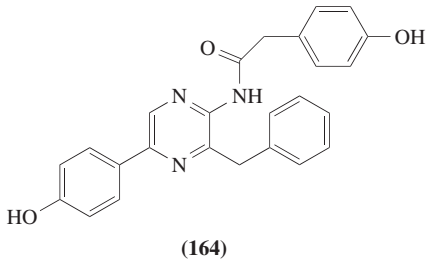
Many bioluminescent organisms are included in Coelenterata (phylum: Cnidaria). The hydrozoan *Aequorea aequorea* (jellyfish) possesses a photoprotein, aequorin, which emits light by the action of Ca^{2+} without molecular oxygen. On the other hand, the anthozoan *Renilla reniformis* (sea pansy) has a luciferin–luciferase reaction system and requires molecular oxygen for light production.[106]

The photoprotein, aequorin (molecular weight 21 400), isolated from the outer margin of the umbrella of the jellyfish *Aequorea victoria* from Friday Harbor, WA, USA, emits blue light in aqueous solution when Ca^{2+} or Sr^{2+} is added in either the presence or absence of molecular oxygen.[113,114] Aequorin consists of two components: an apoprotein (apopaequorin) and a chromophore. The chromophore is made up of coelenterazine (an imidazopyrazine compound (**163**)) and molecular oxygen. The chromophore is attached noncovalently to apoaequorin. The binding of Ca^{2+} to aequorin presumably causes the protein to change its conformation, converting it into an oxygenase, which then catalyzes the oxidation of coelenterazine (**163**) by the bound oxygen, yielding as products light (λ_{max} 470 nm), CO_2, and a blue fluorescent protein (BFP). The BFP is composed of apoaequorin and the oxidized product of (**163**), coelenteramide (**164**). The excited-state coelenteramide (**164**) is the emitter in the reaction. Aequorin may be regenerated from apoaequorin by incubation with coelenterazine (**163**), dissolved oxygen, EDTA, and 2-thioethanol (Scheme 5).





(163)



(164)

Scheme 5

A cDNA library of *Aequorea* was constructed and clones carrying the cDNA for the Ca^{2+}-dependent photoprotein were isolated by the method of colony hybridization using synthetic oligonucleotide probes. The primary structure of the apoprotein (apoaequorin) deduced from the

nucleotide sequence showed that the protein was composed of 189 amino acid residues.[115–117] Intracellular high-affinity Ca^{2+}-binding proteins such as calmodulin, parvalbumin, troponin C, and myosin light chains belong to a superfamily of proteins known a EF-hand proteins. These proteins have either two or four Ca^{2+}-binding regions which show sequence homology with the well-defined helix–loop–helix structures denoted as EF hands in the X-ray structure of carp parvalbumin. The Ca^{2+} dependency of aequorin luminescence indicates the presence of three high-affinity Ca^{2+} sites, making aequorin a candidate for membership in the EF-hand superfamily. Comparison of the amino acid sequence with those of the Ca^{2+}-binding proteins calmodulin and troponin C revealed that three segments of aequorin had properties expected for EF-hand domains. The EF-hand regions had relatively high hydrophilicity, suggesting that the regions are located near the surface of the protein, while the sequence also suggested that the protein had three hydrophobic regions at which the protein may interact with its functional chromophore coelenterazine (**163**).

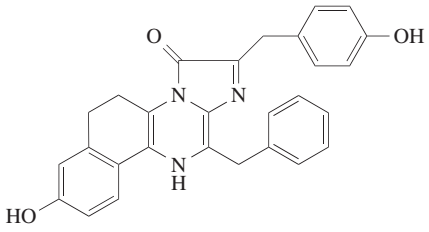
Various expression plasmids for apoaequorin cDNA were constructed and expressed in *Escherichia coli*. Aequorin was regenerated from the expressed apoaequorin by incubating with coelenterazine, 2-mercaptoethanol, and EDTA.[118] Thus, provided coelenterazine is available, the recombinant DNA technique may outweigh the enormous task associated with collecting and processing large numbers of *Aequorea* and purifying the highly unstable aequorin; it was estimated that 5300 specimens of *Aequorea* were required to collect 70–100 mg of aequorin. The initial procedure of the recombinant DNA method for obtaining a highly active preparation of aequorin[118] was, however, time-consuming and the yield was relatively low. These objections were overcome by an improved procedure which consisted of fusing the apoaequorin cDNA to the signal peptide coding sequence of the outer membrane protein A (*ompA*) of *Escherichia coli*.[119] Subsequent expression of the cDNA in *E. coli* results in high-level production of apoaequorin and the release of the protein into the culture medium. The purification of the protein was then easily achieved by acid precipitation and DEAE-cellulose chromatography. The procedure yielded 7.4 mg of recombinant apoaequorin with a purity greater than 95% from 200 ml of culture medium and, on regeneration with coelenterazine, the recombinant aequorin was fully active with Ca^{2+}.

To understand the mechanism of the bioluminescence reaction, a structure–function relationship was studied with respect to modifying certain of its amino acid residues by carrying out oligonucleotide-directed site-specific mutagenesis of apoaequorin cDNA and expressing the mutagenized cDNA in *Escherichia coli*. Amino acid substitution was made at the three Ca^{2+}-binding sites, the three cysteines, and a histidine in one of the hydrophobic regions. Subsequent assay of the modified aequorin showed that the Ca^{2+}-binding sites, the cysteines, and probably the histidine all play a role in the bioluminescence reaction of aequorin.[120] Further study of the modified aequorin with the three cysteine residues replaced by serine revealed that six of seven modified aequorins had reduced luminescence, whereas the seventh with all three cysteines replaced by serine had luminescence activity equal to or greater than that of the wild-type aequorin. The time required for the regeneration of the triply substituted aequorin was substantially increased compared with the time required for the regeneration of the wild-type aequorin. The results suggested that cysteine plays an important role in the regeneration of aequorin but not in its catalytic activity.[121]

The photoprotein aequorin purified by the standard isolation procedure was not homogeneous and consisted of a mixture of closely related photoproteins, i.e., isoaequorins, which have isoelectric points ranging from pH 4.2 to 4.9. Aequorin samples, extracted from 50 000 specimens of *Aequorea*, were separated with sufficient resolution into various molecular forms of aequorin (isoaequorins) by high-performance liquid chromatography on a TSK DEAE-5PW anion-exchange column by using buffers containing sodium acetate to obtain eight different kinds of the photoprotein, i.e., aequorins A–H. The M_r values ranged from 20 100 (aequorin F) to 22 800 (aequorin A), the luminescence activities ranged from 4.35×10^{15} photons mg^{-1} (aequorin A) to 5.16×10^{15} photons mg^{-1} (aequorin F), and the first-order reaction rate constants of luminescence ranged from 0.95 s^{-1} (aequorin A) to 1.33 s^{-1} (aequorin F). As regards sensitivity to Ca^{2+}, aequorin D was the most sensitive, having a sensitivity about 0.4–0.5 pCa units above that of the least sensitive kind (aequorin A).[122]

The light-emitting reaction of aequorin was selectively triggered by Ca^{2+}. Because of its high sensitivity to Ca^{2+}, aequorin is widely used as a Ca^{2+} indicator in various biological systems. To provide useful semisynthetic Ca^{2+}-sensitive photoproteins, chemical modification of the functional part of aequorin was studied by replacing the coelenterazine moiety in the protein with several synthetic coelenterazine analogues. One of the semisynthetic photoproteins, derived from coelenterazine analogue (**165**) with an extra ethano group, showed highly promising properties for the measurement of Ca^{2+} for two reasons: (i) the rise time of luminescence in response to Ca^{2+} was shortened approximately fourfold compared with native aequorin and (ii) the luminescence spectrum

showed two peaks at 405 nm and 465 nm and the ratio of the peak heights was dependent on Ca^{2+} concentration in the range of pCa 5–7, allowing the determination of Ca^{2+} concentration directly from the ratio of two peak intensities (I_{400}/I_{465}). This photoprotein containing the analogue (**165**) was designated *e*-aequorin. The coelenterazine analogue (**166**) with an amino group instead of a hydroxyl group was also incorporated into apoaequorin, yielding a Ca^{2+}-sensitive photoprotein, which indicated that an electrostatic interaction between the phenolate group in the coelenterazine moiety and some cationic center in apoaequorin is not important in native aequorin.[123] Furthermore, 37 coelenterazine analogues were synthesized and incorporated into apoaequorin, yielding 30 semi-synthetic aequorins that had the capacity to emit a significant amount of light in the presence of Ca^{2+} (over 10% of that of natural aequorin). Among them, the photoprotein derived from coelenterazine analogue (**167**) showed by far the highest level of the relative intensity of Ca^{2+}-triggered luminescence of 190 when compared with natural aequorin (relative intensity 1.0). However, since the yield of the incorporation reaction of this photoprotein was low, the overall property of this photoprotein appeared to be not favorable. On the other hand, the photoproteins of *e*-aequorin-type, prepared from the coelenterazine analogues (**168**) and (**169**) containing an ethano bridge, showed fast luminescence reactions, high relative luminescence intensities, and, like *e*-aequorin, the dependency of the spectral distribution of luminescence on the concentration of Ca^{2+}. With the two photoproteins derived from (**168**) and (**169**), the degree of dependence of the luminescence intensity ratio I_{400}/I_{465} on pCa was greater than that with *e*-aequorin, suggesting that these two photoproteins were possibly superior to *e*-aequorin in measuring Ca^{2+} concentration by the ratio method.[124]



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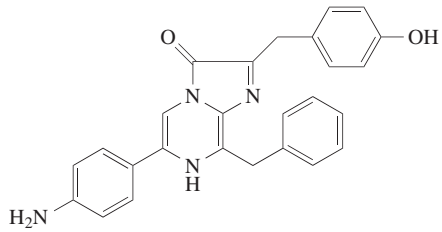

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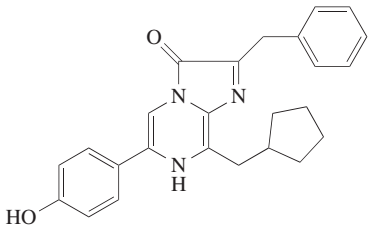

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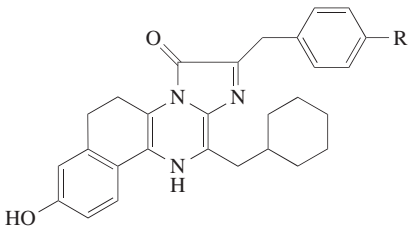
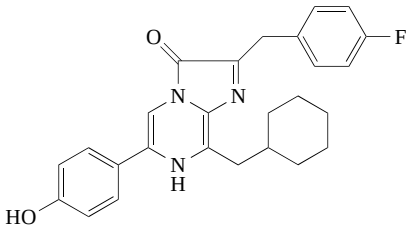


(168) R = OH
(169) R = H

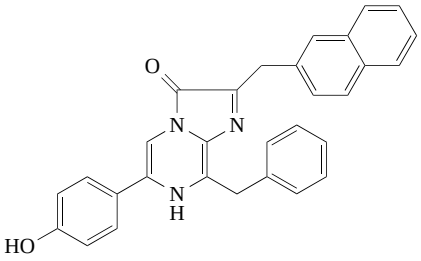
It was suggested that the conformational rigidity of the *p*-hydroxyphenyl group in coelenterazine analogues like (**165**) had some enhancement effects on the light yield, while decreasing light yield was observed for coelenterazine analogues with an alcoholic side-chain due to hydrogen bond formation with one of the nitrogen atoms in the emitter.[125]

The comparison of properties of a recombinant aequorin with those of natural aequorin was studied.[126] Recombinant apoaequorin was obtained by fusing the apoaequorin cDNA to the signal-peptide coding sequence of the outer-membrane Protein A of *Escherichia coli*, subsequent expression of the cDNA in *E. coli*, and purification of the recombinant apoaequorin released into the culture medium by acid precipitation and DEAE-cellular chromatography.[119] In chromatographic behavior the recombinant apoaequorin did not match any of 10 isoaequorins tested, although it was very similar to aequorin J; aequorins I and J[125] were additional isoaequorins isolated subsequent to the initial purification of eight isoaequorins, aequorins A–H.[122] The Ca^{2+}-sensitivity of recombinant aequorin was higher than that of any isoaequorin except aequorin D. The recombinant aequorin exhibited no toxicity when tested in various kinds of cells, even where samples of natural aequorin had been found to be toxic. Properties of four recombinant semisynthetic aequorins (designated

fch-, *hcp*-, *e*-, and *n*-aequorin, respectively), prepared from the recombinant apoaequorin and synthetic analogues of coelenterazine ((**170**), (**167**), (**165**), and (**171**), respectively), were approximately parallel with those of corresponding semisynthetic aequorins prepared from natural apoaequorin as shown in Table 15. Both recombinant *e*-aequorin and natural *e*-aequorin J luminesced with high values of the luminescence intensity ratio I_{400}/I_{465}, although the ratios were not pCa-dependent.



(170)



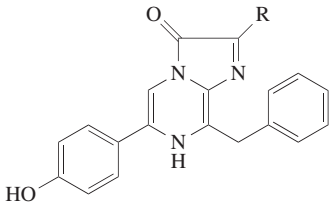
(171)

Table 15 Properties of semisynthetic aequorins derived from recombinant aequorin and natural aequorin J.

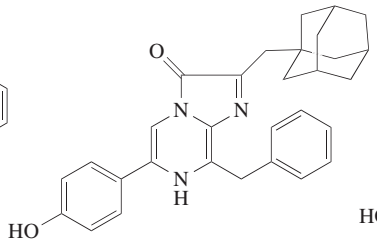
Semisynthetic aequorin	*Coelenterazine part*	*Light-emitting capacity* (10^{12} quanta μg^{-1})	*Light intensity* (10^8 quanta μg^{-1})		
			pCa 7	pCa 6	*Without added* Ca^{2+}
Recombinant aequorin (control)	(**163**)	4.80	2.32	240.0	0.04
Recombinant *fch*-aequorin	(**170**)	3.05	210.0		0.5
Recombinant *hcp*-aequorin	(**167**)	2.23	445.0		0.7
Recombinant *n*-aequorin	(**171**)	1.34	0.60	3.8	0.01
Recombinant *e*-aequorin	(**165**)	2.23	4.1		0.2
Aequorin J (control)	(**163**)	4.50	0.56	50.0	0.03
fch-Aequorin J	(**170**)	3.52	52.0		0.15
hcp-Aequorin J	(**167**)	2.88	120.0		0.24
n-Aequorin J	(**171**)	1.21	0.10	0.6	0.01
e-Aequorin J	(**165**)	2.08	2.1		0.16

A group of semisynthetic *e*-type aequorins showed bimodal luminescence, with peaks at 400–405 nm and 440–475 nm, while all other aequorins luminesced with only one peak in the range 440–475 nm. The cause of the spectral variation was studied by various experiments, and it was suggested that the spectrum of Ca^{2+}-triggered luminescence is strongly affected by the ionic charge on the amide N atom of the coelenteramide that is bound to apoaequorin. When the amine N atom is negatively charged, light is emitted with a 440–475 nm peak. In the case of *e*-type aequorins, the negative charge on the amide N atom is less, resulting in the emission of a 400–405 nm peak from the unchanged form of coelenteramide; the intensity ratio of 400–405 nm peak to 440–475 nm peak is determined by the amount of negative charge resting on the amide N atom of *e*-coelenteramide at the time of light emission. Most of the spectral variations in luminescence and fluorescence can be explained on the basis of ionic and hydrophobic interaction between a coelenteramide and apoaequorin.[127]

Bioluminescence activities of semisynthetic aequorins containing coelenterazine analogues with different substituents at the C-2 position of the imidazopyrazinone ring coelenterazine ((**163**) and (**172**)–(**176**)) were studied and it was evident that a hydroxybenzyl group at the C-2 position was essential for efficient luminescence activity; the photoprotein containing coelenterazine analogues (**173**)–(**176**) gave lower bioluminescence activity than aequorin containing either coelenterazine (**163**) or its close structure analogue (**172**).[128] Coelenterazine analogues possessing either the 2- or 8-adamantylmethyl group (**177**) and (**178**) were also prepared and it was found that the bioluminescence intensity of semisynthetic aequorin and the modified aequorin with three cysteine residues replaced by serine containing an 8-adamantylmethyl coelenterazine analogue (**178**) was stronger than that of natural coelenterazine.[129]



(172) R = CH_2Ph
(173) R = Me
(174) R = Ph
(175) R = CH_2CH_2Ph
(176) R = $CH_2CH_2CH_2Ph$



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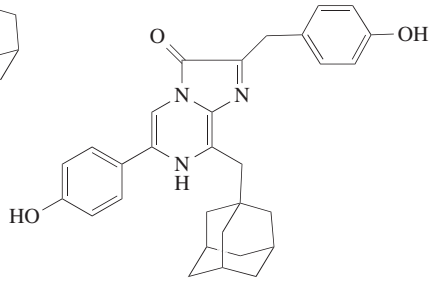
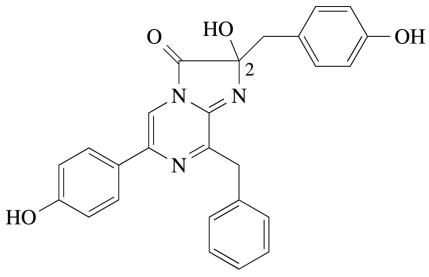


(178)

Shimomura and Johnson[130] reported that reduction of aequorin with $NaHSO_3$ afforded a yellow compound, assigned as the 2-hydroxyl derivative (**179**). Based on this result, Shimomura and Johnson suggested the chromophore coelenterazine as the light-emitting species linking to the protein through a peroxidic bond, as illustrated in Figure 2(a), while Cormier and co-workers[131] proposed the noncovalently bound structure for aequorin (Figure 2(b)).

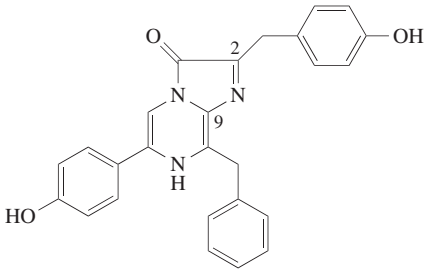


(179)

Figure 2 The functional part structure of aequorin proposed by (a) Shimomura and Johnson and (b) Cormier and co-workers.

Based on the ^{13}C NMR spectra of ^{13}C-enriched aequorins, the proposal represented in Figure 2(a) was supported by Kishi and co-workers.[132] Three ^{13}C-enriched coelenterazines (**180a–c**) were prepared and then incorporated into apoaequorin to yield three ^{13}C-enriched aequorins. During the incubation of coelenterazine (**180a**) into apoaequorin to form aequorin, the ^{13}C NMR showed that the C-2 carbon atom underwent an upfield shift (from 129 ppm to 98 ppm) due to a hybridizational change from sp^2 to sp^3. The chemical shift of -CO*C*H(NH-)R usually occurs in the range δ_C 40–60 and that of -CO*C*(OR^1)(NH-)R in the range 80–100 ppm. The observed chemical shift (98 ppm) for C-2 supported the partial structure (a). On the other hand, the chemical shift of the C-9 carbon of

coelenterazine was shifted downfield during incubation with apoaequorin (from 130 ppm to 148 ppm). An experiment in the presence of $^{18}O_2$ was also carried out. The C-2 signal of the aequorin derived from (**180b**) incubated in the presence of a mixture of $^{18}O_2$ and $^{16}O_2$ was apparently broader than that of (**180b**)-containing aequorin incubated only with $^{16}O_2$ and accompanied by a shoulder which was observed approximately 0.07 ppm upfield from the peak at δ_C 98. The shoulder was attributable to the ^{18}O isotope effect.

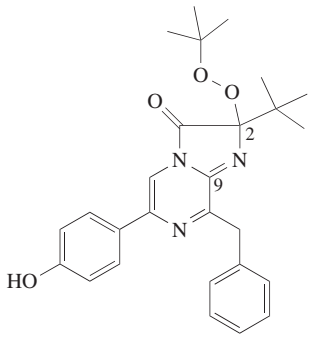


(180a) ^{13}C-labeled doubly at C-2 and C-9

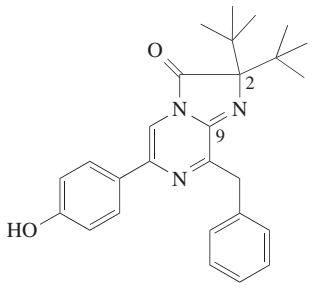
(180b) ^{13}C-labeled singly at C-2

(180c) ^{13}C-labeled singly at C-9

To provide a model for a key intermediate in the bioluminescence of aequorin suggested by Kishi and co-workers,[132] unstable 2-*t*-butyl peroxide (**181**) and stable 2,2-di-*t*-butyl derivative (**182**) were prepared.[133] The chemical shifts of the C-2 and C-9 carbons of these model compounds were δ_C 104.85 and 150.23, respectively, for (**181**) and δ_C 84.83 and 147.88, respectively, for (**182**). The chemical shift of the C-2 carbon of (**181**) appeared 6.85 ppm downfield from the peak of aequorin derived from coelenterazine (**180a**) at δ_C 98,[132] which may have been due to the solvent effect and to the β effect by three methyl carbons in the *t*-butyl group attached to C-2. The chemical shift difference of the C-9 carbon between (**181**) (δ_C 150.23) and (**180a**)-containing aequorin (δ_C 148) was 2.23 ppm. These ^{13}C NMR chemical shift data were consistent with Kishi's proposal that coelenterazine is transformed into the 2-alkyl-2-peroxy-2,3-dihydroimidazo[1,2-*a*]pyrazin-3-one structure in aequorin.

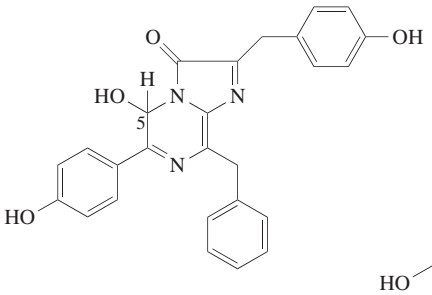


(181)

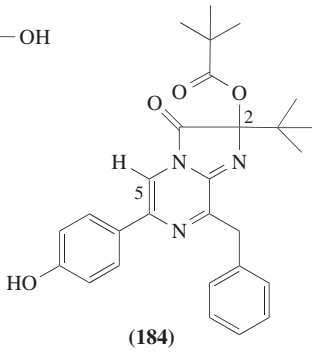


(182)

Concerning the structure of the yellow compound (YC), Teranishi *et al.* reported that YC should have the 5-oxo-structure (**183**) instead of having hydroxyl group at C-2 as reported previously on the basis of preparing model compounds (**184**) and (**185**) oxygenated at C-2 and C-5 positions, respectively, and comparing their absorption spectra.[134] The three absorption spectra of YC,[130] (**184**), and (**185**), similarly showed two maxima at around 430–450 nm and 290–310 nm, while the relative intensity ratio ($\varepsilon_{430}/\varepsilon_{290}$) of YC (ca. 3) corresponded better to that of the 5-oxy compound (**185**) ($\varepsilon_{430}/\varepsilon_{290} = 3.1$) than to that of the 2-oxy compound (**184**) ($\varepsilon_{430}/\varepsilon_{290} = 0.26$). Thus, the 5-oxo structure (**185**) was suggested as a revised structure of YC, being closely related to luciferinol (**161**) derived by oxidation of *Vargula* luciferin (**159**) (Section 8.07.6.1).



(183)



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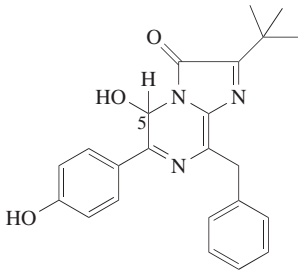
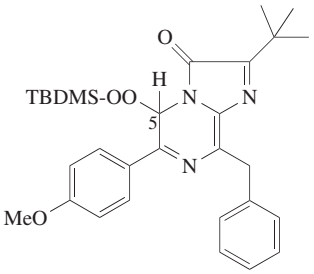
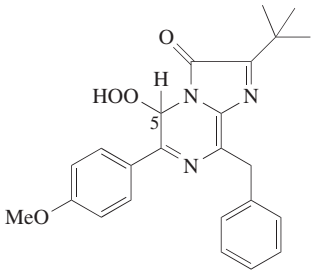


(185)

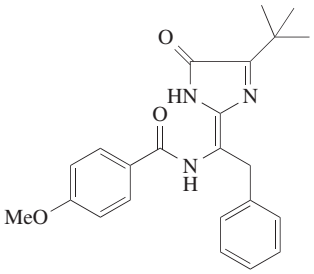
To provide further demonstrations of involvement of 5-oxy coelenterazine analogues in luminescence reactions, synthetic approaches to 5-hydroperoxide were examined. As a result, an unstable *t*-butyldimethylsilyl peroxide of coelenterazine analogue (**186**) was prepared, and this peroxide (**186**) in DMF under either aerobic or anaerobic conditions emitted weak light for 2 days.[135] Furthermore, 5-hydroxyperoxide (**187**) was successfully prepared, which thermally decomposed to give only amide (**188**). From all these results, the structure of YC was suggested as 2-peroxide (**179**).[136]



(186)



(187)



(188)

Chemiluminescence and fluorescence studies of the imidazopyrazine derivatives of coelenterazine in basic aprotic solvents indicated that the excited light-emitter was the amide anion (Figure 3(a), see also Scheme 4),[125] whereas on the basis of luminescence studies of regenerated blue fluorescent proteins (BFP) with coelenterazine analogues including *N*-methyl derivatives, the emitting species was assigned to a pheonolate monoanion (Figure 3(b)).[137] The *O*-methyl and *N*-methyl coelenteramide derivatives (**189**) and (**190**), respectively, were prepared, and the fluorescence emission spectrum of (**189**) in BFP could not be observed while incubation of (**190**) with recombinant apoaequorin yielded a BFP with a fluorescence emission spectrum possessing a peak at 480 nm. This emission maximum was similar to the bioluminescence maximum of aequorin, suggesting that the excited-state emitter in aequorin could not be the amide anion (Figure 3(a)) but must be the phenolate anion (Figure 3(b)).[136]

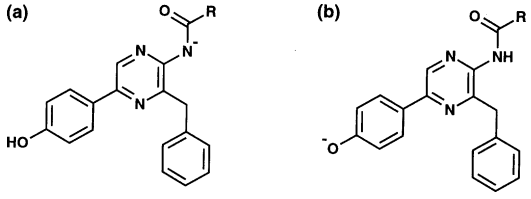
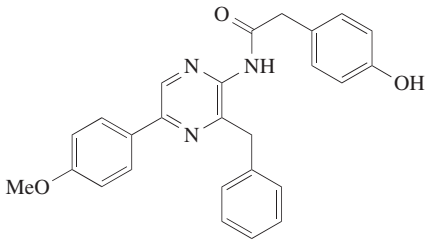
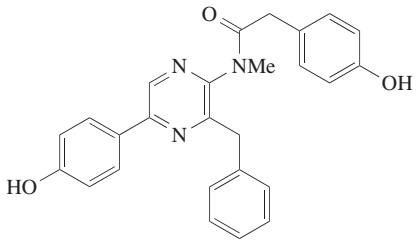


Figure 3 Possible ionized structures of coelenteramide: (a) amide anion, (b) phenolate anion.

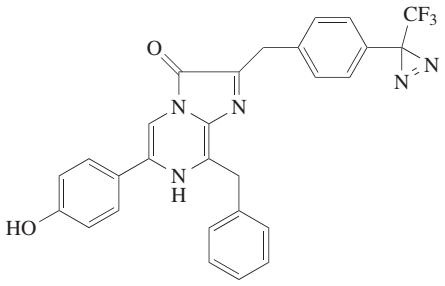


(189)



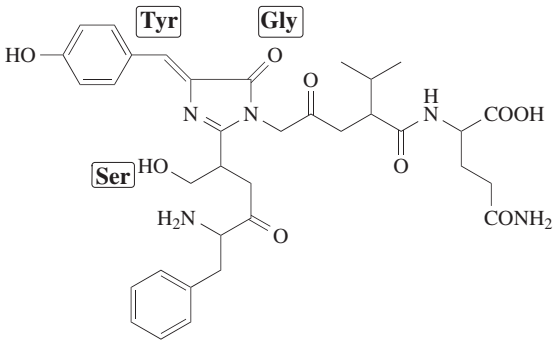
(190)

Photoaffinity labeling is an attractive methodology to characterize and identify functional domains and active sites of biomacromolecules. To examine the bioluminescence mechanism and the nature of the active site and the binding mode between coelenterazine and apoaequorin, a photolabile analogue of coelenterazine with a trifluoromethyl diazirine group (**191**) was prepared and the chemi- and bioluminescence of the analogue (**191**) showed that its behavior was almost identical with that of natural coelenterazine in terms of luminescence characteristics, suggesting that the analogue (**191**) occupied the same active site of aequorin and should be a useful photoaffinity label for probing the detailed structure of aequorin.[138–140]



(191)

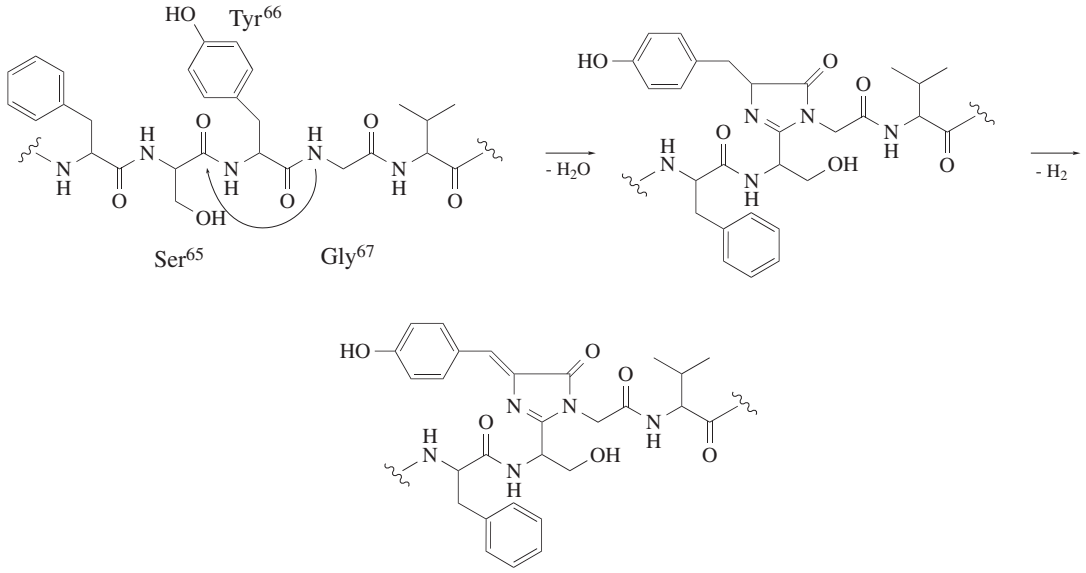
The jellyfish *Aequorea victoria* emits a bluish-green light from the margin of its umbrella. The light is due to not only the photoprotein aequorin described above but also to a green fluorescent protein (GFP).[136] The GFP was known as a single polypeptide chain consisting of 238 amino acid residues and having a M_r of 27 kD, and containing a modified hexapeptide as a chromophore which emits a green light (λ_{max} 508 nm). The GFP chromophore was proposed to be composed of modified amino acid residues within the polypeptide.[141] The structure of the GFP chromophore was elucidated by releasing it as a hexapeptide upon digestion of the protein with papain with the aid of synthesis of model chromophores and modern two-dimensional (2D) NMR spectroscopic studies.[142] As a result, the GFP hexapeptide chromophore was proposed as (**192**) which was formed by a post-translational modification of the tripeptide Ser^{65}-Tyr^{66}-Gly^{67} in the primary structure.



(192)

The chromophore formation through the dehydration–dehydrogenation mechanism in the tripeptide Ser^{65}-Tyr^{66}-Gly^{67} (Scheme 6) was supported by several types of modern mass spectroscopic analyses [ESI (electrospray ionization), MALDI-TOF (matrix-assisted laser desorption/ionization-

time of flight), and MALDI-PSD (post source decay) spectra] of the chromophore-containing peptide obtained from the protease digest of GFP, which suggested the loss of 20 a.m.u. corresponding to the dehydration–dehydrogenation process.[143]



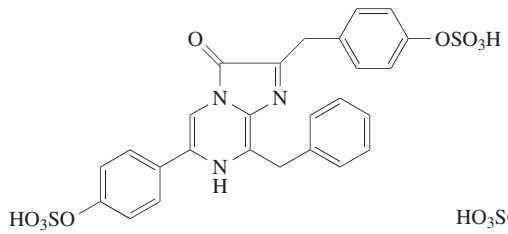
(192)

Scheme 6

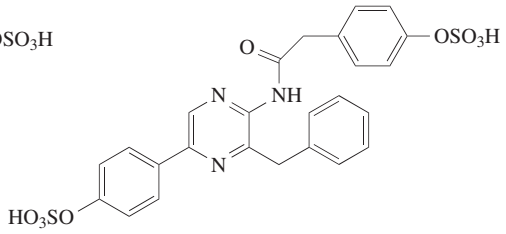
Expression of the cDNA for *Aequorea* GFP in *E. coli* yielded a fused protein with fluorescence excitation and emission spectra virtually identical to those of native GFP, suggesting that the primary structure of the protein underwent modification to form a chromophore during expression.[144] Because of the marked intrinsic fluorescence emitted by GFP, it may also serve as a reporter or marker in gene expression studies.[145]

8.07.6.3 Squid

A luminous squid, *Watasenia scintillan* (Japanese name: hotaru-ika), is caught in large numbers in Toyama Bay, Japan, in a certain period of the year. It has three tiny black spots, which are luminous organs, located at the tip of each of the ventral pair of arms. The chemistry of the chromophore (luciferin) of *Watasenia* squid was studied in the mid-1970s. *Watasenia* oxyluciferin (**193**) and luciferin (**194**) was extracted from the arm photophores, and they were revealed as the disulfates of coelenterazine (**163**) and coelenteramide (**164**), respectively.[146,147]



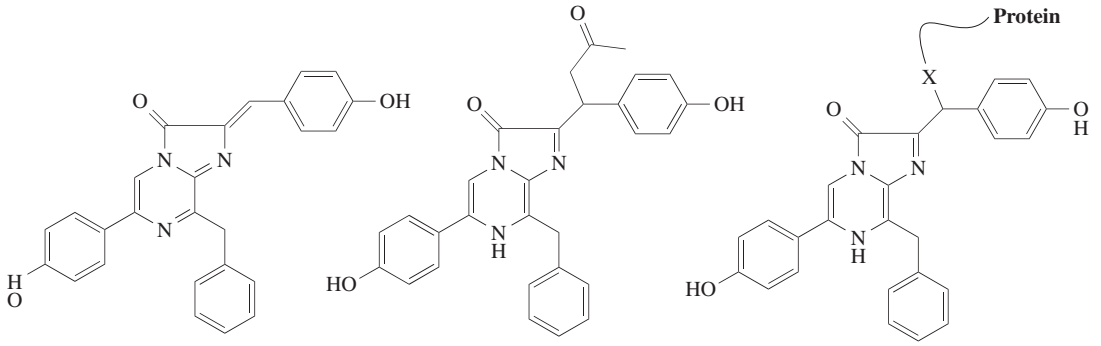
(193)



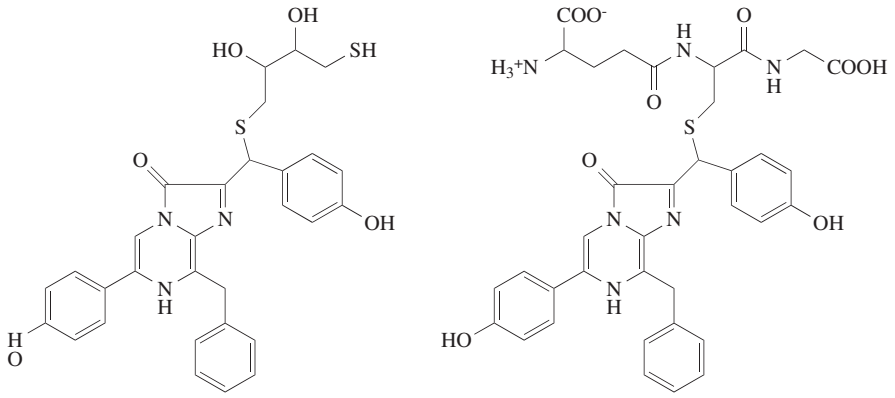
(194)

The bioluminescence system of a different luminous squid, *Symplectoteuthis oualaniensis* (Japanese name: tobi-ika), has been studied extensively.[148] *S. oualaniensis* is an oceanic squid common to the western Pacific and Indian Oceans. The squid possesses a large oval organ (major axis, ca. 2 cm) on the anterodorsal surface of the mantle that emits a bright flash of blue light when stimulated. The organism is yellow and consists of numerous ovate [0.4–1.1 mm (major axis) × 0.3–0.5 mm (minor

axis)] photogenic granules. Studies of the biochemical aspects of the luminescence of *S. oualaniensis* revealed that sodium or potassium ions as well as molecular oxygen were necessary for the luminescence,[149] suggesting involvement of a photoprotein different from aequorin (Section 8.07.6.2). The chromophores of this bioluminescence system were isolated from the acetone-powder of the photogenic organs of *S. oualaniensis* and identified as dehydrocoelenterazine (**195**) and its acetone adduct (**196**). Dehydrocoelenterazine (**195**) was postulated to be equivalent to luciferin in this luminescent system and assumed to be stored in a form of conjugated adduct (**197**).[150,151] Furthermore, the photoprotein in *S. oualaniensis* was extracted in high salt solutions at low temperature.[152] It was found that only the high molecular weight fraction from gel filtration chromatography had luminescence activity, suggesting that the luminescent substance is a photoprotein. No dehydrocoelenterazine (**195**) was, however, detected after the luminescence (470 nm) of the homogenate by addition of KCl, indicating that the bioluminescence of *S. oualaniensis* consumed dehydrocoelenterazine (**195**). On the other hand, three solutions containing dehydrocoelenterazine (**195**), its dithiothreitol (DDT) adduct (**198**), and glutathione (GSH) adduct (**199**) were added, respectively, to the protein fraction after the luminescence, and light emission was immediately observed in all three solutions. These findings recorded the reconstruction of the luminous system in this squid.



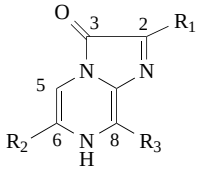
(195) **(196)** **(197)**



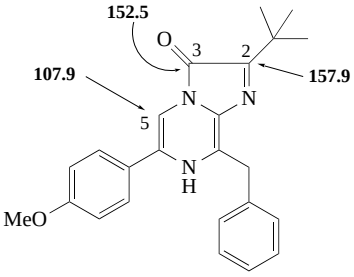
(198) **(199)**

The involvement of a common structure of 3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (**200**) is known in many marine luminescent systems such as *Vargura*, *Watasenia*, and *Aequorea* bioluminescence, and coelenterazine is the most common luciferin among these systems. Although the luminescence mechanism with these luciferins has been well investigated, no direct evidence of the peroxide intermediate (e.g., Scheme 4) was demonstrated until 1995 due to its unstable nature. In 1995, Usami and Isobe succeeded in collecting evidence of the intermediates of luminescence by synthesizing the peroxide at lower temperatures.[153] They prepared a model compound (**201**) with a *t*-butyl group at the C-2 position enriched with ^{13}C atoms at C-2, C-3, and C-5 positions ((**201a**), (**201b**), and (**201c**), respectively), which were photooxygenated in an NMR tube at $-78\,^{\circ}C$ in CF_3CD_2OD-CD_3OD solution and its ^{13}C NMR spectrum was recorded at that temperature. The ^{13}C NMR spectrum revealed that the signal of the starting coelenterazine analogue (**201**) diminished

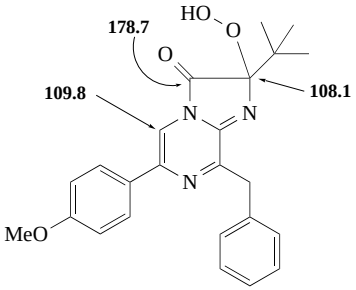
to half intensity after 3 min photo-irradiation, and to 1/5 after a further 3 min irradiation. The first 3 min irradiation produced signals for two newly formed compounds (**202**) and (**203**), while 6 min irradiation resulted in the higher intensity of the signals for (**202**). When the mixture was warmed, its ^{13}C NMR spectrum showed signals due to the stable photoproduct (**204**) corresponding to coelenteramide (**164**). Assignment of the ^{13}C NMR signals of these compounds are summarized in Table 16. These results provide direct evidence for the generation of the peroxide (**202**) and afforded the first proof of 1,2-dioxetanone (**203**) as a luminescence intermediate.[154] Teranishi and co-workers also reported photooxygenation of (**201**) at $-95\,^{\circ}C$ sensitized with polymer-bound Rose Bengal in CD_2Cl_2 solution, in which the peroxide (**202**) was detected as a major product from the ^1H NMR spectrum at low temperature (below $-50\,^{\circ}C$), while dioxetanone (**203**) was not detected and on warming to $-20\,^{\circ}C$ the amide (**204**) was present almost exclusively.[155]



(200)



(201)

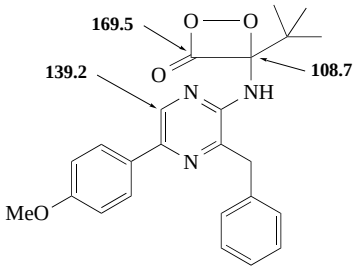


(202)

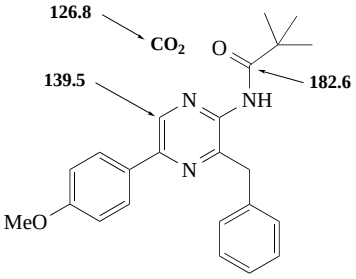
(201a) ^{13}C-labeled singly at C-2

(201b) ^{13}C-labeled singly at C-3

(201c) ^{13}C-labeled singly at C-5



(203)



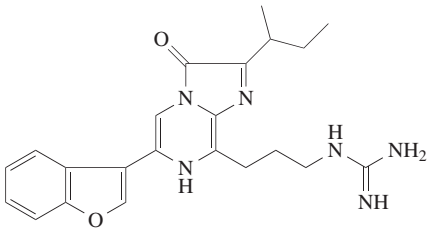
(204)

Table 16 ^{13}C NMR data of the coelenterazine analogue (**201**) under photoirradiation experiments.

	C-2	*C-3*	*C-5*	*Assigned structure*
Before irradiation	157.9	152.5	107.9	(**201**)
After photoirradiation	108.1	178.7	109.8	(**202**)
	108.7	169.5	139.2	(**203**)
After warming	182.6	126.8	139.5	(**204**)

Imidazopyrazinones represented as (**200**) are widely utilized by bioluminescent organisms in the marine environment. Imidazopyrazinones with conjugated substituents at the 6,8-positions were synthesized from the corresponding 3,5-disubstituted-2-aminopyrazines which were prepared from commercially available 2-aminopyrazine by a sequence of reactions including Pd-mediated Stille coupling as a key step.[156] The synthesized imidazopyrazinones showed chemiluminescence. By

applying the same method, a benzofuran analogue (**205**) of the *Vargula* luciferin (**159**) was prepared, which reacted with luciferase to give a luminescence but the light yield was about 1/25, which may be due to its poor chemiluminescence.[157]

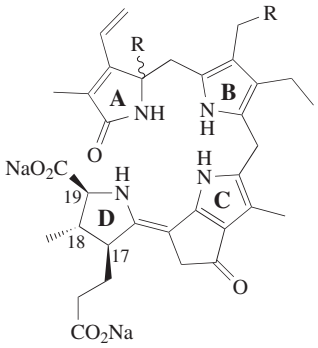


(**205**)

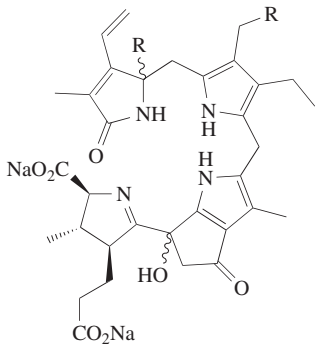
8.07.6.4 Microalgae

Dinoflagellates are ubiquitous microalgae in the oceans and they are responsible for much of the sparkling luminescence elicited at night by disturbing surface waters. The chemical process of light emission involves air oxidation of dinoflagellate luciferin catalyzed by dinoflagellate luciferase. The dinoflagellate bioluminescence system was shown to cross-react with the krill (euphausid shrimp) bioluminescent system,[158] composed of a photoprotein and krill fluorescent substance F. Dinoflagellate luciferin and krill fluorescent substance F share common chemical and spectroscopic properties, and they are both unstable in the presence of oxygen. Krill fluorescent substance F (**206**) was successfully isolated from the krill *Euphausia pacifica* by careful operations using alumina and ion-exchange chromatography at low temperature under an inert atmosphere, and the structures of (**206**) and its air-oxidation product oxy-F (**207**) were elucidated on the basis of chemical degradations and spectroscopic data.[159] Dinoflagellate luciferin (**208**) was isolated from cultured *Pyrocystis lunula*, and its structure was elucidated by comparing the spectroscopic data with those of krill fluorescent substance F (**206**). From the crude extract of luciferin, the air-oxidation product (**209**) with a characteristic blue color was isolated. The nonenzymatic oxidation of dinoflagellate luciferin (**208**) in methanol afforded (**210**) without emission of light, whereas air oxidation in the presence of luciferase proceeded with the emission of light at 474 nm to yield (**211**).[160,161] The absolute stereochemistry of three chiral centers on the D-ring of krill fluorescent substance F (**206**) was established as 17*S*, 18*S*, and 19*S*, respectively, on the basis of chiral HPLC comparison of the ozonolysis product with synthetic compounds, and this result suggested that chlorophylls are the biogenetic origin of krill fluorescent substance F (**206**).[162]

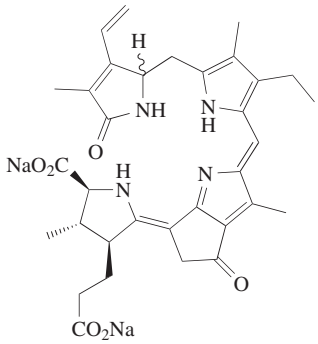
Bioluminescent organisms have thus been investigated extensively and some answers to the question of how they emit light have been obtained, whereas it is still not clear why they emit light.[163] In connection with this subject, the circadian rhythms of microalgae have been studied.[164] In the unicellular marine dinoflagellate *Gonyaulax polyedra*, bioluminescence as well as other biological phenomena including cell division, motility, and photosynthesis exhibits circadian rhythmicity which may persist under constant conditions with a precise period of about 24 h. Using the circadian rhythm of bioluminescence as a bioassay, the period of free-running circadian rhythms in *Gonyaulax* was revealed to be shortened by extracts from mammalian cells. The effect was dose-dependent, accelerating the circadian clock by as much as 4 hours per day. The substance responsible for this effect was isolated from bovine muscle and identified as creatine (**212**). Authentic creatine (**212**) had identical biological effects at micromolar concentrations.[165] A period-shortening substance with similar chemical properties was found also to be present in extracts of *Gonyaulax* itself. The endogenous active substance, termed gonyauline (**213**), was isolated and characterized as a low molecular weight cyclopropanecarboxylic acid.[166] Synthetic (±)-gonyauline (**213**) also had a similar accelerating effect on the period of the circadian clock.[167] The optically active natural (+)-gonyauline (**213**) as well as its enantiomer, (−)-gonyauline, was synthesized by procedures including optical resolution with the aid of brucine, and the absolute configuration of two chiral centers of natural (+)-gonyauline (**213**) was determined as 1*R*, 2*R* by applying the exciton chirality method to the CD spectrum of the bis-α,β-unsaturated ester (**214**), which was prepared from a chirally resolved synthetic intermediate.[168]



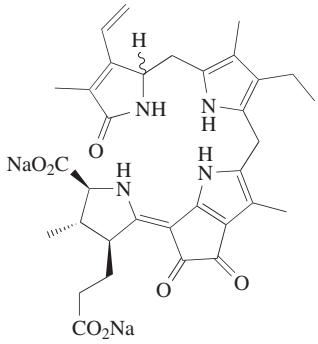
(**206**) R = OH
(**208**) R = H



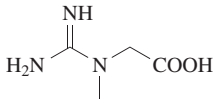
(**207**) R = OH
(**210**) R = H



(**209**)

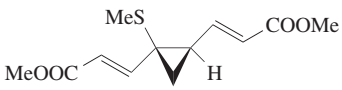


(**211**)



(**212**)

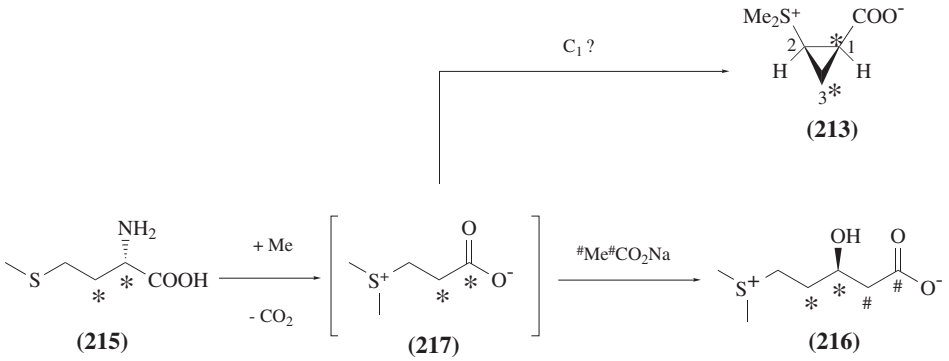
(**213**)



(**214**)

From the structural similarity between gonyauline (**213**) and methionine (**215**) it was estimated that gonyauline (**213**) might be derived directly from methionine (**215**) by methylation and deamination–cyclopropanation reactions accompanied by an inversion of configuration at the C-2 position. Thus, feeding experiments of labeled methionine or its analogues to dinoflagellate *G. polyedra* were carried out. As a result, methyl groups of methionine or *S*-methylmethionine were efficiently incorporated into a sulfonium methyl group of gonyauline (**213**). However, carboxyl carbons of methionine or *S*-methylmethionine at the C-1 position were not incorporated into gonyauline (**213**) even at a high concentration. Thus, it was revealed that gonyauline (**213**) was not derived through direct methylation and deamination–cyclopropanation reactions of methionine (**215**).[169,170] Feeding [2,3-$^{13}C_2$]-methionine, which was synthesized from 2-methylpentane-2,4-diol and [1,2-$^{13}C_2$]-acetonitrile, to *G. polyedra* afforded ^{13}C-labeled gonyauline (**213**) at C-1 and C-3 positions, suggesting that gonyauline (**213**) were derived biogenetically from methionine (**215**) through decarboxylation and elongation of C_1 carbon (Scheme 7).[170] During these biogenetical studies of gonyauline (**213**), another sulfonium compound, gonyol (**216**), was found to be accumulated in *G. polyedra*, and its structure was elucidated by spectroscopic methods and confirmed by chemical synthesis.[169] Feeding experiments showed that gonyol (**216**) was also biogenetically derived from methionine (**215**), but the carboxyl carbon of methionine (**215**) was not incorporated into gonyol (**216**). However, the C-1 and C-2 of

gonyol (**216**) were labeled with [1,2-$^{13}C_2$]-sodium acetate in the presence of methionine (**215**). Gonyol (**216**) obtained by the feeding experiment with [2,3-$^{13}C_2$]-methionine was enriched with ^{13}C at C-3 and C-4 positions. These results implied that gonyol (**216**) might be biogenetically derived from methionine (**215**) and acetate through dimethyl-β-propiothetin (**217**) or its analogous intermediates (Scheme 7). Although gonyol (**216**) was found as a minor component in *G. polyedra* under normal culture conditions, other dinoflagellates such as *Amphidinium* sp. and *Symbiodinium* sp. contained gonyol (**216**) at various levels from a trace to one of the major components. It seemed likely that dinoflagellates contain various sulfonium compounds and dimethyl-β-propiothetin (**217**) might be a common precursor for a methionine cascade to sulfonium compounds including gonyauline (**213**) and gonyol (**216**).



Scheme 7

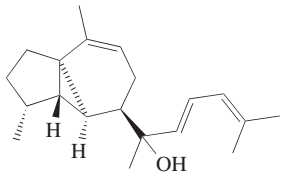
8.07.7 CHEMICAL DEFENSE INCLUDING ANTIFEEDANT ACTIVITY

There are many soft-bodied and benthic organisms in the marine environment. These organisms appear not to have physical defense mechanisms, while most of them contain unusual secondary metabolites and these secondary metabolites are assumed to have a defensive function. These defensive functions are termed "chemical defense." A number of good reviews have been published on this subject (e.g., on chemical defense of tropical marine algae,[171] nudibranchs,[172] alcyonarian corals,[173] fish,[174] and others[175–178]). In this section, we survey several reports on this subject.

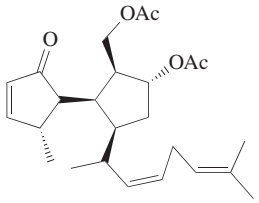
8.07.7.1 Algae

The major function of chemical defense by marine natural products may be feeding deterrence. From the neutral methanol extract of the brown alga *Dilophus okamurai*, two spatane-type diterpenes (**84**) and (**85**) were isolated as inhibitors of the settlement and metamorphosis of the swimming larvae (veliger) of the abalone *Haliotis discus hannai*.[88] The diterpenes (**84**) and (**85**) were also found to be strongly active feeding deterrents for the young abalone. For this bioassay, the Avicel plate method developed by Sakata and co-workers was used (see Section 8.07.2.2). The ethanol solutions (25 μl) of a standard phosphatidylcholine (PC) (10 μg) and the samples, which were prepared by mixing 100 μg of each of the fractions and pure compounds with PC (10 μg), were applied with a microsyringe onto the sample zone (25 mm in diameter) on an Avicel plate. The feeding-deterrent activity of each sample was evaluated by comparing the number of biting traces left on the plates with that of the standard PC. Further studies on feeding-deterrent substances of this alga led to the isolation of seven active compounds related to spatane-type diterpenoids (**218**)–(**224**).[179–181] The feeding-deterrent activity of these compounds was evaluated by the Avicel plate method to reveal the following observations. Compounds (**84**), (**85**), (**219**), and (**222**) exhibited comparably strong feeding-deterrent activity, and compounds (**220**) and (**224**) exhibited moderate activity. Compounds

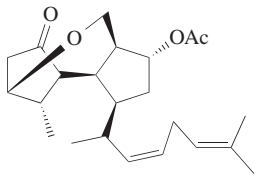
(**218**) and (**223**) showed weak activity, while compound (**221**) showed very weak activity. It was interesting that although compounds (**218**) and (**223**) exhibited weak feeding-deterrent activity against the young abalone *Haliotis discus hannai*, these metabolites were found to be the strongest feeding deterrents against the sea urchin *Strongylocentrotus nudus*.[181]



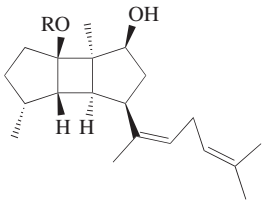
(**218**)



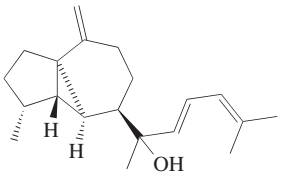
(**219**)



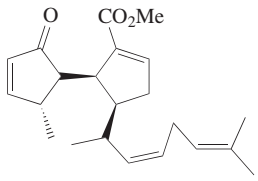
(**220**)



(**221**) R = H
(**222**) R = Ac

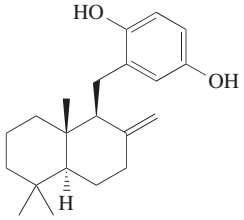


(**223**)

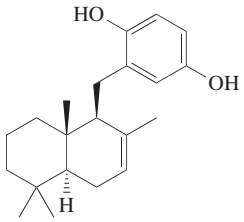


(**224**)

From the brown alga of the genus *Dictyopteris*, collected at Akita Prefecture, Japan, five sesquiterpenoid derivatives, zonarol (**225**), isozonarol (**226**), zonarone (**227**), isozonarone (**228**), and chromazonarol (**229**) were isolated as feeding deterrents against abalones, and the feeding-deterrent activity of these sesquiterpenoids was considerably stronger than that of diterpenes obtained from *Dilophus okamurai*.[182]



(**225**)



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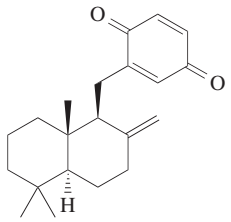
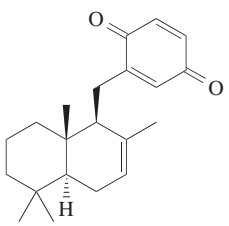
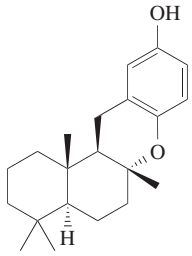


(**227**)

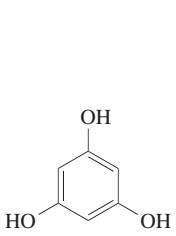


(**228**)



(**229**)

Feeding deterrents of brown alga of the order Laminariales against abalones or sea urchins were identified as water-soluble phenols such as phloroglucinol (**230**) and its oligomers (**231**) and (**232**).[182]



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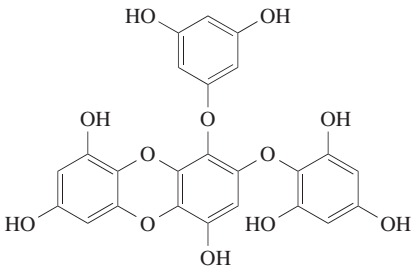

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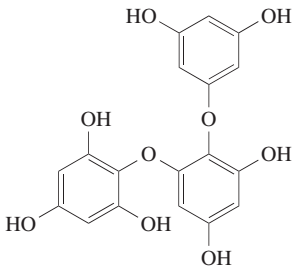
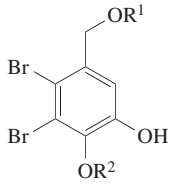
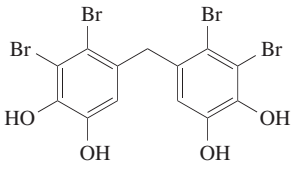


(232)

The red alga *Rhodomela* sp. contained bromophenols (**233**)–(**235**) as feeding deterrents against sea urchins, but a co-isolated sulfate (**237**) was inactive, while feeding deterrents of red alga of the genus *Laurencia* against sea urchins was identified as bromine-containing diterpenes (**238**)–(**243**).[182]



(233) $R^1 = R^2 = H$
(234) $R^1 = Me$, $R^2 = H$
(237) $R^1 = R^2 = SO_3^-K^+$



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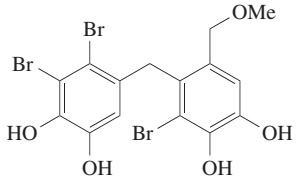
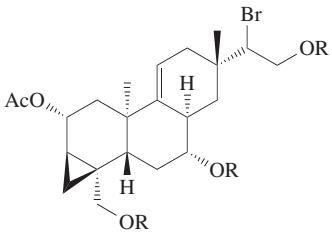
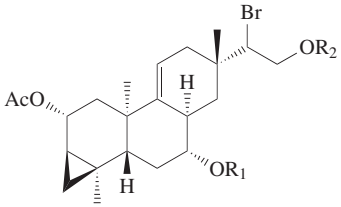


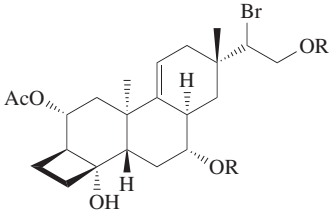
(236)



(238) R = H
(239) R = Ac

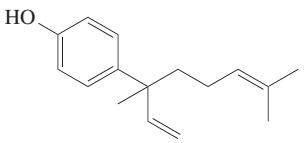


(240) $R^1 = R^2 = H$
(241) $R^1 = H$, $R^2 = Ac$



(242) R = H
(243) R = Ac

Three prenylated phenols, sporochnols A–C (**244**)–(**246**), were isolated from the Caribbean marine alga *Sporochnus bolleanus*.[183] The major metabolite (**244**) showed significant feeding deterrence toward herbivorous fishes. When sporochnol A (**244**) was incorporated (at 80% of its natural yield) into agar strips containing palatable, freeze-dried algae, it reduced parrotfish consumption of treatment strips by a significant 27% compared with otherwise equivalent control strips. This compound, however, had no effect on feeding by the sea urchin *Diadema antillarum* or the amphipod *Cymadusa filosa*. Because herbivory by reef fishes appears to be the major factor selecting for herbivore defense in tropical seaweeds, it is not unusual to find seaweed defensive metabolites that are effective primarily against fish. Thus, from this observation, the sporochnols appear to function as defensive agents in *Sporochnus bolleanus*.



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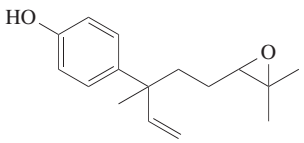
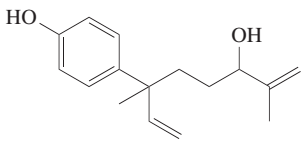
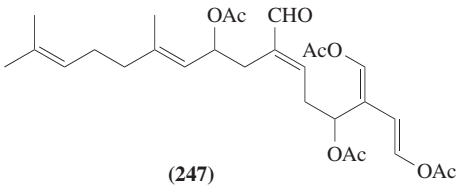


(245)

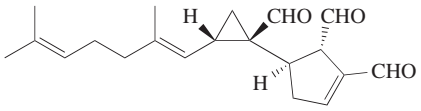


(246)

One type of facultative defense of marine organism that has been recognized is the production of predator-induced defenses. This type of defense operates when an attack by a predator acts as a cue for stimulating the synthesis of new or additional defensive compounds. Another type of related facultative or inducible chemical defense is the rapid conversion of one secondary metabolite to another more potent defensive compound upon injury to the organism. The latter type of defense mechanism eliminates the need for an organism to maintain high levels of very deterrent and biologically active substances which may be toxic to the organism itself. Thus, the risk of autotoxicity is minimized. As an example of this type of chemical defense, activation of chemical defense in the tropical green algae *Halimeda* spp. was described.[184] *Halimeda* spp. are among the most common seaweeds on tropical reefs and these seaweeds produce diterpenoid feeding deterrents; the major metabolites were halimedatetraacetate (**247**) and halimedatrial (**248**). It was observed that most species of *Halimeda* on Guam immediately convert the less-deterrent secondary metabolite halimedatetraacetate (**247**) to the more potent feeding deterrent halimedatrial (**248**) when plants are injured by grinding or crushing. This conversion would therefore occur when fish bite or chew *Halimeda* plants. Extracts from injured plants contained higher amounts of halimedatrial (**248**) and were more deterrent toward herbivorous fish than extracts from control plants.



(247)

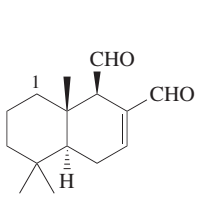


(248)

8.07.7.2 Mollusks

Naked nudibranch mollusks exhibit a series of defensive strategies against potential predators, which include the use of chemicals obtained either from the diet or by *de novo* biosynthesis.[172] Two Mediterranean species of *Dendrodoris* nudibranchs have elaborated a very sophisticated defensive strategy against predators. Polygodial (**249**), which was initially known as an antifeedant for insects of the African plant *Warburgia stuhlmanni*,[185] was identified as a defensive allomone of two Mediterranean nudibranchs, *Dendrodoris limbata* and *D. grandiflora*.[186] Polygodial (**249**) was revealed to be an antifeedant to fish, anorectic to insects, and hot-tasting to humans, and these biological activities are most probably due to the simultaneous interaction of both the aldehydic groups with primary amine moieties.[187,188] Polygodial (**249**) was found to be toxic to *D. limbata*,[189] and it was suggested that a related sesquiterpenoid olepupuane (**250**) was the masked form of the allomone present in the animal. The anatomical distribution of the drimane sesquiterpenoids such as (**249**) in different sections and egg masses of the mollusk was investigated. As a result, drimane sesquiterpenoids were found only in the yellow mantle border, in the gills, in the hermaphrodite glands, and in the egg ribbons.[190] TLC analysis of the mantle border revealed the presence of polygodial (**249**), clearly deriving from olepupuane (**250**). Compounds (**249**) and (**250**) were completely absent in other organs. Drimane esters (**251**) with C_{18}–C_{20} fatty acids and euryfuran (**252**) were localized in hermaphrodite glands and egg masses, while the gills possessed 7-deacetoxy-olepupuane (**253**). No drimane sesquiterpenoids were detected in the mucous secretion of *D. limbata*. Analogous studies on *D. grandiflora* showed almost identical results. It seems likely that *Dendrodoris* mollusks have elaborated a very effective mechanism to secure their own survival. Related compounds are localized in different organs of the mollusk, and perform different biological roles. 7-Deacetoxy-olepupuane (**253**) could be the precursor of the defensive allomones, olepupuane (**250**) and polygodial (**249**), whereas the esters (**251**) might play a role during the reproductive cycle.[190]

The ascoglossan mollusk *Cyerce cristallina* has a typical defensive behavior known as autotomy. This animal possesses aposematically colored dorsal appendices called "cerata." When the mollusk is attacked by predators, the cerata are detached from the mantle and exhibit prolonged contractions while secreting large amounts of a toxic mucous secretion. After the autotomic process, the animal provides a striking example of regeneration by completely reproducing the cerata within only 7–10 days. In connection with this phenomenon, seven pyrones, cyercenes A, B, and 1–5 (**254**)–(**260**), were isolated from the cerata of the mollusk *C. cristallina*,[191] and the possible correlation between these pyrone metabolites and the process of chemical defense and regeneration was studied by investigation of the tissue distribution, biological activity, and biogenesis of cyercenes.[192]



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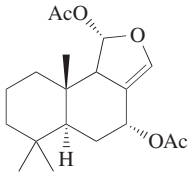

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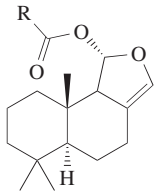
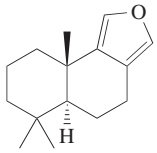
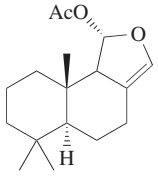


(251)

$R = C_{18} - C_{20}$



(252)



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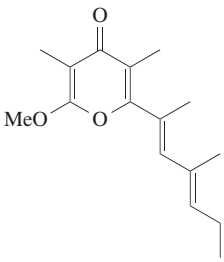

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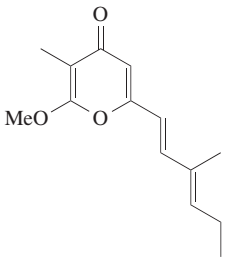

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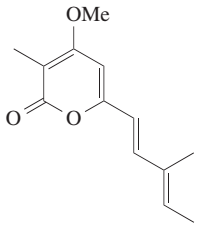

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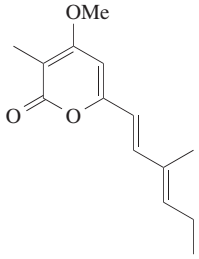

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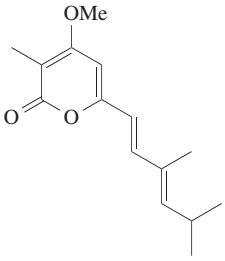

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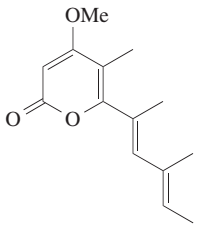

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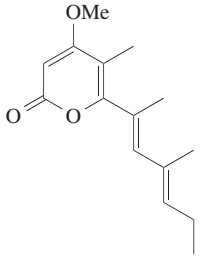
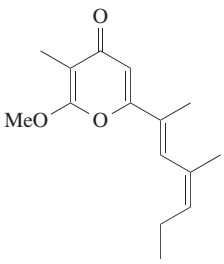


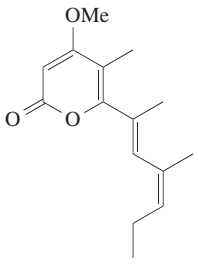
(260)

HPLC analysis showed differences in chemical composition between the *C. cristallina* mantle, cerata, digestive gland, and mucous secretion. No cyercene was contained in the digestive gland, while only cyercenes 1 (**256**), 2 (**257**), and 3 (**258**) were found in the mantle. The mucus contained all cyercenes except cyercene A (**254**), and cyercene A (**254**) was only present in the cerata, which contained all seven compounds. The presence of cyercene A (**254**) only in the regenerating tissue, the cerata of *C. cristallina*, may suggest the possible involvement of this compound in the quick regenerative mechanisms. The regeneration-stimulating activity of cyercenes was assayed by a model test of cell growth and differentiation factors, using the *Hydra vulgaris*, which had a comparable speed (normally 8 days) in the regeneration of its head and tentacle to that of *C. cristallina* cerata (7–10 days). Cyercene A (**254**) exhibited 69% enhancement of the *H. vulgaris* average tentacle number at 15 μg ml^{-1}, suggesting that compound (**254**) plays a biological role as a pivotal growth-inducing factor. It was, however, reported that other molecules that act as the main inducing factors of the rapid regeneration of *C. cristallina* may be present in hydrophilic extracts of the mollusk.[191] The cyercenes were contained in the toxic mucus secretion of *C. cristallina*, thus suggesting that these compounds may be involved in the chemical deterrence. Two pyrones (**261**) and (**262**) struc-

turally similar to cyercenes were isolated from the Australian ascoglossan *Cyerce nigricans*, but they were reported to lack the potent ichthyodeterrent properties.[193]



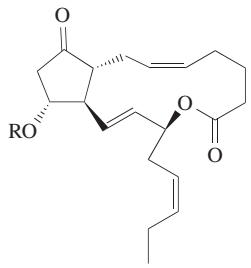
(261)



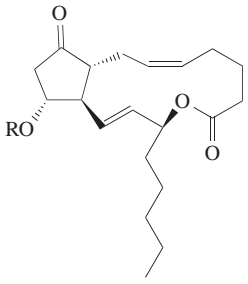
(262)

The ichthyotoxicity of the mucus secretion of *C. cristallina* as well as the purified cyercenes was examined by using the mosquito fish *Gambusia affinis* to reveal that all five cyercenes tested (compounds (**254**), (**255**), and (**257**)–(**259**)) and the mucus crude extract were toxic at a concentration of 10 μg ml^{-1}. Thus, cyercenes may be involved in chemical defense in *C. cristallina*. The complete absence of cyercenes and any other related metabolites in the extract of the digestive gland suggested that these compounds were not derived from dietary sources but were biosynthesized *de novo* by *C. cristallina*, and this suggestion was confirmed by an isotope incorporation experiment. The cerata of the mollusks were detached and the "naked" mollusks were incubated in seawater containing [^{14}C]-sodium propionate. Incorporation of almost 60% of the radioactivity was observed in the fractions containing the seven cyercenes isolated by the feeding experiment.[192]

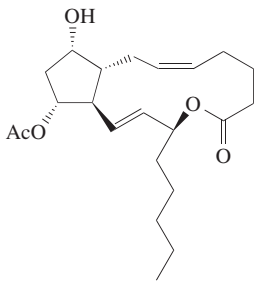
The opisthobranch mollusk *Tethys fimbria* also detaches the dorsal appendices (cerata) during the behavioral defense mechanism known as autotomy. From this nudibranch mollusk prostaglandin(PG)-1,15-lactones of the E series, PGE_3-1,15-lactone 11-acetate (**263**), PGE_2-1,15-lactone (**264**), and PGE_3-1,15-lactone (**265**), were isolated, and these were the first naturally occurring prostaglandin-1,15-lactones.[194] These lactones were contained in the mantle and the cerata of this mollusk, and prostaglandin-1,15-lactones of the F series (**266**) and (**267**) together with PGE_2-1,15-lactone 11-acetate (**268**) were also obtained from the mantle and cerata of the same animal.[195] HPLC analysis of the extracts of both the mantle and the cerata showed the presence of free acids, PGE_2 (**269**) and PGE_3 (**270**).



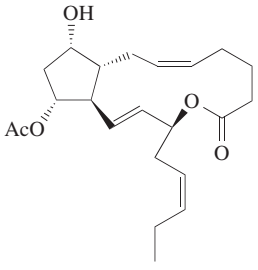
(263) R = H
(265) R = Ac



(264) R = H
(268) R = Ac



(266)



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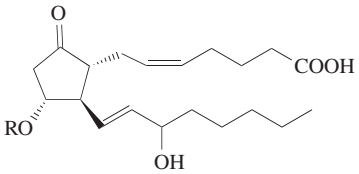
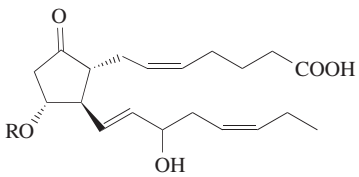


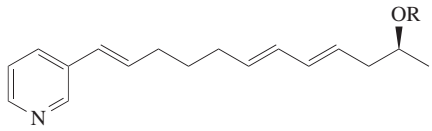
(269)



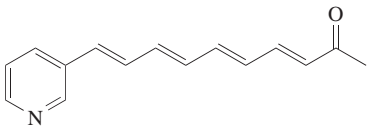
(270)

Thus, the biogenetic relationship between PG-1,15-lactones and PG-free acids and their biological roles in *T. fimbria* were investigated.[196] Incorporation experiments on [^{3}H]-PG-free acids revealed that PG-1,15-lactones are (i) synthesized from PG-free acids mainly in the mantle of this animal; (ii) mostly transferred into the cerata; and (iii) converted back into PG-free acids mainly upon detachment of the cerata from the molested mollusk and during their spontaneous contractions. This appears to be a unique mechanism by which PGs, in the form of structurally related compounds, are stored *in vivo* ready to be released in response to a mechanical stimulus to effect their biological action. HPLC analysis showed the absence of PG-free acids and the presence of PG-1,15-lactones in the defensive mucus secretion of *T. fimbria*. Ichthyotoxic activity assay against the mosquito fish *Gambusia affinis* revealed that PG-free acids did not exhibit any toxicity while the 1,15-lactones were toxic at concentrations of 1–10 μg ml^{-1}. From these findings, the nudibranch *T. fimbria* has developed a very economical way of exploiting PG lactones for more than one purpose: (i) as defense allomones in the mollusk defensive secretion; (ii) as inactive precursors of bioactive PG-free acids within the cerata; and (iii) as bioactive lactones in tissues other than the cerata and the mantle, as might be the case for PGF lactones, which are likely to play a role in the control of oocyte production and/or fertilization.[197]

From the Mediterranean cephalaspidean mollusk *Haminoea navicula* two 3-alkylpyridines, haminols A (**271**) and B (**272**) were isolated,[198] and these compounds had structural analogy with the alarm pheromones, in particular with navenone A (**273**), isolated from the Pacific Algajidae *Navanax inermis*.[199] Haminols A (**271**) and B (**272**) also induced alarm response at a concentration of 0.3 mg for (**271**) and 0.1 mg for (**272**).[198]

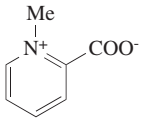


(**271**) R = H
(**272**) R = Ac



(**273**)

The common bright yellow Antarctic lamellarian gastropod *Marseniopsis mollis* was examined for the presence of chemical defense mechanism, and homarine (**274**) was isolated as a major component of ethanolic extracts of this animal.[200] Further HPLC analysis of the mantle, foot, and viscera verified the presence of homarine (**274**) in all body tissues at concentrations ranging from 6 to 24 mg g^{-1} dry tissue.



(**274**)

Filter paper disks treated with homarine (**274**) at 0.4 and 4 mg/disk were rejected by the sea star *Odontasier validus*, which was a conspicuous macroinvertebrate predator of the shallow Antarctic benthos. The highest concentration of homarine (**274**) tested not only caused feeding deterrence, but in several sea stars a flight response was noted. Homarine (**274**) was not detected in the Antarctic ascidian *Cnemidocarpa verrucosa*, a presumed primary prey of *M. mollis*. Homarine (**274**) was, however, contained in the epizooites that foul the tunic (primarily the bryozoans and hydroids), suggesting *M. mollis* may ingest and derive its chemicals from them. Since the vestigial internalized shell of *M. mollis* is considered as a primitive condition, the identification of homarine (**274**) as a feeding deterrent may lend support to the hypothesis that chemical defense evolved prior to shell loss in shell-less gastropods.

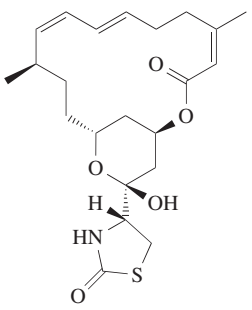
8.07.7.3 Sponges

Among various marine organisms, marine natural product chemists have found that sponges (Porifera) constitute one of the most interesting sources of bioactive substances. The high frequency

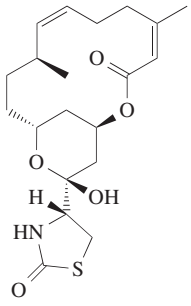
of bioactive secondary metabolites in these primitive filter-feeders may be interpreted as the chemical defense of sponges against environmental stress factors such as predation, overgrowth by fouling organisms, or competition for space. Toxic or deterrent sponge metabolites are, consequently, frequently found in habitats such as coral reefs, and sponges growing exposed are usually more toxic than those growing unexposed.[201]

The palatability of crude organic extracts of 71 species of Caribbean sponges from reef, mangrove, and grassbed habitats was examined by laboratory feeding assays employing the common Caribbean wrasse *Thalassoma bifasciatum*. The majority of sponge species (69%) yielded deterrent extracts, but there was considerable inter- and intraspecific variability in deterrency. Reef sponges generally yielded more deterrent extracts than sponges from mangrove or grassbed habitats. There was no relationship between sponge color and deterrency, suggesting that sponges are not aposematic.[202] Sponge species with high concentrations of spicules in their tissues, and with variable spicule morphologies, were examined to assess the palatability to predatory reef fish, revealing that the presence of spicules did not alter food palatability relative to controls for any of the sponges tested. Analysis of the ash content, tensile strength, protein, carbohydrate, lipid content, and total energy content showed that the tissues of palatable sponges were not different from those of chemically deterrent species with regard to the mean ash content, mean tensile strength, protein content, carbohydrate content, and total energy content, but the tissues of chemically defended species did have a higher mean lipid content than those of palatable species.[203]

A striking example of the chemical defense of sponges through accumulation of toxic or deterrent secondary metabolites against fish was provided by the vividly red-colored sponge *Latrunculia magnifica* that was found in the Gulf of Aquaba (Red Sea). This sponge was avoided by fish that readily accepted other cryptic sponges when exposed. When *L. magnifica* was squeezed, the sponge exuded a reddish fluid that caused fish to flee immediately from the vicinity. It was observed that the sponge toxins contained in this juice led to excitation of fish (*Gambusia affinis*) after 5–10 min, jumping, partial paralysis, turning over onto their backs, hemorrhage of the gills, and finally death. Chemical analysis of the sponge afforded the macrocyclic lactones, latrunculins A (**275**) and B (**276**), that were responsible for the strong ichthyotoxicity of the sponge.[204] The toxicity of (**275**) and (**276**) was shown to be at least due to inhibition of acetylcholinesterase.

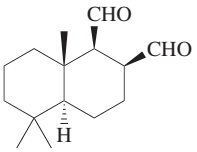


(275)



(276)

From a Southern Australian sponge *Dysidea* sp. a sesquiterpene dialdehyde (**277**) was isolated together with related sesquiterpenes (**278**)–(**280**). The dialdehyde (**277**) is a 7,8-dihydro derivative of the sesquiterpene polygodial (**249**), which is known as a defensive allomone of nudibranchs (see Section 8.07.7.2). The antifeedant properties of polygodial (**249**) were thought to arise from the double condensation of the dialdehyde functionality with primary amines (e.g., lysine) to form pyrroles (e.g., (**281**)). Although the dialdehyde (**277**) does not incorporate an α,β-unsaturated aldehyde moiety such as in polygodial (**249**), it seemed feasible that (**277**) could condense with primary amines in much the same way as (**249**). Thus, it was proposed that the dialdehyde (**277**) may serve as an antifeedant for the *Dysidea* sp. and other sesquiterpene co-metabolites (**278**)–(**280**) may serve as "inactive" reserves or metabolite by-products, with (**277**) acting as the primary defensive allomone.[205] No biological experimental data, however, were described to support this proposal.



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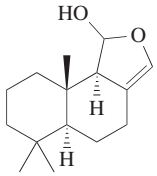

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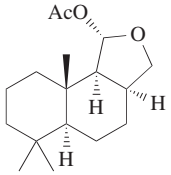

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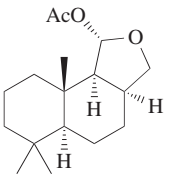

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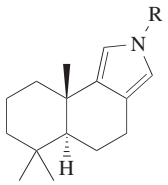
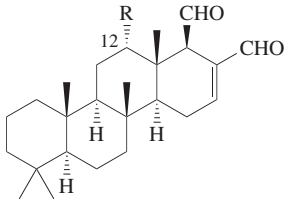


(281)

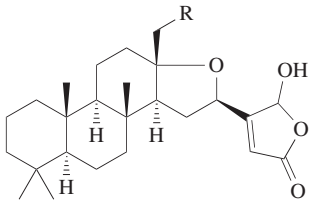
Two dialdehyde sesterterpenoids, 12-deacetoxyscalaradial (**282**) and scalaradial (**283**), were isolated from a marine sponge *Cacospongia mollior* collected near Naples.[206] These two dialdehydes showed interesting features in their bioactivities. Both compounds possess the same dialdehyde functionality as polygodial (**249**).

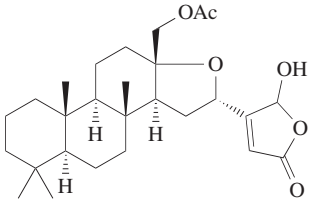


(282) R = H
(283) R = OAc

Polygodial (**249**) is known to have suitable distances between the two aldehyde groups and an adjacent intracycle unsaturation, and to exhibit antifeedant activity and a hot taste for humans (see Section 8.07.7.2). The scalaradial molecule (**283**), which embodies all these structural features, was tasteless and displayed the same antifeeding effects on fish as polygodial (**249**), but at twice the concentration of (**249**). On the other hand, 12-deacetoxyscalaradial (**282**) showed a similar biological response to polygodial (**249**). On comparing the activities of (**282**) and (**283**), the two compounds were active in the fish feeding inhibition bioassay at concentrations of 30 μg cm^{-2} and 60 μg cm^{-2}, respectively, while (**282**) only was hot to the taste. These results showed that molecular size was not a restrictive factor in these activities, but pointed out the specific importance of the substituent at C-12 in (**282**) and (**283**), or in the equivalent C-1 position of a supposed polygodial derivative. The presence of a bulky substituent in this position, such as the acetoxy group in (**283**), may inhibit the biological activity of the metabolite, either by altering the conformation of the nearby aldehyde group, or altering the surface complementarity between the molecule and the binding site of the receptor.

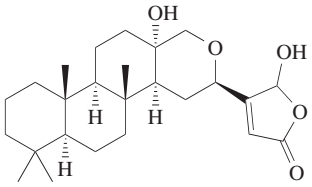
Chemical studies of the Caribbean sponge *Cacospongia* cf. *linteiformis* (order Dictyoceratida, family Thorectidae) led to the isolation and characterization of a number of bioactive cyclic sesterterpenes without dialdehyde functionality, namely lintenolides A–E (**284**)–(**288**). Antifeedant tests of the *Carassius auratus* fish indicated for lintenolides A–E (**284**)–(**288**) a high activity at a concentration of 30 μg cm^{-2} of food pellets, suggesting their potential role as feeding deterrents.[207,208]

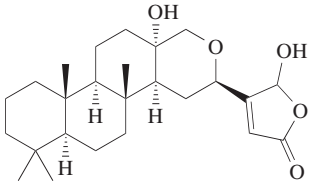




(284) R = OAc
(286) R = H

(285)

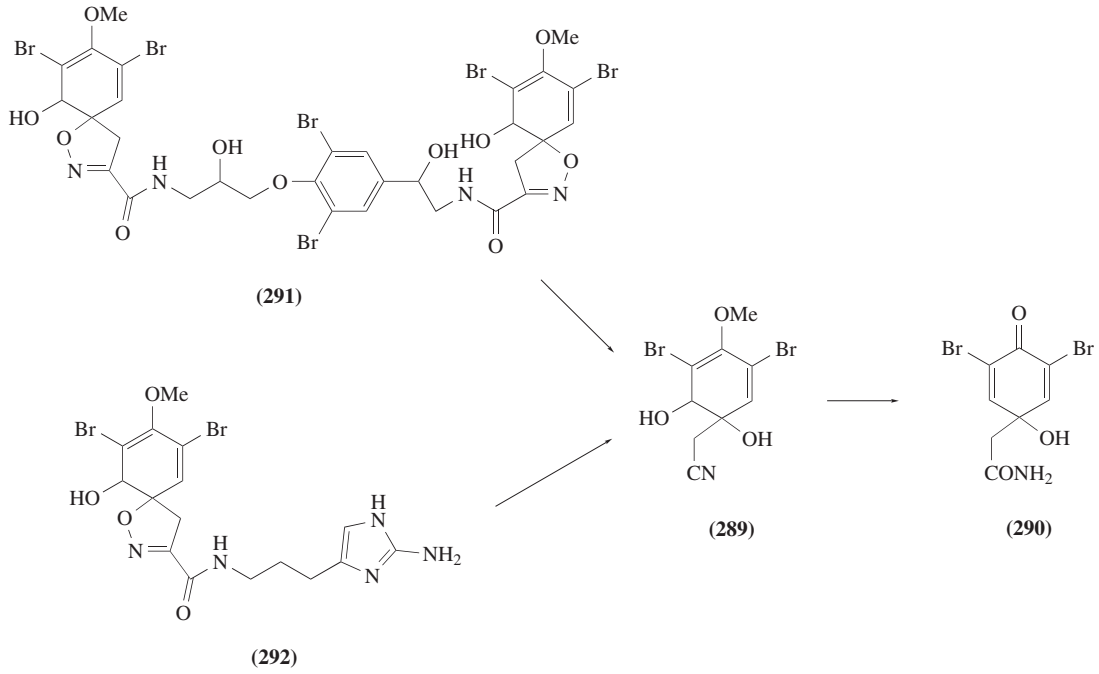




(287)

(288)

Sponges of the order Verongida are rich sources of brominated secondary compounds, which are biogenetically thought to be derived from bromotyrosine. The Mediterranean sponge *Verongia aerophoba*, found around the Canary Islands, was exceptionally rich in brominated compounds, and it was revealed that the bioactive sponge constituents aeroplysinin-1 (**289**) and the dienone (**290**) are biotransformation products which originate from the biologically inactive or weakly active precursors isofistularin-3 (**291**) and aerophobin-2 (**292**) by enzymatically catalyzed conversions following breakdown of the cellular compartmentation (Scheme 8).[209]



(291)

(289)

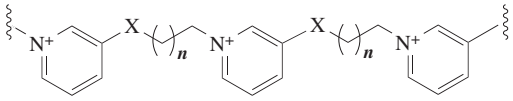
(290)

(292)

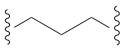
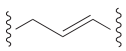
Scheme 8

The enzymatically catalyzed biotransformation processes of (**291**) and (**292**) into (**289**) and (**290**) were suggested by the following observations. (i) HPLC analysis of the 100% MeOH, 50% aqueous MeOH, or 100% H_2O extracts of the *V. aerophoba* sponge showed that (**291**) and (**292**) were the dominating brominated constituents in the 100% MeOH extract in which (**289**) and (**290**) were completely missing, whereas in the 50% aqueous MeOH extract the amounts of (**289**) and (**290**) increased and both (**291**) and (**292**) decreased, and in the aqueous extract (**290**) was dominant. (ii) In the absence of freeze-dried sponge tissue (**291**) as well as (**292**) proved stable when incubated in the presence of MeOH, 50% aqueous MeOH, or 100% H_2O. When exogenous (**291**) or (**292**) was added to lyophilized sponge tissue, the amount of (**290**) formed increased by 20–30% compared with controls lacking exogenously supplied substrate. (iii) No conversion of (**291**) or (**292**) was observed when the assay was carried out using heated sponge at 90 °C for 5 min prior to incubation. This enzymatic conversion was of special interest with regard to the biological activities of these bromotyrosine metabolites. The products of biotransformation, (**289**) and (**290**), were antibiotically active against several Gram-positive and Gram-negative bacteria, whereas the substrates, (**291**) and (**292**), proved to be completely inactive in the same experiments. In addition, (**289**) and (**290**) were found to exhibit pronounced cytotoxicity toward human carcinoma cells (Hela cells) with similar IC_{50} (50% inhibitory concentration) values (3.0–3.2 μM) for both compounds, while compounds (**291**) and (**292**) were revealed to be less cytotoxic (IC_{50} values: 8.5 μM and 99 μM, respectively). The antibiotic activity and the cytotoxicity of (**289**) and (**290**) could be relevant for the chemical defense of *V. aerophoba*, suppressing, for example, an overgrowth of fouling organisms. These compounds are an example of a stress-induced defense mechanism by the enzymatic conversion of preformed biologically inactive storage compounds into highly active defense metabolites. On the other hand, samples of *V. aerophoba* from different islands were collected and HPLC analysis of their extracts revealed that the pattern of brominated compounds was almost superimposable, indicating *de novo* biosynthesis by the sponge or by endosymbiotic microorganisms rather than uptake by filter feeding. The only differences observed between the different samples analyzed were with regard to the total concentrations of brominated compounds which varied from 7.2% to 12.3% of the dry weight, depending on the collection site.[210]

During examination of Porifera collected along the coast of the Bahamas, strong antifeedant activity was discovered in a crude extract of the sponge *Amphimedon compressa*. The MeOH-toluene (3:1) extract of this sponge was extracted successively with EtOAc and Bu^nOH, and the Bu^nOH-soluble material showed strong antifeedant activity. Chromatographic purification of the *n*-BuOH-soluble fraction yielded a polymeric pyridinium alkaloid named amphitoxin (**293**).[211] In laboratory feeding experiments, purified amphitoxin (**293**) deterred feeding of the fish *Thalassoma bifasciatum* at a concentration of 1 mg ml^{-1}, which was one-sixth of its natural concentration; this strong bioactivity suggested a role in the chemical defense of *A. compressa*.

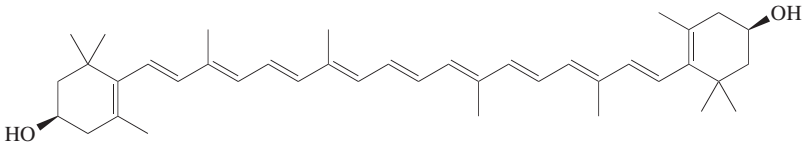


(293)

X =  or  (ratio about 1:1)

$n = 5$ (5 is a mean value in saturated chains)

As part of a study of the origins of biologically active substances in marine sponges, zeaxanthin [(3*R*,3′*R*)-dihydroxy-*β*,*β*-carotene (**294**)], which is one of the widely distributed carotenoids in marine organisms, was found to be produced by two species of marine bacteria *Flexibacter* sp., associated with the marine sponge *Reniera japonica*.[212] This carotenoid was also detected in the host sponge, suggesting the transport of zeaxanthin (**294**) from the microorganisms to the host. As zeaxanthin (**294**) plays the role of a quencher and scavenger for active species of oxygen, it was presumed that the sponge accumulates the bacterial product as a defense substance against the active oxygen species produced under irradiation by strong sunlight. It was thought that the bacteria are symbionts of the host sponge and act by obtaining the solid substrate and medium needed for settlement and growth from the host, and by producing and transmitting the biologically active substance to the host.

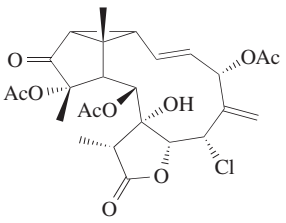


(294)

Scientific dredging operations in Prydz Bay, Antarctica, over the summer of 1990/1991, yielded many collections of marine sponges, including two specimens of the Antarctic marine sponge *Tedania charcoti* from geographically distinct locations. One specimen was collected from trawling around 67 °S, 71 °E at a depth of 439 m and at a water temperature of −2.1 °C, while a second *T. charcoti* was obtained from trawling around 67 °S, 78 °E at a depth of 251–266 m and at a water temperature of −1.6 °C. The aqueous ethanol extract of both these sponges proved to exhibit potent antibacterial properties, inhibiting the growth of strains of the bacteria *Staphylococcus aureus*, a *Micrococcus* sp., a *Serratia* sp., and *Escherichia coli*, as well as the ability to modulate protein phosphorylation in chicken forebrain. Detailed chemical investigations revealed the biological activity to be due to extraordinarily high levels of both cadmium and zinc.[213] It is noteworthy that *Tedania charcoti* possesses the ability to sequester both cadmium and zinc from seawater, where they are present in only trace amounts, as well as to tolerate the accumulation of extraordinarily high levels of these toxic heavy metals. It may be speculated that the high concentration of cadmium and zinc accumulated by *T. charcoti* serves as a natural antibiotic and/or an antifouling agent and/or a toxic defense against predation. It was therefore suggested that there is a possibility that at least some biologically active responses of extracts of marine organisms may be due to inorganic rather than organic substances.

8.07.7.4 Other Invertebrates

The Caribbean encrusting gorgonian octocoral *Erythropodium caribaeorum* deters predation by reef fish. The crude lipid-soluble extracts of *E. caribaeorum* incorporated into carrageenan food strips at the same volumetric concentration as it occurred at gorgonian tissues deterred feeding of a natural assemblage of fish on the reef from which the gorgonian had been collected. Fractionation of the crude extract revealed that the feeding-deterrent effects were present in a fraction containing chlorinated diterpenoids such as erythrolides A (**295**), B (**296**), and D (**297**), while a fraction contained sesquiterpene hydrocarbons in which erythrodiene (**298**) was found as a major compound. Of the three diterpenes, only (**296**) and (**297**), when assayed independently, inhibited feeding of reef



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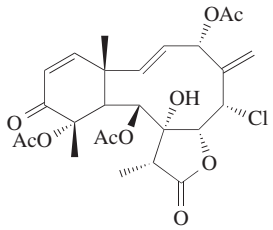

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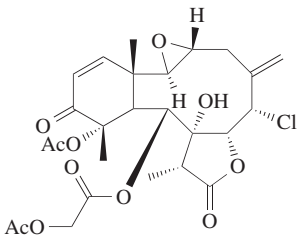

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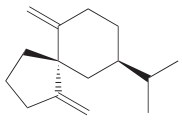
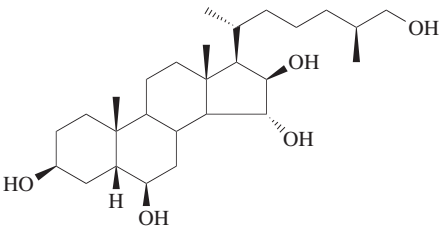


(298)

fish at the natural concentrations found in *E. caribaeorum*. Thus, the erythrolides appear to defend *E. caribaeorum* from reef predators, while the function of sesquiterpene hydrocarbons like (**298**), which occur in high concentrations in the lipid-soluble extracts of many octocorals, remained unclear.[214]

The starfish *Luidia clathrata* of the northern Gulf of Mexico showed feeding-deterrent properties against marine fish. The body-wall tissues of *L. clathrata* were rejected by the pinfish *Lagodon rhomboides* significantly more frequently than control tissues. Pellets containing EtOH body-wall extract (3 mg ml^{-1} agar) and krill were rejected significantly more often than control pellets containing only krill. Pellets containing body-wall extracts at 0.75 mg ml^{-1} agar were consumed with equal frequency to control pellets. The fish antifeedant activity noted for pellets containing natural tissue concentrations of body-wall extract of *L. rhomboides* indicated the presence of compounds that deter fish predators. From the EtOH extract of the starfish *L. rhomboides* 10 new polyhydroxysteroids (**299**)–(**308**) were isolated, and these polyhydroxysteroids were likely to be responsible for fish antifeedant activity, but the comparatively large amount of compound required



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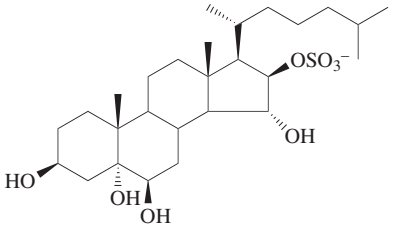

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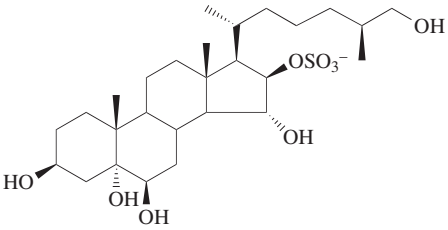

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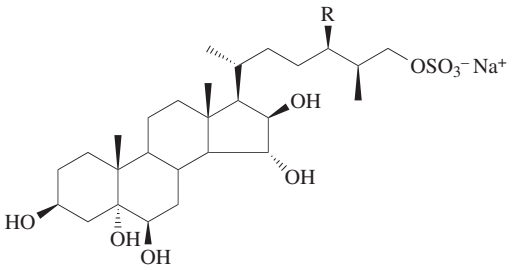
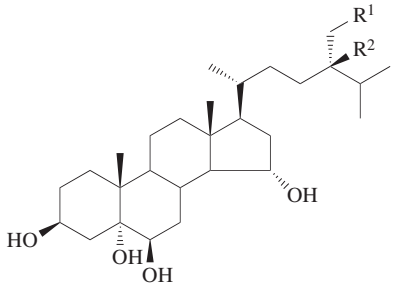
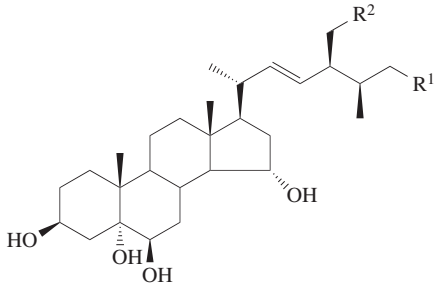


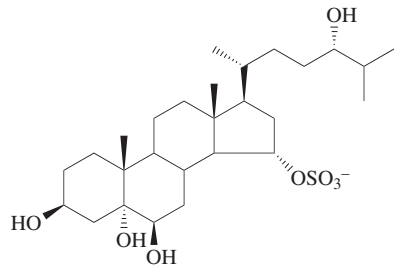
(302) R = H
(303) R = Me



(304) $R^1 = CH_2OSO_3^- Na^+$, $R^2 = H$
(307) $R^1 = OSO_3^- Na^+$, $R^2 = OH$



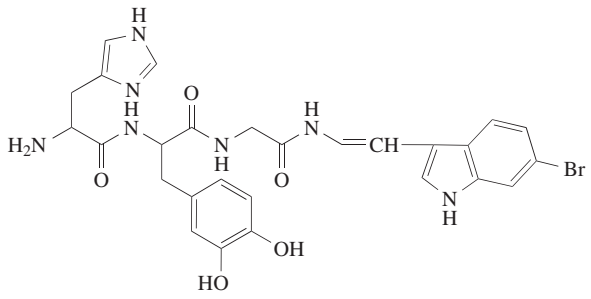
(305) $R^1 = OSO_3^- Na^+$, $R^2 = H$
(306) $R^1 = H$, $R^2 = OSO_3^- Na^+$



(308)

in the fish antifeedant assay precluded this determination. In addition, the EtOH body-wall extract of the starfish *L. rhomboides* significantly inhibited attachment of competent cyprid larvae of the barnacle *Balanus amphitrite* and larvae of the bryozoan *Bugula neritina* at concentrations of 3.0, 0.6, and 0.12 mg ml^{-1} of seawater. The EtOH body-wall extract of *L. rhomboides* also inhibited the growth of two species of Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, at a concentration of 0.75 mg per disk. Purified polyhydroxysteroid compounds tested were all active in inhibition assay of the settlement of larvae, while the antibacterial activity was not necessarily observed in all purified polyhydroxysteroid compounds tested, thus indicating that some polyhydroxysteroids may display a broad spectrum of bioactivity, while others may have specific functional roles.[215]

It has been proposed that hemocytes play important roles in the defense mechanisms of ascidians. Two antibacterial substances, halocyamines A (**309**) and B (**310**) were isolated from the hemocytes of the solitary ascidian *Halocynthia roretzi*,[216] and they were tetrapeptide-like substances containing one bromine atom and were present only in the hemocytes. Both possess antimicrobial activity against several kind of Gram-positive bacteria and yeasts, and against a highly antibiotic-sensitive strain of Gram-negative bacterium. They also showed cytotoxic activity against some cultured mammalian cells. Halocyamine A (**309**) inhibited *in vitro* the growth of two fish RNA viruses, infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus. Pretreatment of RNA virus with halocyamine A (**309**) reduced the infectivity of the virus toward host cells. The growth of marine bacteria *Achromobacter aquamarinus* and *Pseudomonas perfectomarinus* was also inhibited by halocyamine A (**309**) but that of *Alteromonas putrefaciens* and *Vibrio anguillarum* was not. These results suggest that halocyamines may have a role in the defense mechanisms of *H. roretzi* against marine viruses and bacteria.[217]



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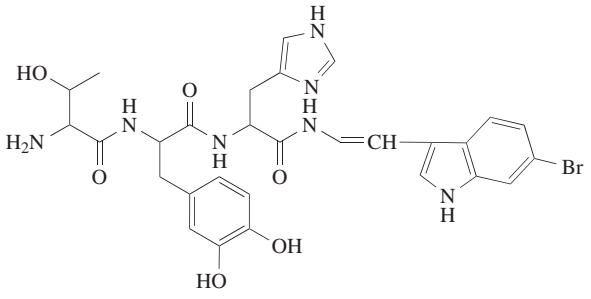
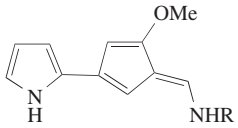


(310)

Research with the marine ascidian *Atapozoa* sp. and its nudibranch predators showed the direct chemical link in predator–prey associations involving ascidians and physically vulnerable mollusks and has demonstrated the *in situ* ichthyodeterrent properties of the *Atapozoa* secondary metabolites.[218] Bipyrrole metabolites such as tambjamines A (**311**), C (**312**), E (**313**), and F (**314**) were found in the organic extract of *Atapozoa* sp. and its nudibranch predators of the genus *Nembrotha*.

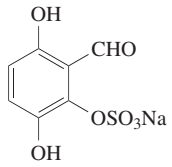
The unpalatability of *Atapozoa* larvae to coral reef fish and the presence of deterrent quantities of tambjamine C (**312**) in these larvae, along with *in situ* feeding deterrent properties of tambjamine class alkaloids, provides convincing evidence for the chemical protection of *Atapozoa* larvae. Microscopic examination of *Atapozoa* revealed that its intense pigmentation is confined to the granular



(311) R = H
(312) R = $CH_2CH(Me)_2$
(313) R = CH_2CH_3
(314) R = CH_2CH_2Ph

amebocyte blood cells. Thus, the large quantity (0.5–1.7% dry weight) of the brightly yellow-colored tambjamines should therefore reside within these blood cells. A transmission electron microscope investigation of *Atapozoa* larvae, performed expressly for the purpose of identifying bacterial symbionts, failed to find any significant quantities of bacteria within the granular amebocytes or in any other part of this ascidian. Thus, *Atapozoa* seem to be capable of the *de novo* biosynthesis of the tambjamines.[219]

A sulfated polyhydroxy benzaldehyde, polyclinal (**315**), was isolated from extracts of the temperate colonial ascidian *Polyclinum planum* and the structure was solved by an X-ray crystallographic study. The distribution of polyclinal (**315**) within three distinct regions of the ascidian colonies was investigated. The colonies were dissected into the stalk, the pulpy inner mesenchyme, and the zooid-rich surface layer. The concentration of polyclinal (**315**) in these different colony parts, based on wet weights, were determined to be 5.8×10^{-5} g g^{-1}, 7.8×10^{-4} g g^{-1}, and 2.5×10^{-3} g g^{-1} in the stolons, the cortex, and the zooid-rich outer layers of the colonies, respectively. The higher concentration of polyclinal (**315**) in the zooid-rich surface layer of the colonies suggested that polyclinal (**315**) may function as a chemical defense against predators, which would be consistent with the observation that predator-deterrent gorgonian secondary metabolites were also distributed in the outer more accessible portions of the colonies.[220]



(315)

8.07.7.5 Fish

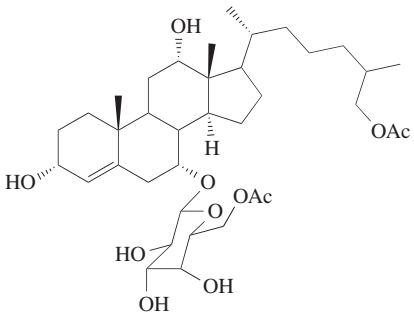
Soles of the genus *Pardachirus* are characterized by their chemical defense against predation with a copious and ichthyotoxic secretion being discharged upon disturbance. Two chemical classes of bioactive entities, namely, steroid monoglycosides and amphiphilic peptides, were isolated from each secretion of two different biological species.[174] Toxic material contained in the mucosal secretion of the Pacific sole *Pardachirus pavoninus* was investigated to isolate three ichthyotoxic and strongly surfactant peptides, which were named pardaxin P-1 (**316**), P-2 (**317**), and P-3 (**318**).[221]

(**316**) Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu

(**317**) Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Ile-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Gly-Gln-Glu

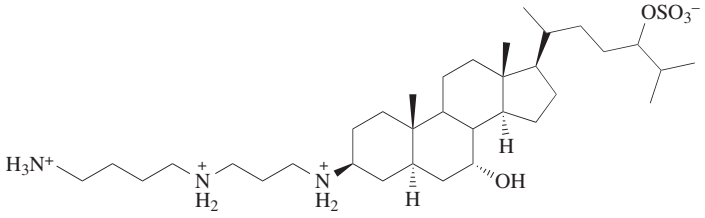
(**318**) Gly-Phe-Phe-Ala-Phe-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu

These peptides have nearly identical sequences and contain both an unusually larger number of serine residues (including a Ser-27–Ser-28–Ser-29 segment) and a large number of hydrophobic residues such as leucines and isoleucines. These 33 amino acid polypeptides fold into ordered structures in trifluoroethanol–water solution and in micelles but adopt a random-coiled structure in aqueous solution. The complete proton NMR spectrum of pardaxin P-2 (**317**) was assigned in CF_3CD_2OD/H_2O (1:1) solution, and the 3D structure was elucidated with distance-restrained molecular dynamics calculations. It was demonstrated that peptide segments within the 7–11 and 14–26 residue stretches were helical while residues at the C- and N-terminus exist predominantly in extended conformations in solution. The dipeptide 12–13 segment connecting the two helices exists as a bend or a hinge allowing the two helices to be oriented in an L-shaped configuration. From these studies pardaxin P-2 (**317**) was shown to adopt a novel amphiphilic helix (7–11)-bend (12–13)-helix (14–26) motif with Pro-13 forming the focal point of the turn or bend between the two helices.[222] The structure and activity of pardaxin and analogues were studied by assaying 13 synthetic pardaxin analogues for their ability to interact with model membranes of phosphatidylcholine. As a result, it was found that (i) an amphipahtic α-helix from isoleucine-14 to leucine-26 was responsible for most of the membrane-perturbing properties of pardaxin; (ii) a hydrophobic N-terminal region enhanced the activity of the isoleucine-14 to leucine-26 α-helix by binding the pardaxin molecule to the lipid bilayer; (iii) a bend centered around ^{12}Ser–^{13}Pro appeared to create overall amphipathicity for the two different helical regions of pardaxin, but this contributed only slightly to potency; and (iv) the C-terminal amino acids were unimportant for membrane-perturbing activity and may be present only to enhance transportation in an aqueous environment prior to membrane binding in the native system.[223] The mechanism of action of pardaxin as well as steroid glycosides such as mosesin-1 (**319**) was investigated based on the permeabilization assay method of a phospholipid bilayer to suggest that it may well be attributed to nonspecific derangement of animal cell membrane without binding to any particular biomolecule. The N-terminal region of pardaxins was revealed not only to function as the strong binding region, but also to enhance the deranging activity (putatively of the α-helical region at the middle of the 33 amino acid sequence) by a factor of 6 when bound. On the other hand, defensive steroid glycosides like (**319**) were shown to be indifferent to the cholesterol content in the lipid bilayer in their permeabilizing action, being unlike the conventional hemolytic saponins. In addition, different kinetics between the steroid glycosides and pardaxins implied that the action of the former is rather transient while that of the latter is persistent, resulting in all-or-none rupture of liposomes. An extent of synergism, possibly with such differently allotted functions in the defensive action, was also shown between the two chemical entities.[224] The conformations of synthetic peptides of different length corresponding to the amino-terminal, central, and carboxyl-terminal regions of pardaxin were studied by circular dichroism spectroscopy.[225] The peptide segments that adopt an ordered conformation showed a similar conformation when present in the entire toxin as suggested by proton magnetic resonance data,[222] and the amino-terminal and central regions of the toxin was indicated to play a role in initiating and maintaining an ordered conformation of pardaxin.



(319)

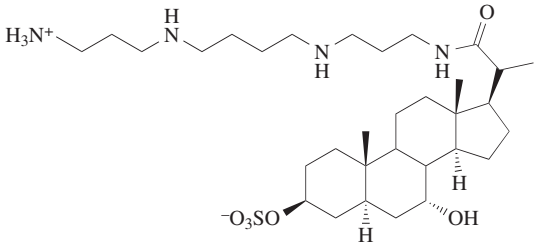
A water-soluble cationic steroidal antibiotic, named squalamine (**320**), was isolated from the dogfish shark *Squalus acanthias*. This aminosterol antibiotic (**320**) exhibited potent bactericidal activity against both Gram-positive and Gram-negative bacteria. In addition, squalamine (**320**) was fungicidal and induced osmotic lysis of protozoa. Squalamine (**320**) is an aminosterol characterized by condensation of an anionic bile salt intermediate with spermidine, which is without precedent in vertebrates. The discovery of squalamine (**320**) implicated a steroid as a potential host-defense agent in vertebrates.[226]



(320)

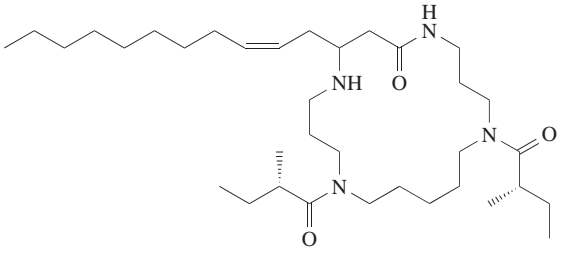
Squalamine (**320**) was distributed in many tissues of the shark. The liver and gall bladder, the organs in which bile salts are synthesized and stored for secretion into the gastrointestinal tract, were the richest sources identified (4–7 μg g^{-1} of tissue). However, both the spleen and the testes of this animal were also relatively rich sources of squalamine (**320**), each containing ~2 μg g^{-1}. The stomach (1 μg g^{-1}), the gills (0.5 μg g^{-1}), and the intestine (0.02 μg g^{-1}) yielded smaller amounts. It was clear that squalamine (**320**) was also present in organs that were not engaged in the synthesis of bile salts for digestive functions. It was not certain whether a single organ such as the liver was the principal site of synthesis of squalamine (**320**), and it was also possible that squalamine (**320**) was not synthesized by the shark, but rather, derived from an exogenous source present in the shark's food chain. This unusual steroid (**320**) would appear to be the product of an unknown biochemical pathway involving the condensation of spermidine with a steroid. It may be speculated that squalamine (**320**) serves as a systemic antimicrobial agent in this animal, but not direct evidence of this speculation was provided. The biosynthetic pathway of squalamine (**320**), its role in host defense, and its expression after injury and infection have yet to be investigated.

Syntheses of squalamine (**320**) and its mimics were investigated.[227] The mimic (**321**), which possessed a pendant sulfate and a spermidine group at the opposite placement to those of (**320**), exhibited potent antibiotic properties against a broad spectrum of microorganisms.[228] In addition, the mimic (**321**) possessed unusual ionophoric properties and recognized negatively charged phospholipid membranes; (**321**) favored the transport of ions across negatively charged bilayers over ones that are electrically neutral.[229]



(321)

A lipophilic ichthyotoxin from the defensive mucous skin secretion of soapfish *Diploprion bifasciatum* and *Aulacocephalus temmincki*, named lipogrammistin-A (**322**), was isolated and chemically characterized. Lipogrammistin-A (**322**) not only differed in its chemical type from all other known defense substances of fish, but also was the first macrocyclic polyamine lactam found in the animal kingdom. It showed toxicity to medaka fish and to mice, and hemolytic activity to rabbit erythrocytes. A synthetic study of (**322**) was also performed for further chemical and biological investigation of this compound.[230]



(322)

8.07.8 MARINE TOXINS

8.07.8.1 Cone Shells

8.07.8.1.1 Conus geographus

The cone shells that inhabit tropical and subtropical seas are carnivorous gastropods which catch prey organisms such as fish and shellfish by shooting a venom-containing harpoon-like radular tooth. The venom of one species of cone shell (anboina, *Conus geographus*) is so potent that occasionally it is responsible for human fatalities. Thus, the anboina is called "habu-gai" (shellfish that is dangerous like a venomous snake) and is feared in the Okinawan Island of Japan. On the other hand, cone shells are loved by shell collectors all over the world because of their beautiful colors and shapes. The family Conidae includes approximately 400 species, most of which inhabit the Okinawan Islands.

Cone shells are divided into three groups according to their major prey organisms. A majority feed on various marine worms such as polychaetes (vermivorous); a smaller number prey on shellfish (molluscivorous); and a few feed on small bottom fish (piscivorous). Each species elaborates a venom that reflects the dominant target animals. The venoms of molluscivorous cone shells are highly venomous to gastropods, but are almost ineffective on mice. On the other hand, the venoms of piscivorous Conidae are fatal to both fish and mice. Further, the symptoms in mice differ depending on the species of cone shell involved. These observations suggest a variety of bioactive principles of cone shell venoms among various species of Conidae.

In the early 1950s, Kohn started ecological studies on cone shells,[231] and Endean *et al.* expanded these ecological studies to the toxicology, pharmacology, and biochemistry of Conidae venoms using crude venoms.[232] In 1980, Kobayashi *et al.* started the screening of venoms from about 30 species of cone shells, collected in Okinawa, Japan, by monitoring the pharmacological action on isolated mammalian muscle preparations including skeletal, cardiac, and smooth muscles.[233] In the course of this project, Kobayashi *et al.* have isolated numerous new bioactive compounds with a variety of chemical and pharmacological properties from representative species of worm-, shellfish-, and fish-eating cone shells, and have shown that most of these new compounds are peptide toxins acting on ion channels of the cell membrane.

Among various carnivorous gastropods, cone shells are in a unique position because of the highly specialized radular teeth used as their feeding apparatus. Herbivorous gastropods have hundreds of tiny chitinous radular teeth arranged as in a grater; they shave their algal food with these radular teeth. In the case of cone shells, on the other hand, one central radular tooth has been developed to become a harpoon. The venom apparatus of cone shells consists of a muscular venom bulb, a long coiled venom duct, a radular sheath, radular teeth, and a proboscis. The venom produced in the venom duct is transferred to the radular sheath, where hollow radular teeth are charged with the venom. The bulb contains no venom and is hence thought to provide a pump that transfers the venom from the duct into the radular sheath, which has tens of harpoon-like chitinous radular teeth. The teeth are set one by one along the pharynx to the proboscis, where the foremost tooth is held with its pointed tip forward like a spear. Stinging is accomplished through the thrust of a radular tooth by the proboscis into the prey organism. A tooth is used only once, and if it fails to shoot the prey, that tooth is discarded and a new one from the radular sheath is charged with the venom. The length of radular teeth ranges from several millimeters to 1 cm. The radular tooth has a barb near the tip which prevents it from slipping out once it has taken hold.

Conus geographus is called "anboina" as the animal's habitat is the Gulf of Anbon, Indonesia. The shells are about 10 cm in length. *C. geographus* is highly toxic and responsible for human injury. Fatal cases have been reported from Okinawa, Japan. Collectors are usually stung while swimming with their catch in a net bag on their side or while attempting to examine the shell markings. Stings by *C. geographus* immediately produce severe pain and numbness at the injured area. Then numbness spreads around the mouth and to the extremities. In most cases, this is accompanied by vomiting, dizziness, lachrymation, hypersalivation, and chest pain. In severe cases dyspnea, dysphagia, aphonia, blurring of vision, motor incoordination, generalized pruritus, respiratory paralysis, and death follow.

It has been reported that the crude venom of *C. geographus* causes an inhibitory effect on the contractile response of stimulated skeletal muscle. Kobayashi and co-workers isolated two peptide toxins composed of 22 amino acid residues including 3-hydroxyprolines by monitoring the pharmacological activity.[234] The primary structures of the two peptides named geographutoxins I and II (GTX I and II, (**323**) and (**324**), respectively) are very similar to each other.[235] They differ by four

amino acid residues, at positions 8, 14, 18, and 19 from the C terminal. The substitution of the amino acids was explained by single-base substitution in the triplet codons except for the substitution at residue 18 of Gln in GTX I by Met in GTX II. Distinctive features of the primary structures are a basic amino acid cluster in the middle of the sequence, and a Cys–Cys sequence in both N and C terminal regions. The LD_{50} (50% lethal dose) values (i.p.) of GTX I and II in mice are 340 μg kg^{-1} and 110 μg kg^{-1}, respectively.

(**323**) Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2

(**324**) Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Arg-Lys-Cys-Lys-Asp-Arg-Arg-Cys-Lys-Hyp-Met-Lys-Cys-Cys-Ala-NH_2

GTX II (**324**) at very low concentrations inhibits twitch responses of skeletal muscle.[236] The IC_{50} value of GTX II (3×10^{-8} M) is somewhat higher than that of tetrodotoxin (1×10^{-8} M). GTX II inhibited action potentials of skeletal muscle without change in the resting potential. Surprisingly, action potentials of the crayfish giant axon, neuroblastoma N1E-115 cells, and cardiac muscle were not affected by GTX II even at high concentrations.[237]

Toxin-binding studies have shown that tetrodotoxin and saxitoxin bind at a common receptor site on sodium channels, which has been designated neurotoxin receptor site 1. GTX II inhibits [^{3}H]saxitoxin binding to sodium channels in skeletal muscle homogenates and T-tubular membranes in close agreement with concentrations that block muscle contraction.[238] However, [^{3}H]saxitoxin binding to membranes of the superior cervical ganglion is not affected by GTX II even at high concentrations. Scatchard analysis of [^{3}H]saxitoxin binding to T-tubular membranes revealed a primarily competitive mode of inhibition of saxitoxin binding by GTX II. These results indicate that GTX II interacts competitively with saxitoxin in binding at neurotoxin receptor site 1 of the sodium channel in a highly tissue-specific manner. Previously no differences had been found experimentally between muscle and nerve sodium channels because of their electrophysiological and pharmacological similarity. Thus, GTX II is the first reagent that discriminates at this site between nerve and muscle sodium channels. GTX II is used to identify these tissue-specific regions of the sodium channel structure at neurotoxin receptor site 1.[239]

Olivera and co-workers isolated from *C. geographus* peptide toxins of three classes, named α-, μ-, and ω-conotoxins.[240,241] α-Conotoxins (GI, GIA, GII, and MI) are composed of 13–15 amino acid residues including two S-S bridges per molecule and are homologous with one another (Table 17). These peptides cause postsynaptic inhibition at the neuromuscular junction and competitively inhibit α-bungarotoxin and curare alkaloid binding to nicotinic acetylcholine receptors. Many pharmacological studies have revealed that α-conotoxins are nicotinic receptor blockers. μ-Conotoxins GVIIIA, GVIIIB, and GVIIIC with 22 amino acid residues are rich in hydroxyproline. These peptide toxins block sodium channels in muscle much more effectively than those in nerve. Geographutoxins I and II (GTX I and II) may be identical with GVIIIA and GVIIIB, respectively. ω-Conotoxins GVIA, GVIB, GVIC, GVIIA, and GVIIB, called "shaker peptides," were also isolated from *C. geographus*. These peptides, consisting of 25–29 amino acid residues, induce a persistent tremor when injected intracerebrally in mice. The transmitter release at the neuromuscular junction of skeletal muscle was blocked by ω-conotoxins GVIA, GVIB, and GVIC, whereas it was not affected by other ω-conotoxins (GVIIA and GVIIB). Electrophysiological experiments indicate that ω-conotoxin GVIIA acts by blocking calcium channels without affecting the propagation of action potentials in the nerve terminal.[242] ω-Conotoxins may provide a valuable tool for defining structural differences among the calcium channel subtypes in muscle and nerve.

The *Conus* peptides are used in hundreds of research laboratories for a wide variety of physiological and pharmacological investigations in both vertebrate and invertebrate nervous systems. Some *Conus* peptides such as ω-conotoxin GVIA have become well-established neurobiological tools, and syntheses of these peptides have also been investigated. For example, μ-conotoxin GIIIA, a 22-amino acid peptide paralytic toxin inhibiting the muscle voltage-activated sodium channels, was synthesized by a solid-phase method. No purification of intermediates was necessary for the synthesis, and a simple air oxidation of the deprotected crude peptide gave the desired toxin.[243] Three photoreactive derivatives of μ-conotoxin GIIIA were also prepared as photoaffinity labeling reagents for muscle-type sodium channels. The reagents competitively inhibited the binding of saxitoxin to the eel sodium channel with K_i values of 11–18 nM. The introduced chromogenic phenyldiazirine group on the toxin was photolyzed efficiently, and spectroscopic properties of the

Table 17 Amino acid sequences of conotoxins.

α	GI	E C C N P A C G R H Y S C[a]
	GIA	E C C N P A C G R H Y S C G K
	GII	E C C H P A C G K H F S C[a]
	MI	G R C C H P A C G K N Y S C[a]
μ	GVIIIA	R D C C T Hy Hy K K C K D R Q C K Hy Q R C C A[a]
	GVIIIB	R D C C T Hy Hy R K C K D R R C K Hy M K C C A[a]
	GVIIIC	R D C C T Hy Hy K K C K D R R C K Hy L K C C A
ω-"Shape-Blocker"	GVIA	C K S Hy G S S C S Hy T S Y N C C R – S C N Hy Y T K R C – – Y[a]
	GVIB	C K S Hy G S S C S Hy T S Y N C C R – S C N Hy Y T K R C – – Y G
	GVIC	C K S Hy G S S C S Hy T S Y N C C R – S C N Hy Y T K R C
ω-"Shaker"	GVIIA	C K S Hy G T Hy C S R G M R D C C T – S C L L Y S N K C R R Y
	GVIIB	C K S Hy G T Hy C S R G M R D C C T – S C L S Y S N K C R R Y
	MVIIA	C K G K G A K C S R L M Y D C C T G S C R – – S G K C[a]
"Sleeper"	GV	G E γ γ L Q γ N Q γ L I R γ K S N[a]

[a] The α-carboxyl is known to be amidated; absence of [a] indicates that no assignment has been made. A (Ala), C (Cys), D (Asp), E (Glu), F (Phe), G (Gly), H (His), K (Lys), L (Leu), N (Asn), P (Pro), Q (Glu), R (Arg), S (Ser), T (Thr), Y (Tyr), γ (γ-carboxylglutamate), Hy (*trans*-4-hydroxyproline).

reagents demonstrated that irradiation and detection can be performed in a spectral region where the absorptions due to most biological macromolecules are negligible.[244]

Structural studies of multiple disulfide cross-linked *Conus* peptides have been conducted. The three intramolecular SS linkages of geographutoxin I (GTX I, μ-conotoxin GIIIA) were examined to show that the SS bridges were between cysteines Cys3 and Cys15, Cys4 and Cys20, and Cys10 and Cys21, indicating that GTX I has a rigid conformation consisting of three loops stabilized by these three SS linkages.[245] The 3D structure of conotoxin GIIIA was determined in aqueous solution by 2D proton NMR and simulated annealing based methods. On the basis of 162 assigned nuclear Overhauser effect (NOE) connectivities obtained at the medium field strength frequency of 400 MHz, conotoxin GIIIA is characterized by a particular folding of the 22-amino acid peptide chain, which is stabilized by three disulfide bridges arranged in a cage at the center of a discoidal structure of approximately 20 Å diameter. The seven cationic side-chains of lysine and arginine residues project radially into the solvent and form potential sites of interaction with the skeletal muscle sodium channel for which the toxin is a strong inhibitor. These results may provide a molecular basis to elucidate the remarkable physiological properties of this neurotoxin.[246]

The amino acid sequence of μ-conotoxin GIIIA (geographutoxin I) was modified by replacing each residue with Ala or Lys to elucidate its active center for blocking sodium channels of skeletal muscle. NMR and CD spectra were virtually identical between native and modified toxins, indicating the similarity of their conformation including disulfide bridges. The inhibitory effect of these modified peptides on twitch contractions of the rat diaphragm showed that Arg at the 13th position and the basicity of the molecule are crucial for the biological action. The segment Lys11–Asp12–Arg13 is flexible, and this may represent a clue to the subtle fit of Arg13 to the specific site of sodium channels. Since known ligands to sodium channels, such as tetrodotoxin, anthopleulin-A, etc., contain guanidino groups as a putative binding moiety, Arg may be a general residue for peptide toxins to interact with the receptor site on sodium channels.[247] Studies of the solution structures of α-conotoxin GI as well as μ-conotoxin GVIA have been carried out using 2D NMR spectroscopy and various structural calculations.[248–250]

Olivera and co-workers also isolated sleeper peptides called conatokins from *C. geographus* (Table 17), which causes a sleep-like state in mice when injected intracerebrally. The conatokins contain 4–5 residues of γ-carboxyglutamate (Gla),[241] and the most well-characterized one, conatokin-G or GV, has five Gla residues[251] and blocks glutamate receptors of NMDA (*N*-methyl-D-aspartate) subtype.[252]

8.07.8.1.2 Other Conus *toxins*

Conus striatus, a piscivorous member of the Conidia, is not sufficiently venomous to be of potential danger to humans, but several severe cases of poisoning by *C. striatus* have been reported.

Kobayashi *et al*. have isolated a glycoprotein (molecular weight 25 000) named striatoxin (StTX) from this venom as a cardiotonic component.[253] Application of StTX to the isolated guinea-pig left atria caused a concentration-dependent inotropic effect; the effect of StTX was reversed in the presence of tetrodotoxin. In the atria, StTX provoked action potentials with a plateau phase of long duration without affecting the maximum rate of rise and amplitude of the action potential and the resting membrane potential. This prolongation was also reversed by tetrodotoxin. These results suggest that StTX causes prolongation of the action-potential duration probably due to slowed inactivation of sodium inward currents and that this may result in an increase in Ca^{2+} availability in cardiac muscle cells. This could explain the cardiotonic action of StTX. Furthermore, in mouse neuroblastoma cells StTX slowed sodium channel inactivation without affecting the time course of activation of the channels, and the voltage dependence of activation was shifted to more negative membrane potentials. The binding of saxitoxin or α-scorpion toxin to sodium channels was not affected by StTX, while that of batrachotoxin was slightly enhanced by the toxin. It may be concluded that StTX interacts with a new receptor site on sodium channels at which specific effects on channel inactivation can occur.[254] In the isolated guinea-pig ileum, StTX caused rhythmic transient contraction followed by relaxation, which was reversed by tetrodotoxin and a low-Na^+ medium. Pharmacological studies on StTX suggested that the contraction of the ileum induced by the toxin is due to the excitation of cholinergic nerves, while the relaxation is mediated through nonadrenergic inhibitory nerves.[255] The minimum lethal dose of StTX in a fish, *Rhodeus ocellatus smithi*, is 1 μg g^{-1} body weight.

The venom of *Conus magus* is lethal to fish and mice, as are those of *C. geographus* and *C. striatus*, although the average size of *C. magus* is only about 5 cm long and smaller than *C. geographus* and *C. striatus*. A myotoxin (MgTX), a peptide toxin with an estimated molecular weight of 1500, was isolated from the venom of *C. magus* by gel filtration.[256] MgTX elicits an increase in contractile force of the isolated guinea-pig ileum. The mode of action of MgTX is similar to that of the cardiotonic glycoprotein StTX isolated from *C. striatus*. Another active component (MAC) in the venom of *C. magus* was also purified and characterized as a protein of molecular weight between 45 000 and 65 000.[257] MAC exhibits rhythmic transient contractions of the guinea-pig ileum followed by relaxations and a marked increase in the contractile force of the guinea-pig left atria, as in the case of StTX. On the other hand, two peptide toxins, MI and MVIIA, were isolated from the Philippine *C. magus* by Olivera and co-workers.[241] The chemical and pharmacological properties of MI and MVIIA are different from those of MAC and MgTX isolated from the Okinawan *C. magus*, but resemble those of GII and GVIIA isolated from the Philippine *C. geographus*. MI and MVIIA are classified as α- and ω-conotoxins, respectively. MI showed no rhythmic contractions of the guinea-pig ileum, suggesting that the pharmacological properties of MgTX and MI differ from each other. Olivera and co-workers described the purification and first biochemical characterization of an enzymatic activity in venom from *C. magus*, and this enzyme, named conodipine-M, was a novel phospholipase A_2 with a molecular mass of 13.6 kDa, being comprised of two polypeptide chains linked by one or more disulfide bonds.[258]

Conus eburneus is a vermivorous member of the Conidae, which feeds on polychaete worms. The average size of the shell is about 6 cm long and they inhabit sandy seashores. From the venom of the Okinawan *C. eburneus* was purified a vascoactive protein (molecular weight 28 000) named eburnetoxin (ETX).[259] ETX induces contractions of isolated rabbit aorta, resulting from a remarkable increase in Ca^{2+} permeability in the cell membrane.

Conus tessulatus is a vermivorous member of the Conidae as is *C. eburneus*; the average size of the shell is approximately 5 cm long. A vasoactive protein (molecular weight 26 000) designated tessulatoxin (TsTX), was isolated from the venom of the Okinawan *C. tessulatus*.[260] The pharmacological action of TsTX is quite similar to that of ETX, the vasoactive substance from *C. eburneus*. Lazdunski and co-workers isolated a protein toxin consisting of two subunits (molecular weights 26 000 and 29 000) from the venom of the Madagascan *C. tessulatus*.[261] This protein toxin activates the Na^+–Ca^{2+} exchange system by increasing Na^+ permeability of the cell membrane to result in increased Ca^{2+} permeability of cardiac muscle cells, or to induce contraction of smooth muscle.

Conus textile is a molluscivorous member of the Conidae and its prey organisms are closely related molluscivorous Conidae. The average size of the shell is comparable to that of *C. geographus*. Kobayashi and co-workers examined the toxicity of the Okinawan *C. textile* venom against mice, but found that no mouse died even after intraperitoneal injection of a large amount of the venom. The human fatalities recorded in the literature, therefore, may result from the venomous sting of *C. geographus* rather than from that of *C. textile*. Arachidonic acid was isolated from the venom of *C. textile* as a principle of the powerful transient contraction of the isolated guinea-pig ileum. The

amount of arachidonic acid in the contents of the venom duct was estimated to be as much as 6 mg g^{-1} wet weight,[262,263] but the role of arachidonic acid in the venom remains uncertain.

From the snail *Conus imperialis*, which preys on polychaete worms, a peptide ligand for nicotinic acetylcholine receptors was isolated by Olivera and co-workers.[264] This peptide, named α-conotoxin ImI, was highly active against the neuromuscular receptor in frogs but not in mice. α-Conotoxin ImI had a sequence that shows striking sequence differences from all α-conotoxins of fish-hunting *Conus*, but its disulfide-bridging was similar. It was suggested that cone venoms may provide an array of ligands with selectivity for various neuronal nicotinic acetylcholine receptor subtypes.

The venom of *Conus nigropunctatus*, a piscivorous member of the Conidae, contained a new peptide toxin, named conotoxin NgVIA,[265] affecting sodium current inactivation, that competes on binding with δ-conotoxin TxVIA purified from *C. textile* venom.[266] The primary structure of conotoxin NgVIA had an identical cysteine framework and similar hydrophobicity as δTxVIA but differed in its net charge. These toxins may enable analysis of the functional significance of the receptor sites in gating mechanisms of sodium channels.

From the venom of the snail-hunting species *Conus marmoreus*, two new peptides, μO-conotoxins MrVIA and MrVIB, were isolated and characterized.[267] These toxins potently blocked voltage-sensitive sodium currents. μO-Conotoxin MrVIA was chemically synthesized and proved indistinguishable from the natural product. The μO-conotoxins showed no sequence similarity to the μ-conotoxins. μO-Conotoxins are as potentially important to sodium channel subtype differentiation as the ω-conotoxins are to calcium channels.

From the milked venom of the eastern Pacific piscivorous species, *Conus purpurascens* (the purple cone), three peptides that cause paralysis in fish were isolated, and the sequence and disulfide bonding pattern of one of these, αA-conotoxin PIVA, was disclosed. The peptide was chemically synthesized in a biologically active form. Electrophysiological experiments and competition binding with α-bungarotoxin demonstrated that αA-conotoxin PIVA acts as an antagonist of the nicotinic acetylcholine receptor at the postsynaptic membrane.[268] *C. purpurascens* uses two parallel physiological mechanisms requiring multiple neurotoxins to immobilize fish rapidly: a neuromuscular block and an excitotoxic shock. Among them, κ-conotoxin PVIIA inhibits the ''shaker'' potassium channel, while δ-conotoxin PVIA delays sodium-channel inactivation. In general, the evolution of excitotoxins may be influenced by how well the prey is initially tethered. A sea snake that has securely impaled a small fish with venom fangs only uses a neuromuscular block, whereas sea anemones, which initially contact their prey with a single tentacle, have convergently evolved Na^+ and K^+ channel-targeted excitotoxins analogous to those used by *C. purpurascens*.[269]

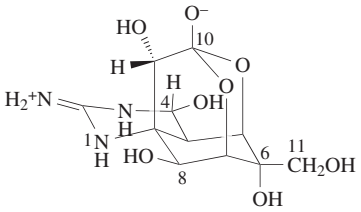
8.07.8.2 Tetrodotoxin and Saxitoxin

8.07.8.2.1 Tetrodotoxin

Tetrodotoxin (TTX (**325**)) is one of the best known marine natural products, being a toxin responsible for the fatal food poisoning caused by puffer fish.[270,271] TTX (**325**) possesses a unique chemical structure and exhibits potent neurotoxicity by specifically blocking the sodium channels of excitable cell membranes. The structure of TTX was elucidated simultaneously by an American and two Japanese groups in 1964.[272–274] The total synthesis of (±)-TTX was accomplished by Kishi *et al.* in 1972.[275,276] The etiology of TTX (**325**) is of interest because of the wide distribution of the toxin among genetically unrelated animals (Table 18).

Table 18 Distribution of TTX in animals.

	Organisms
Amphibians	newts, frogs
Fish	puffers (*Fugu*), breams (*Ypsiscarus*, *Scarus*), gobies (*Pomacanthus*)
Molluscs	gastropods (*Babylonia*), Octopoda
Echinoderms	starfish (*Astropecten*)
Anthropods	crabs (*Zosimus*)
Annelids	lugworms (*Pseudoptamilla*)
Nemerteans	Anopla (*Lineus*)
Flatworms	Polyclada
Red algae	Cryptonemiales (*Jania*)



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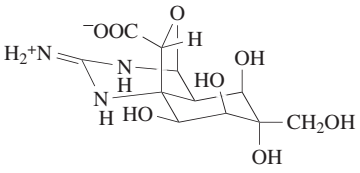
The marked variations in toxin concentrations in TTX-containing animals according to individual, region, and season suggested the exogenous origin of the toxin in those animals. This was further supported by the observation that puffers raised with artificial baits are unable to develop toxicity; TTX concentrations in cultured puffer fish, *Fugu rubripes*, were determined by indirect competitive enzyme immunoassay with a monoclonal antibody against the toxin, and the average concentrations in skin, muscle, liver, and vescera were 48.9, 11.6, 2.6, and 6.4 ng g^{-1}. These levels were very low and well within accepted tolerances.[277] The primary source of TTX was therefore investigated and demonstrated to be a marine bacterium.[278] The bacterium isolated from a red alga was first assigned to *Pseudomonas* sp., then to *Alteromonas* sp., and finally placed in a new species, *Shewanella alga*. The toxin was identified by Fast Atom Bombardment Spectrometry (FABMS), fluorometric HPLC, dose–survival responses in mice, and degradation into 2-amino-6-(hydroxymethyl)-8-hydroxyquinazoline. Subsequently, a broad spectrum of bacteria were reported to produce the toxin, such as *Vibrio* sp. from a xanthid crab *Atergatis floridus*[279] and *Pseudomonas* sp. from the skin of a puffer fish *Fugu poecilontus*.[280] The amount of toxin in the bacteria was, however, so small that chemists were not able to conduct biosynthetic studies.

In spite of the long history of TTX research, little is known about the biosynthetic origin of this unique molecule. The feeding of radioactive general metabolic precursors to toxic newts and their symbiotic bacteria failed to effect the incorporation of radioactivity into TTX.[281] As an alternative approach, the search for molecules which may provide clues for the biosynthetic pathway of TTX has been extensive. A highly sensitive HPLC method for analyzing TTX has been developed. This analyzer separated analogues on a reversed-phase column and detected fluorescent products produced by heating in sodium hydroxide solution.[282]

A number of new TTX analogues were isolated from puffers, newts, and a frog: tetrodonic acid (**326**), 4-epiTTX (**327**), and 4,9-anhydroTTX (**328**) from Japanese *Takifugu pardalis* and *T. poecilonotus*,[283] 11-norTTX-6(*R*)-ol (**329**) from *Fugu niphobles*,[284] and 11-oxoTTX (**330**) from Micronesian *Arothron nigropunctatus*.[285] From the Okinawan newt *Cynops ensicanda* 6-epi-TTX (**331**) and 11-deoxyTTX (**332**) were isolated,[286] and in this study the structural determination of



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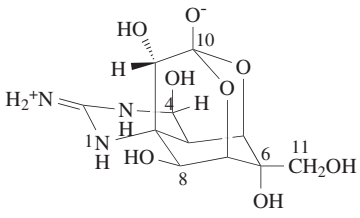

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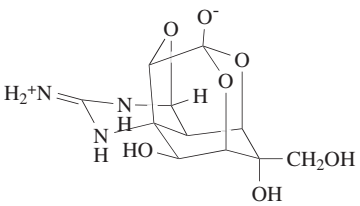

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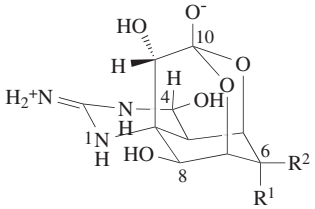
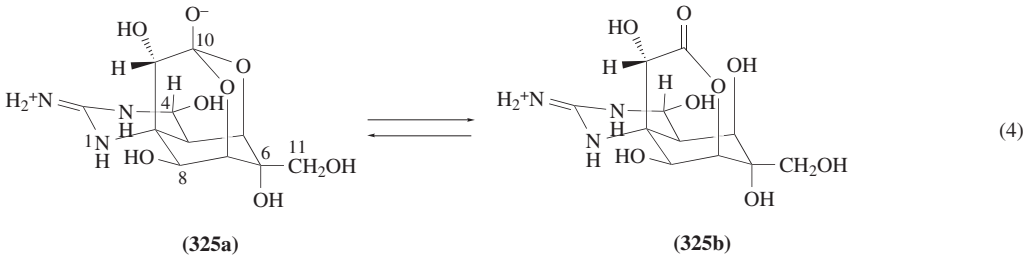


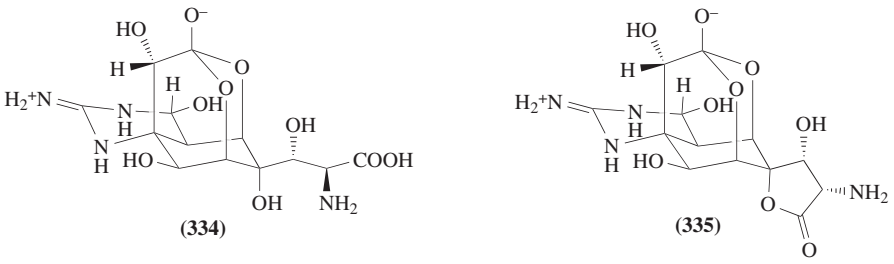
(329) $R^1 = H$, $R^2 = OH$
(330) $R^1 = OH$, $R^2 = CH(OH)_2$
(331) $R^1 = CH_2OH$, $R^2 = OH$
(332) $R^1 = OH$, $R^2 = Me$
(333) $R^1 = OH$, $R^2 = H$

these TTX analogues was achieved mainly through NMR measurements. The poor resolution of ^{1}H and ^{13}C NMR spectra of TTX caused by hemiacetal-lactone tautomerism (Equation (4)) was markedly improved by the addition of CF_3COOD (1%) to the solvent (4% CD_3COOD/D_2O) to allow the ^{1}H and ^{13}C signals to be firmly assigned by ^{1}H-^{1}H and ^{13}C-^{1}H COSY measurements.



(325a) (325b) (4)

A Costa Rican frog, *Atelops chiriquiensis*, contained TTX (**325**) and chiriquitoxin (**334**). Chiriquitoxin (**334**) was first isolated in 1975 and was as potent as TTX (**335**) in lethality to mice and in blocking the voltage-gated sodium channel, whereas other analogues such as (**326**)–(**328**) have markedly reduced biological activities.[287] It was found that analogues modified at C-6 or C-11 such as (**329**)–(**332**) and (**334**) still retained significant potency to block sodium channels. The structure of chiriquitoxin (**334**) was determined to be a TTX analogue with a glycine molecule attached to C-11. The high potency of chiriquitoxin (**334**) suggested the sodium channel protein had specific binding sites for the C-12 NH_2 and/or the C-13 CO_2H. In addition, functionalities within the channel which interact with the guanidinium and the C-9 and C-10 hydroxyls of TTX (**325**) may be operative. ^{1}H and ^{13}C NMR data indicated that, under acidic conditions, H-12 of chiriquitoxin (**334**) and its 6,13-lactone derivative (**335**) slowly exchanged within one month with deuterium of solvents. However, the configuration at C-12 did not change.

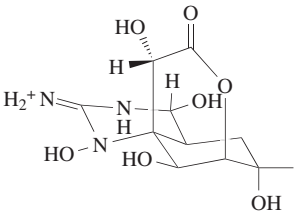


(334) (335)

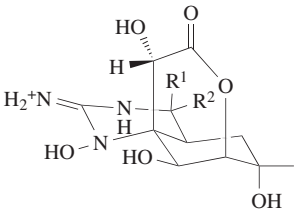
From the southern puffer *Arothron nigropunctatus*, collected in Okinawa, 11-nor-TTX-6(*S*)-ol (**333**) was isolated, and the structure was assigned by NMR data and confirmed by preparation of (**333**) from (**325**).[288] Oxidation of TTX (**325**) with H_5IO_6 followed by reduction with $NaBH_3CN$ afforded two products in the ratio of 1:6, the minor product being assigned as 11-norTTX-6(*R*)-ol (**329**), while the major one was concluded to be its epimer at C-6, 11-norTTX-6(*S*)-ol (**333**).

The *N*-hydroxyl and ring-deoxy derivative of TTX, 1-hydroxy-5,11-dideoxyTTX (**336**) was isolated from the Californian newt *Taricha torosa*.[289] Compound (**336**) was the first 10,7-lactone type analogue of TTX. In addition, 5,6,11-trideoxyTTX (**337**) and its 4-epimer (**338**) were isolated from the puffer fish *Fugu poecilonotus* collected at Shimonoseki, Japan.[290] The discovery of these TTX analogues may be highly significant with respect to the biosynthesis of TTX. Biosynthesis of TTX supposedly involves arginine, analogous with saxitoxin, and a C-5 unit derived from either amino acid, isoprenoids, shikimates, or branched sugars. The occurrence of 6-epiTTX (**331**) and 11-deoxyTTX (**332**) may render an isoprenoid unit a favorable candidate, because it possesses both sp^2 carbons available for Diels–Alder-type condensation and a methyl that remains in (**332**). 11-DeoxyTTX (**332**), 11-norTTX-6-ols (**329**) and (**333**), and 11-carboxyaminomethyl derivative (**334**) were considered to be compounds at different stages of progressive oxidation or their derivatives as in the case of chiriquitoxin (**334**). While structural variations found in (**329**) and (**332**)–(**334**) are limited to the 6,11-positions, i.e., the branching portion, compounds (**336**)–(**338**) were TTX derivatives which lack a ring oxygen function. It seemed, therefore, likely that TTX was formed by the

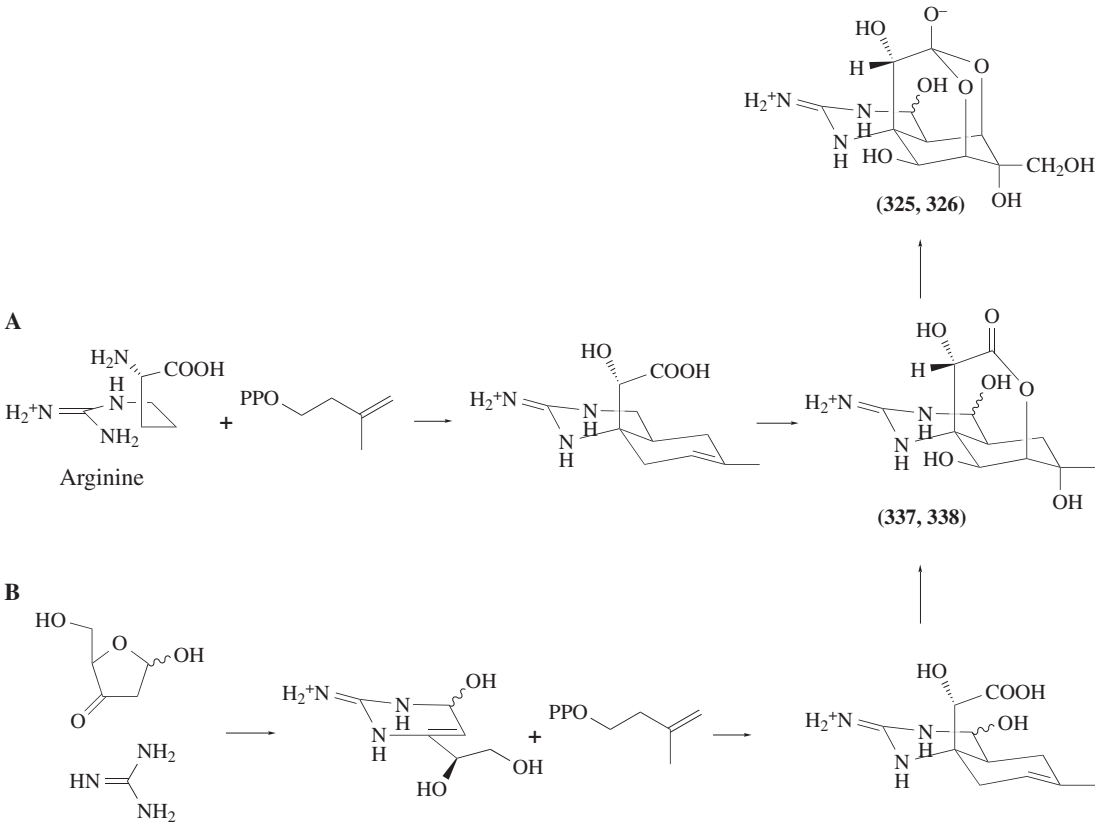
stepwise oxidation of an alicyclic system, and one of the speculations that TTX was formed by the condensation of arginine and a branched sugar was excluded. The discovery of 5,6,11-trideoxyTTX (**337**), which possesses a C-5-methylene and C-6-methine in the cyclohexane ring, also supported the isoprenoid origin (Scheme 9, route A). Alternatively, the 2-aminoperhydropyrimidine portion of TTX (**325**) could be constructed from guanidine and 2-deoxy-3-oxo-D-pentose. Condensation of the product with an isoprenoid would also lead to (**337**) (Scheme 9, route B). Co-occurrence of (**337**) and its 4-epimer (**338**) as an equimolar mixture seemed to favor route B, which is not stereoselective with regard to C-4-OH. If oxidation of C-4 occurred enzymatically after ring formation as in route A, the resulting product would be generated more stereospecifically. However, epimerization at C-4 occurs rather easily.



(336)



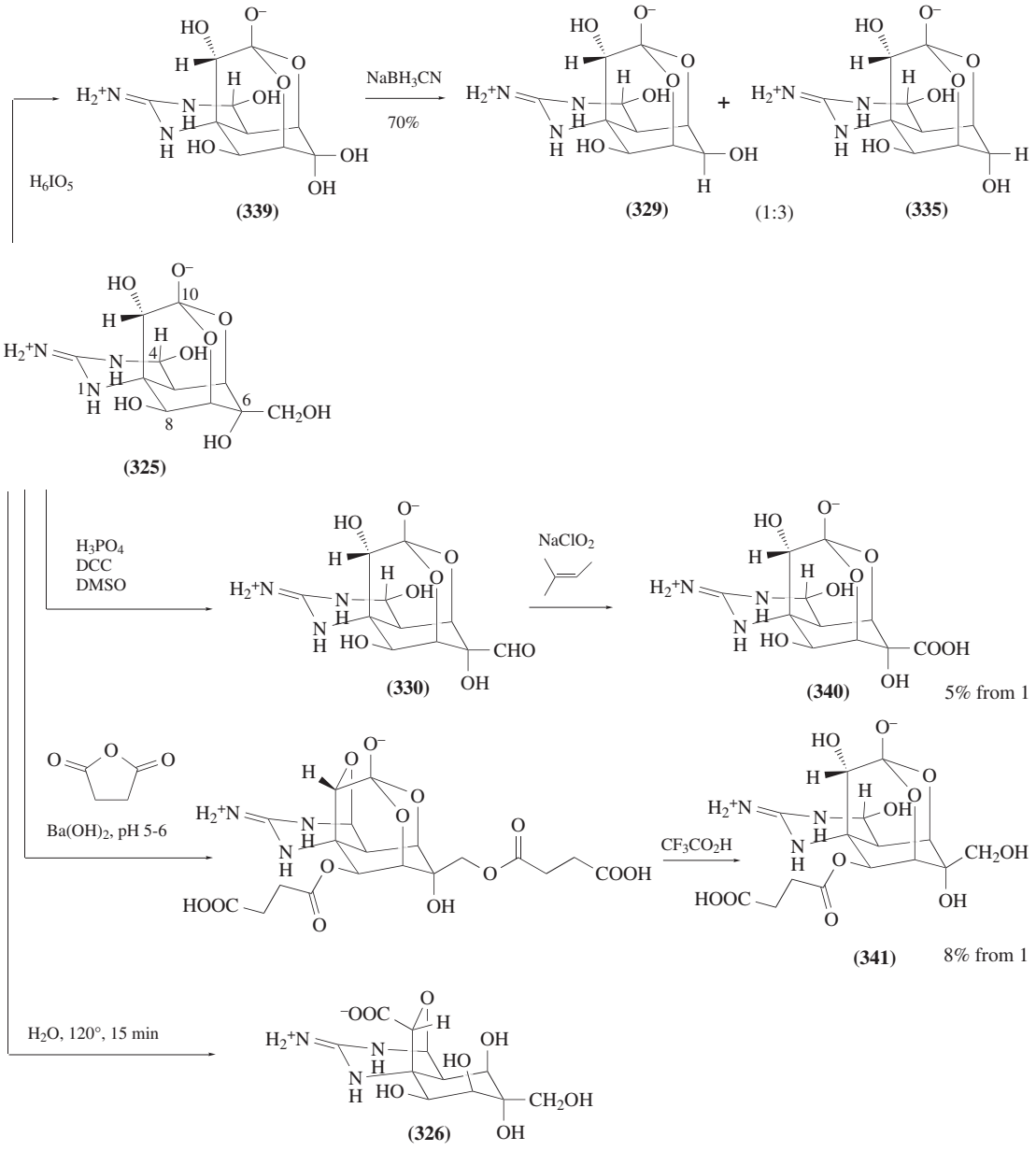
(337) $R^1 = H$, $R^2 = OH$
(338) $R^1 = OH$, $R^2 = H$



Scheme 9

Several derivatives of TTX analogues were prepared for determination of their binding potencies to sodium channels.[291] 4-EpiTTX (**327**), 4,9-anhydroTTX (**328**), 6-epiTTX (**331**), 11-deoxyTTX (**332**), and chiriquitoxin (**334**) were obtained from natural sources, while tetrodonic acid (**326**),

11-norTTX-6(*S*)-ol (**329**), 11-oxoTTX (**330**), 11-norTTX-6(*S*)-ol (**333**), 11-norTTX-6,6-diol (**339**), TTX-11-carboxylic acid (**340**), and TTX-8-*O*-hemisuccinate (**341**) were prepared chemically (Scheme 10).



Scheme 10

Isolation, chemical reaction, and purities of the final products were monitored by fluorometric HPLC as mentioned above. The quantity of each derivative was determined by ^{1}H NMR spectroscopy by comparison of the signal intensity of H-4 with that of Bu^{t}OH added to the solution as an internal standard (0.000 38% Bu^{t}OH in 3.96% CD_3COOD-D_2O). Purities of the derivatives proved to be more than 99.3% by HPLC analyses. Their binding potencies to the sodium channel protein were examined by competitive binding assays against ^{3}H-saxitoxin (STX), which shares the same binding site with TTX (**325**) (Table 19).

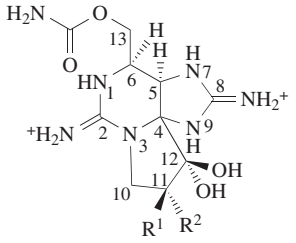
Table 19 ^{3}H-STX binding inhibition activities of TTX analogues.

Analogues	IC_{50} (nM)	K_i (nM)
(**325**)	13.7±1.5	1.89±0.21
(**326**)	> 3000	> 340
(**327**)	384±45	43.3±5.1
(**328**)	1142±256	129±20
(**329**)	186±25	25.7±3.5
(**330**)	11.6±1.8	1.60±0.25
(**331**)	295±35	40.7±4.8
(**332**)	279±38	38.5±5.2
(**333**)	161±25	22.2±3.5
(**334**)	6.90±1.9	0.774±0.22
(**339**)	13.7±1.1	1.54±0.13
(**340**)	> 3000	> 340
(**341**)	2553	287

Compounds (**330**), (**334**), and (**339**) retained the high potency of (**325**). The potencies of compounds (**327**), (**329**), and (**331**)–(**333**) were decreased to 1/20–1/40 of (**325**). Moreover, the channel binding potency was almost lost in compounds (**326**), (**328**), (**340**), and (**341**). These results were almost consistent with the stereostructural locations of charged amino acid groups around the binding site of (**325**) deduced from site-directed mutagenesis[292] and the photoaffinity labeling experiments of the sodium channel[293] (see Section 8.07.8.2.3).

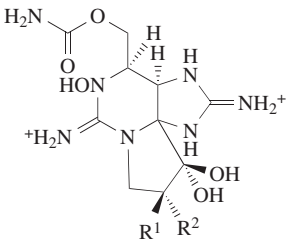
8.07.8.2.2 Saxitoxin

Saxitoxin (STX (**342**)) and its analogues are well-known paralytic shellfish poisons (PSP),[294,295] and are the most serious threat to public health among all seafood poisonings. The economic damage caused by accumulation of the toxins in shellfish is immeasurable. STX (**342**) and its analogues are produced by a variety of organisms, including dinoflagellates of the genus *Alexandrium* (formerly *Gonyaulax* or *Protogonyaulax*), *Gymnodinium*, and *Pyrodinium*, a freshwater blue-green alga *Aphanizomenon flos-aquae*, and the red alga *Jania* sp. The presence of STX (**342**) was found in the common Atlantic mackerel *Scomber scombrus*.[296] The occurrence of this toxin in taxonomically varied species as well as quantity differences within the same species suggests the presence of common vectors. STX (**342**) was identified as the toxin in the culture broth of a bacterium isolated from the dinoflagellate *Protogonyaulax tamarensis*.[297] Further confirmation was expected to substantiate PSP-producing bacteria.[298]

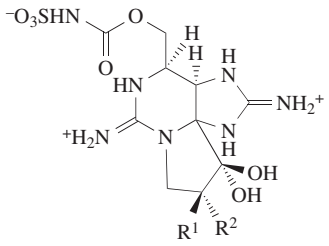


(**342**) $R^1 = R^2 = H$
(**344**) $R^1 = H$, $R^2 = OSO_3^-$
(**346**) $R^1 = OSO_3^-$, $R^2 = H$

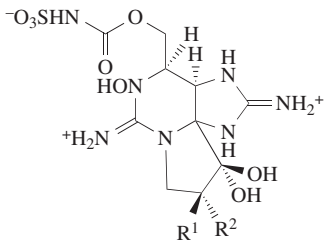
Detection of STX analogues was facilitated by the use of a fluorometric HPLC analyzer, which measures fluorescence derived from oxidation products.[299] The toxins can be divided into two groups, viz., those with and without a hydroxyl group at the N-1 position: saxitoxin (**342**) and neosaxitoxin (**343**). Both groups were further diversified by the presence of 11-*O*-sulfate (**344**)–(**347**) or *N*-sulfate (**348**)–(**353**), the absence of carbamoyl groups (**354**)–(**356**) and the absence of oxygen at C-13 (**357**)–(**359**).[294,295]



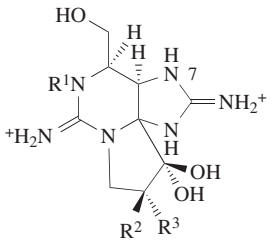
(343) $R^1 = R^2 = H$
(345) $R^1 = H$, $R^2 = OSO_3^-$
(347) $R^1 = OSO_3^-$, $R^2 = H$



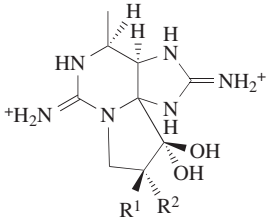
(348) $R^1 = R^2 = H$
(350) $R^1 = H$, $R^2 = OSO_3^-$
(352) $R^1 = OSO_3^-$, $R^2 = H$



(349) $R^1 = R^2 = H$
(351) $R^1 = H$, $R^2 = OSO_3^-$
(353) $R^1 = OSO_3^-$, $R^2 = H$



(354) $R^1 = H$, $R^2 = H$, $R^3 = OSO_3^-$
(355) $R^1 = H$, $R^2 = OSO_3^-$, $R_3 = H$
(356) $R^1 = OH$, $R^2 = R^3 = H$



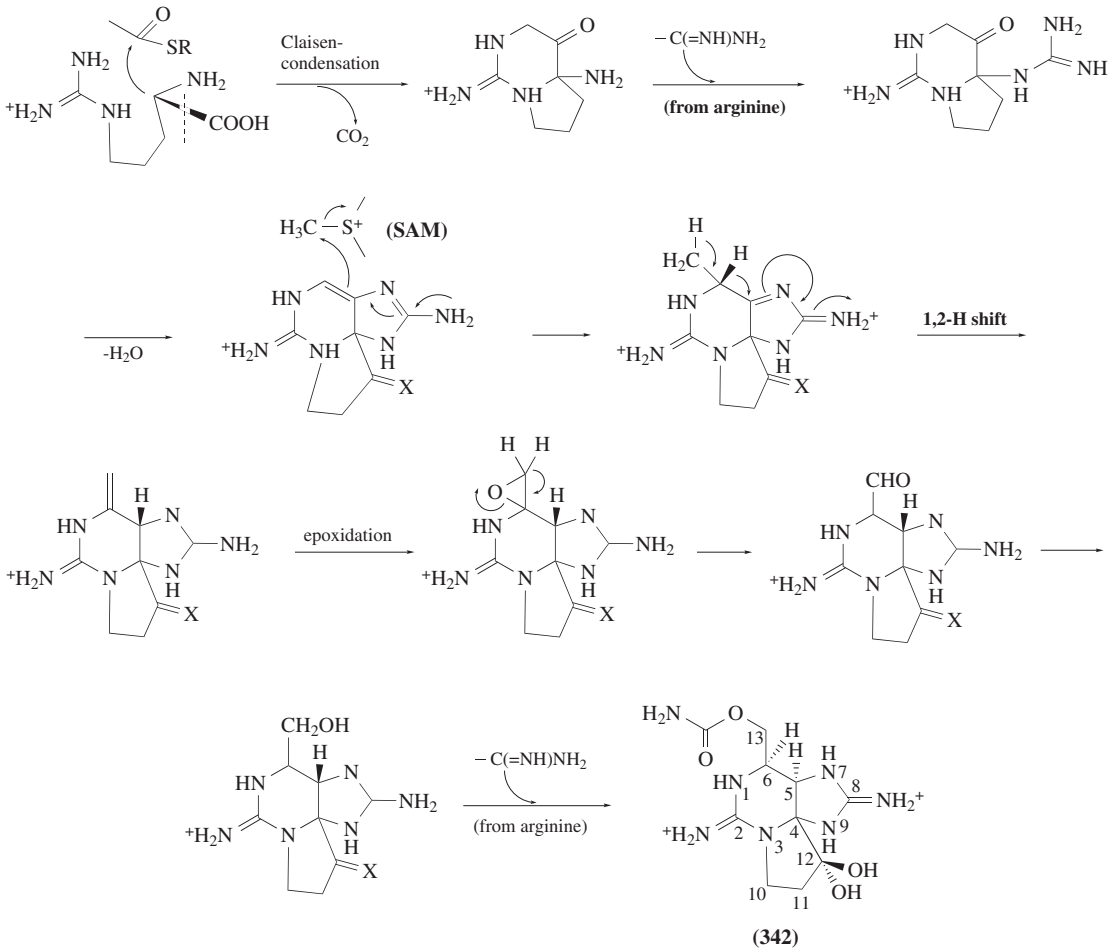
(357) $R^1 = R^2 = H$
(358) $R^1 = H$, $R^2 = OSO_3^-$
(359) $R^1 = OSO_3^-$, $R^2 = H$

The biosynthesis of STX analogues was extensively investigated by feeding experiments of labeled precursors into *Alexandrium tamarense* and *Aphanizomenon flosaquae*.[295] The carbamoyl group and two guanidinium moieties were shown to be derived from the guanido group of arginine by the feeding experiment of [*guanido*-^{14}C]arginine. The feeding of [1,2-$^{13}C_2$]acetate showed that C-5 and C-6 came from one acetate unit. When [2-^{13}C-2-^{15}N]arginine was fed to the organism, the connectivity of ^{13}C–^{15}N was incorporated intact in neosaxitoxin (**343**). Thus, it was suggested that the skeleton of the tricyclic ring system was formed by a Claisen-type condensation of an acetate unit or its equivalent onto the amino-bearing α carbon of arginine followed by decarboxylation, introduction of a guanidine moiety, and cyclization (Scheme 11). Incorporation of ^{13}C atom was observed at the C-13 position by feeding with [*methyl*-^{13}C]methionine, and feeding experiments with [2-^{13}C-2-$^{2}H_3$]acetate established that the hydrogen found at C-6 was not from the acetate methyl group and that a deuterium atom on the acetate methyl group was shown to have rearranged to the C-5 position. Moreover, feeding [*methyl*-^{13}C,$^{2}H_3$]methionine demonstrated that only one of the methionine methyl hydrogens was left on the side-chain methylene group. From these observations, it was proposed (Scheme 11) that the side-chain carbon (C-13) was derived from a methionine methyl group by the electrophilic attack of *S*-adenosylmethionine (SAM) on a dehydro intermediate followed by hydride migration and elimination of a proton. The resulting exomethylene group was likely to be first converted into an epoxide followed by opening to an aldehyde and subsequent reduction into a hydroxymethyl group; these processes explain the retention of only one deuterium atom. The carbamoyl group may then be introduced to the C-13 carbinol position by use of the guanido group of arginine to build up the STX-type toxins.

Thus, three molecules of arginine may be required to construct one STX molecule. In some organisms, the toxin concentrations in the cell reach 60 pg/cell, which represents an enormous portion of the total organic content of the organisms. The biological significance of these compounds is often discussed and ascribed to self-defense, but the toxigenicity of the organism varies from strain to strain even within the same species with no apparent effect on their chance of survival.[295]

8.07.8.2.3 Sodium channels and TTX/STX

Tetrodotoxin (TTX (**325**)) and saxitoxin (STX (**342**)) are specific and potent blockers of the sodium channel, which is essential for the generation of action potentials in excitable cells. Studies[300] have suggested that these positively charged toxins are bound close to the extracellular orifice of the channel in a region containing negatively charged groups. The complete primary structure of



Scheme 11

the sodium channel protein from eel electroplax was first deduced from cDNA in 1984,[301] followed by those from rat brain, skeletal muscle, and heart. These proteins are highly homologous with each other, and each contains four internally homologous domains (repeats I–IV), each of which has six proposed transmembrane helices (segments S1–S6).[300] Single-point mutation of the rat sodium channel that replaced glutamic acid residue 387 (E387) with a glutamine reduced its sensitivity to TTX and STX by more than three orders of magnitude and reduced its single-channel conductance, whereas its macroscopic current properties were only slightly affected.[302] Another mutation that neutralized the aspartic acid residue 384 (D384) to asparagine almost completely eliminated ionic currents. Thus, residues D384 and E387 are suggested to be located at the extracellular mouth or inside the ion-conducting pore of the channel. D384 and E387 belong to the short segment SS2 in the region between the hydrophobic segments S5 and S6 in repeat I. In each of four repeats the S5–S6 region was thought to contain two short segments, SS1 and SS2, that may partly span the membrane as a hairpin and the SS2 segments were postulated as forming part of the channel lining. The effects on toxin sensitivity and single-channel conductance of site-directed mutations in the region encompassing the SS2 segment of each of the four repeats were investigated, focusing mainly on charged residues. As a result, the sensitivity to TTX and STX of the sodium channel was revealed to be strongly reduced by mutations of specific amino acid residues in the SS2 segment of each of the four internal repeats. These residues were found in two clusters of predominantly negatively charged residues and were located at equivalent positions in the SS2 segment of the four repeats. On the other hand, mutations of other amino acid residues in the SS2 and adjacent regions caused minor or insignificant changes in toxin sensitivity. These observations suggested that these two clusters of predominantly negatively charged residues, probably forming ring structures, take part in the extracellular mouth and/or the pore wall of the sodium channel (Figure 4).[292]

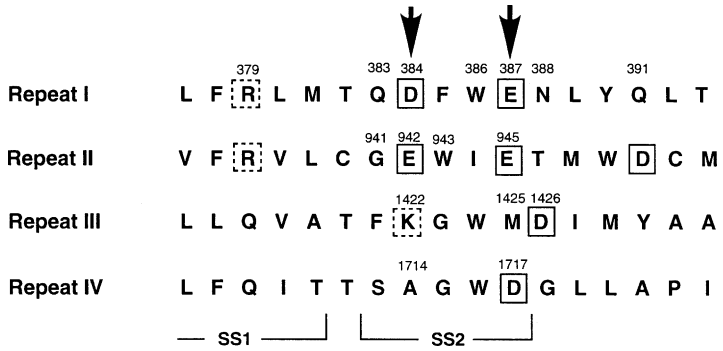
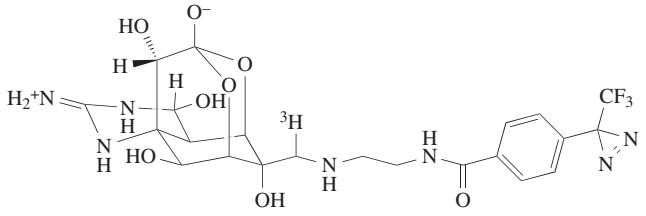


Figure 4 Regions encompassing the SS2 segment of the four repeats of the rat sodium channel. The positions of the SS1 and SS2 segments and the numbers of the amino acid residues mutated are indicated. Negatively charged residues are boxed with solid lines, and positively charged residues with broken lines. The positions of the clusters of residues that were identified as major determinants of toxin sensitivity are indicated by arrows.

It was also proposed that TTX and STX bind to the sodium channels with three points, at least, including the negatively charged groups. Photoaffinity labeling may play an important role in identification of toxin/ligand binding regions within the primary structures of ion channels.[293] Several photoactive TTX derivatives including (diazirino)trifluoroethylbenzoyl TTX (**360**)[303] were prepared and specifically labeled the electroplax sodium channel protein of 250 kDa.



(360)

At least 43% of the labeled sites with a photoactive TTX derivative (**360**) were identified by probing protease-digested labeled fragments with several sequence-directed antibodies. They were located in the loop between segments S5 and S6 of repeat IV, as well as the region containing transmembrane segment S6 and adjacent extracellular and cytoplasmic sequences in repeat III. No photolabeled fragments were detected in the corresponding region of repeat I. These results suggested that C-11 of TTX where the photoreactive moiety was attached oriented to the region between S5 and S6 in repeats III and IV, and the guanidinium group of TTX which was apparently directed opposite to C-11 oriented most likely to the negative charge cluster of repeats I and II (Figure 5).[293]

As discussed in Section 8.07.8.2.1, TTX and its analogues, either isolated from natural sources or prepared by chemical modification of TTX, were tested for binding potencies to sodium channels with the use of [^{3}H]-STX as a competitor (Table 19). Inhibition of ionic bonds between the guanidium group of TTX and acidic clusters in repeats I and II resulted in complete loss of the channel-blocking activity. Oxidation of 11-CH_2OH to CO_2H also caused complete loss of the activity, probably due to repulsion between 11-CO_2H and Asp-1717 in repeat IV. The hemilactal structure was important for ionic interaction with Lys-1422 in repeat III. The effect of epimerization at C-6 was less significant. Reduction of hydrophilic 11-CH_2OH to hydrophobic 11-Me reduced hydrogen bond formation with Asp-1717 and thus resulted in reduced activity. These results of structure–activity relationship studies support the stereostructural locations of charged amino acid groups around the binding site deduced from the site-directed mutagenesis[292,302] and the photoaffinity labeling experiments[293] (Figure 4).

Why are puffer fish not intoxicated by their carrying tetrodotoxin? There are two possible answers to this question. Mutations appear to have occurred in sodium channel molecules of puffer fish to differentiate them from those of TTX-sensitive sodium channels of other animals. Sodium channels of puffer fish showed the lowest affinity to STX, while those of bonito showed higher affinity, and

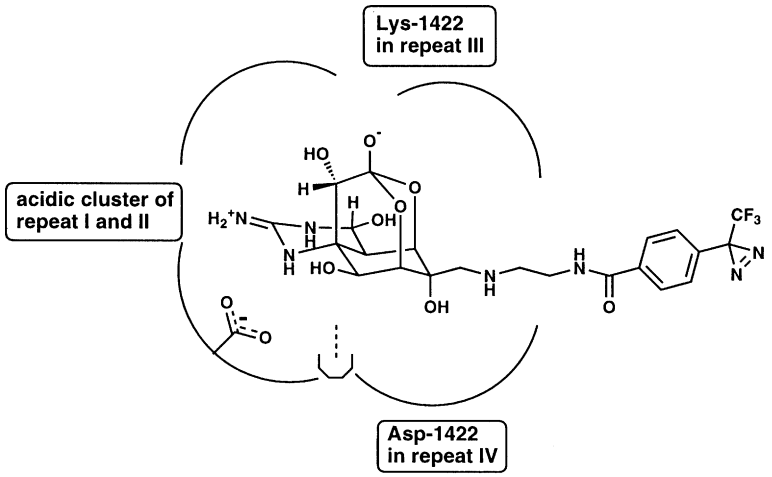


Figure 5 A model of the relative positions of photoreactive TTX analogue (**360**) and sodium channels.

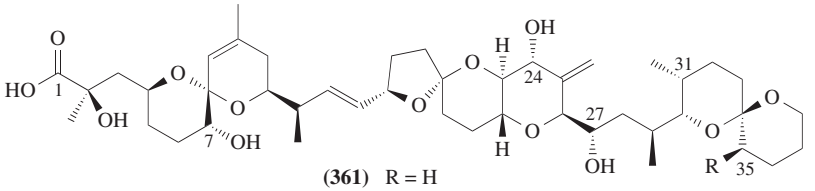
those of rat brain showed the highest. The resistance of puffers to STX (=TTX) would thus be explained. Sequential analysis of cDNA of the sodium channel of puffer fish muscle revealed that the amino acid sequences of the binding site (site-1) differ at three points from those of the rat brain sodium channel. The mutation presumably is responsible for the self-resistant system of puffer fish against their own TTX.[304] The second possible explanation of the resistance mechanism against TTX in puffer fish may be the possible existence of other stronger TTX-binding proteins. The number of TTX-binding proteins in the muscle of puffer fish was estimated to be much greater than that of sodium channel proteins estimated from the binding of brevetoxin (BTX), while in the brain of puffer fish the numbers of TTX-binding proteins and sodium channel proteins (estimated from BTX-binding) were equal.[305] This finding suggested the presence of TTX-binding protein different from sodium channels in the muscle of puffer fish, and it may be speculated that the protein functions in a detoxification pathway for TTX of puffer fish. In connection with this observation, a bullfrog, *Rana catesbeiana*, having low STX-sensitivity, contained in its plasma a soluble component of unknown function that specifically binds to [^{3}H]-STX.[306] The protein responsible for this activity was named saxiphilin and was purified and shown to have a molecular weight of 89 kDa. The function of this protein is unknown but it may possibly be part of a species-specific detoxification mechanism for STX; when STX is administered to the frog, saxiphilin traps the toxin and, consequently, the sodium channels of the frog are not affected.

8.07.8.3 Diarrhetic Shellfish Poisoning

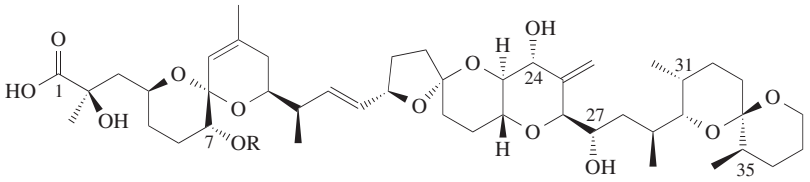
In 1976, mussel poisoning of *Mytilus edulis* broke out in Miyagi Prefecture, Japan. This shellfish poisoning disease was called diarrhetic shellfish poisoning (DSP) since the prominent human symptoms were gastrointestinal disorders such as diarrhea, nausea, vomiting, and abdominal pain. DSP was associated with ingestion of bivalves such as mussels, scallops, or clams which fed on toxic dinoflagellates. DSP was widely distributed in various places in Japan and Europe. The number of known DSP cases in Japan since 1976 exceeds 1300 despite the existence of extensive surveillance; 5000 cases were reported in the single year of 1981 in Spain and there were 400 cases in 1983 in France. Thus, it is a serious problem for public health as well as for the shellfish industry. Although no red tide was sighted during the infestation period, the regional and seasonal variation of shellfish toxicity strongly suggested a planktonic origin of the toxins. The causative organisms were identified as several dinoflagellates of the genus *Dinophysis*, and three kinds of polyether toxins, i.e., okadaic acid and its analogues, pectenotoxins, and yessotoxin, were identified as DSP toxins.[271]

8.07.8.3.1 Okadaic acid and dinophysistoxin

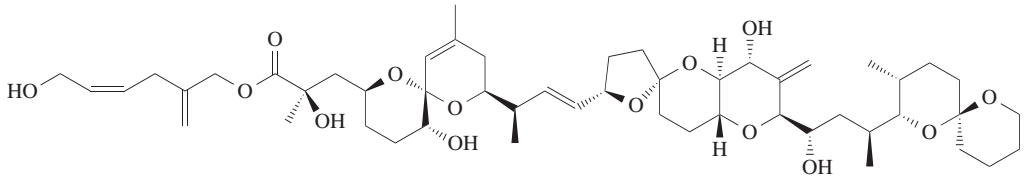
Okadaic acid (OA (**361**)) was first isolated from the sponge *Halichondria okadai*[307] and subsequently it was revealed that the acid was also detected in dinoflagellates *Prorocentrum lima* and *Dinophysis* sp. The toxin responsible for DSP caused by the mussel *Mytilus edulis* was identified as 35-methylokadaic acid, which was named dinophysistoxin 1 (DTX-1 (**362**)).[308] Detection of okadaic acid derivatives was carried out with high sensitivity by a fluorometric HPLC analysis of 9-anthryldiazomethane ester derivatives.[309,310] Okadaic acid (**361**) was toxic (LD_{50} 192 μg kg^{-1}, mouse, i.p.) and showed potent cytotoxicity with ED_{50} values of 1.7 ng ml^{-1} and 17 ng ml^{-1} against P388 and L1210 cells, respectively. In addition, (**361**) was discovered to act as an inhibitor of protein phosphatases 1 and 2A, and widely utilized as a tool for biochemical and pharmacological studies.[311] A series of OA/DTX-related compounds were thus far known. Among them, DTX-3 (**363**)[312] was an unsaturated fatty acid ester attached on 7-OH of DTX-1, and was identified as a main toxic constituent of Japanese scallops, while a saturated acid ester, 7-*O*-palmitoyl DTX-1 (**364**),[313] also obtained from the mussel, was not toxic. The unsaturation in the acyloxy group seemed to play a substantial role in the bioactivity of the toxin. From the dinoflagellate *Prorocentrum lima*, a diol ester (**365**) of okadaic acid was isolated[313] and found to be more toxic (125 μg kg^{-1}, mouse, i.p.) than okadaic acid (**361**). From a mussel digestive gland collected at Bantry Bay, Ireland, where *Dinophysis* sp. occurred and DSP-activity was observed, an isomer of okadaic acid, named dinophysistoxin 2 (DTX-2 (**366**)),[314] was isolated together with okadaic acid (**361**). An underivatized extract of mussel was examined by an ion spray LC–MS method.[315] Extensive spectroscopic studies on (**366**) revealed that this compound bore no methyl group on C-31 but a methyl group was present on C-35. Compound (**366**) had comparable activity to (**361**) and (**362**) in the rat toxicity bioassay.



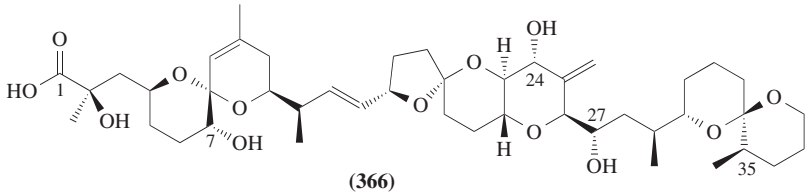
(**361**) R = H
(**362**) R = Me



(**363**) R = acyl [$C_{14:0}$ (13%), $C_{16:0}$ (29%), $C_{18:3}$ (3%), $C_{18:4\omega3}$ (9%), $C_{20:5\omega3}$ (23%), and $C_{22:6\omega3}$ (29%)]
(**364**) R = palmitoyl



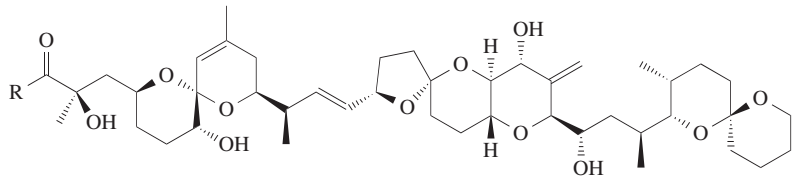
(**365**)



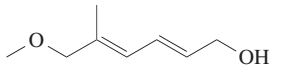
(**366**)

From the cultures of the dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum*, diol esters (**367**)–(**369**) were obtained.[316] It was observed that the cellular concentration of okadaic acid (**361**) decreased with time in *Prorocentrum* sp. while the concentration of the esters increased, suggesting that the esterified products may be shunt metabolites, produced at a certain stage in the

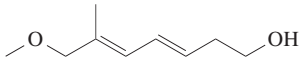
growth cycle, or when the concentration of okadaic acid (**361**) in the cell reached a critical level. It was found that the methyl ester of okadaic acid (**361**) did not show inhibitory activity of protein phosphatases 1 and 2A, suggesting that a free carboxylic acid was essential for the activity. The diol esters (**367**) and (**368**) were also found to be inactive in the inhibition of phosphatases, further supporting the idea that a free carboxyl group is essential for the activity. Water-soluble sulfated DSP toxin derivatives have been isolated; DTX-4 (**370**)[317] from *Prorocentrum lima* and DTX-5a (**371**) and -5b (**372**)[318] from *Prorocentrum muculosum*. The sulfated ester (**370**) was toxic in the mouse bioassay,[317] but its phosphatase inhibition activity was weaker by almost an order of magnitude.[318] The occurrence of these sulfate esters may support the idea that the less active sulfate esters of DSP toxins were the form in which the toxin was stored within, and excreted from, the dinoflagellate cell. Okadaic acid (**361**) was observed in the medium of *P. lima* cultures, suggesting that after exiting the cell the sulfate form was hydrolyzed to the biologically active form, which could not reenter the *Prorocentrum* cell.



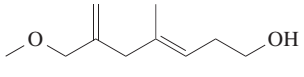
(**367**) R =



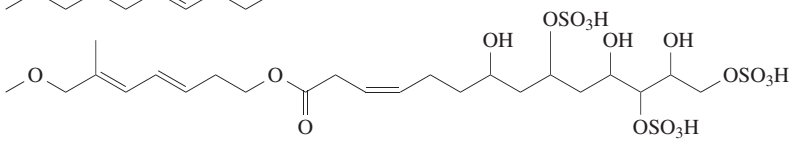
(**368**) R =



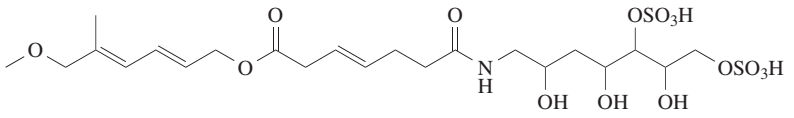
(**369**) R =



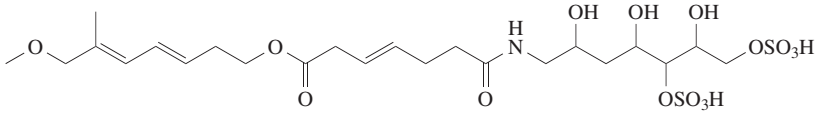
(**370**) R =



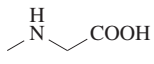
(**371**) R =



(**372**) R =

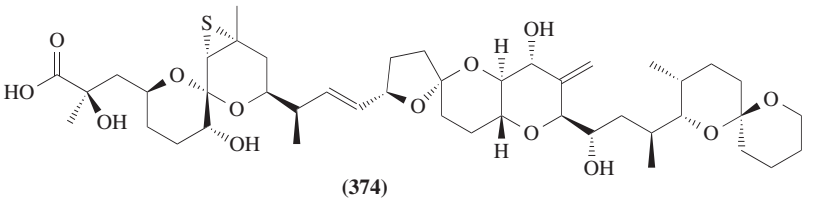


(**373**) R =

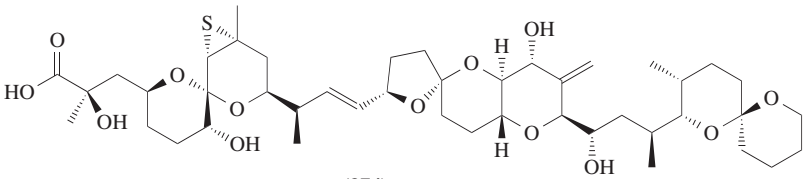


Okadaic acid (**361**) was revealed to be a potent tumor promoter of a new class, different from those belonging to the class of TPA (phorbol-12-tetradecanoate-13-acetate)-type tumor promoters.[319] TPA-type promoters were known to activate protein kinase C, while okadaic acid (**361**) inhibited dephosphorylation of proteins, predominantly serine/threonine residues. Both types of tumor promoters eventually cause the accumulation of essentially the same phosphorylated proteins, which may be involved in tumor promotion. The structure–activity relationships (SAR) within a series of okadaic acid derivatives were investigated by evaluating 17 okadaic acid derivatives as possible tumor promoters by means of three biochemical tests: inhibition of specific [^{3}H]-okadaic acid binding to a particulate fraction of mouse skin containing protein phosphatases, inhibition of protein phosphatase activity, and induction of ornithine decarboxylase in mouse skin.[320] The results indicated that the carboxyl group as well as the four hydroxyl groups at C-2, C-7, C-24, and C-27 of okadaic acid were important for activity. Acanthifolicin (**374**), an episulfide-containing polyether isolated from the sponge *Pandaros acanthifolium*,[321] also gave a positive response in the three biochemical tests as strong as those of okadaic acid (**361**). Glycookadaic acid (**373**), a conjugated form of (**361**) with glycine, proved to be an anticachexia substance.[322] Elucidation of the

interrelationship between the fine structure and the activity of (**361**) was studied by NMR, and the formation of a flexible cavity between the C-1 carboxyl group and the C-24 hydroxyl group (Figure 6) was proposed.



(**374**)



(**374**)

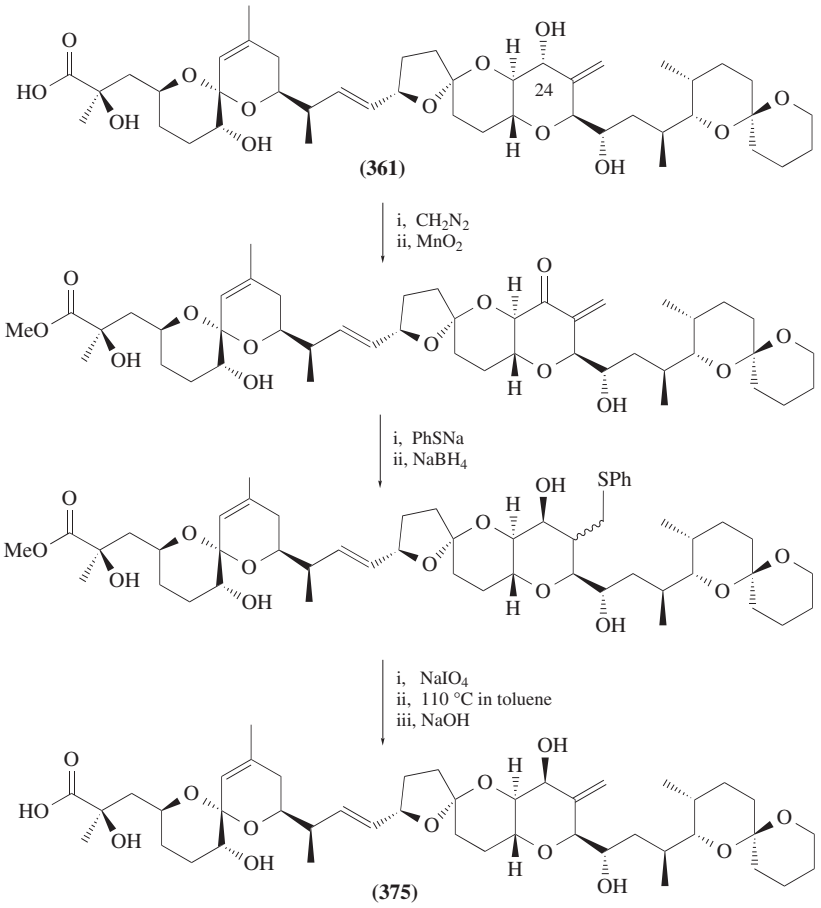
Figure 6 Flexible cavity hypothesis of okadaic acid.

Based on this proposal, a derivative (**375**), the C-24 hydroxyl group of which was epimerized, was prepared (Scheme 12), and was revealed to be less cytotoxic than (**361**) by two orders of magnitude.

The proposed structure of (**361**) including the flexible cavity (Figure 6) was supported by lactonization of okadaic acid (**361**) to give a 3-*O*-methylthiomethyl-1,24-olide (**376**) (Scheme 13). The results of SAR studies on okadaic acid derivatives are summarized in Figure 7,[323] which suggests that (i) the presence of the double bond at C-9/C-10 and the methyl group on C-35 is not important for activity, and (ii) the C-1 carboxyl group and four hydroxyl groups play certain roles in showing the activity. These studies showed that an intramolecular hydrogen bond between the C-1 carboxyl group and the C-24 hydroxyl group was very strong. The triacetate of okadaic acid was chromatographically more polar than (**361**), providing a supportive observation for the strong hydrogen bond network at the C-1/C-24 positions. The tetra-*O*-methyl derivative and C-24 epimer of (**361**), both of which cannot construct the C-1/C-24 hydrogen bond, had considerably reduced activities.

The crystal structures of the *o*-bromobenzyl ester of okadaic acid (**361**)[307] and acanthifolicin (**374**)[321] corresponded well to each other. Three-dimensional structures of okadaic acid (**361**) in organic and aqueous solutions (CD_3OD-$CDCl_3$ or $NaOD/D_2O$) were analyzed on the basis of long-range carbon-proton coupling constants which were measured by using hetero half-filtered TOCSY and phase-sensitive HMBC techniques,[324] revealing that the conformations in organic and aqueous solutions (Figure 8) resembled each other and that obtained for the crystalline *o*-bromobenzyl ester of (**361**).[307] The methodology using the $^{2,3}J_{C,H}$ and $^{3}J_{H,H}$ values proved to be powerful in elucidation of conformations and configurations of acyclic or macrocyclic portions in natural products of this size.

Several studies on the biosynthesis of okadaic acid and dinophysistoxins reported on the basis of feeding experiments with labeled precursors to the cultures of the dinoflagellates *Prorocentrum* sp. First, the ^{13}C NMR signals of okadaic acid (**361**) were assigned by analyses of 2D NMR spectra, and [1-^{13}C], [2-^{13}C], or [1,2-$^{13}C_2$] sodium acetate was added to cultures of *Prorocentrum lima*.[325] The ^{13}C NMR spectra of labeled compounds showed a 15-fold enhancement of the signal intensities of the labeled carbons. Out of the 44 carbons of (**361**), signals for 39 carbons (carbons other than C-1, C-2, C-37, C-38, and C-44) were enhanced, according to Torigoe and Yasumoto's report.[325] Investigation of the biosynthetic origin of dinophysistoxin-1 (**362**) by addition of [1-^{13}C], [2-^{13}C], or [1,2-$^{13}C_2$] sodium acetate to artificial cultures of *P. lima* revealed enhancement of all carbons except

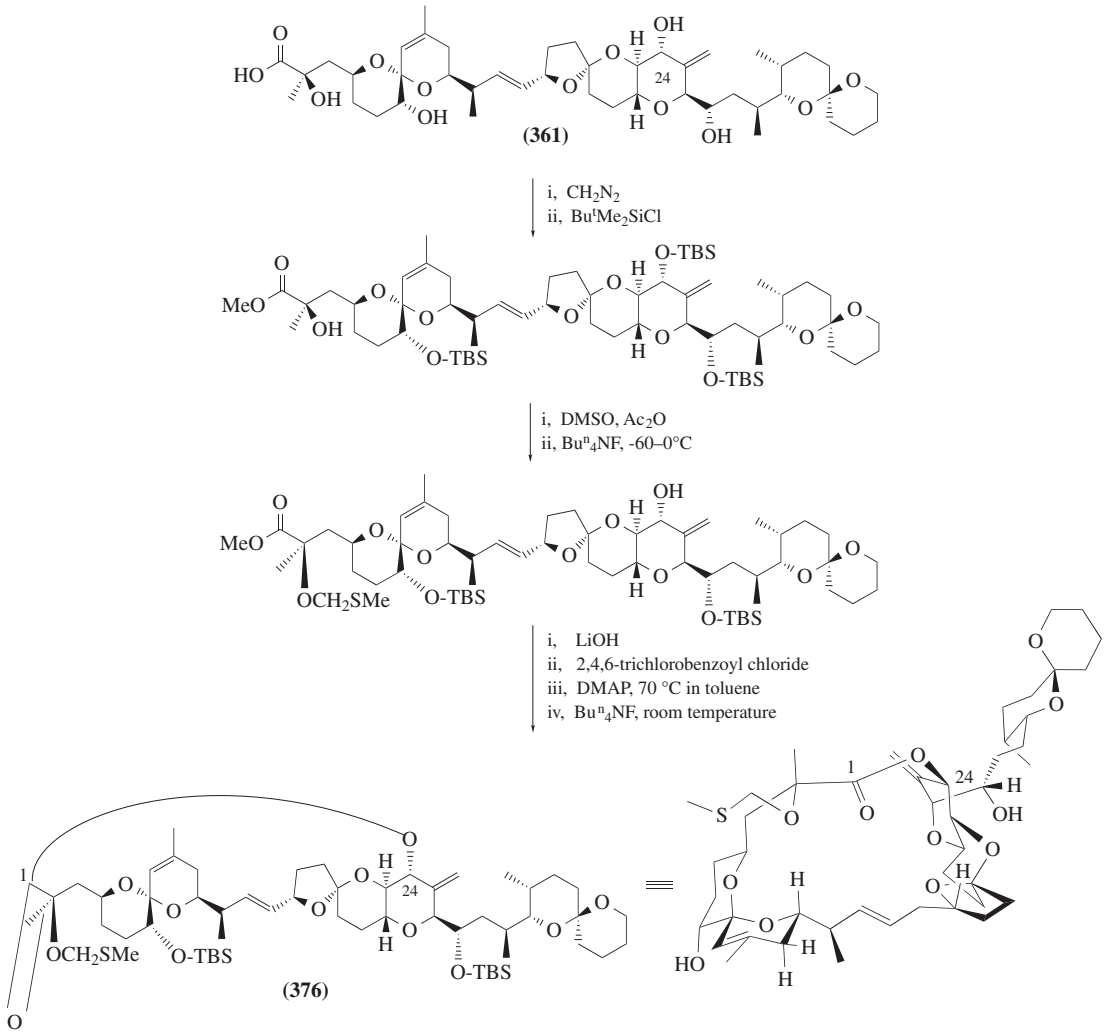


Scheme 12

for the two at C-37 and C-38.[326] Incorporation patterns of intact acetate units to (**361**) or (**362**) are shown in Figure 9. This labeling pattern suggested a type of mixed polyketide involving building blocks formed through dicarboxylic acids such as malonate, succinate, and 3-hydroxyl-3-methylglutarate (Figure 10). In Figure 10, possible biosynthetic precursors of okadaic acid (**361**) and DTX-1 (**362**) proposed by Torigoe and Yasumoto[325] (Figure 10(a)) and Norte *et al.*[326] (Figure 10(b)) are shown. Both Figures 10(a) and 10(b) contain fragments proposed to be derived from classical polyketides. Those in Figure 10(b) contain several methyl branches derived from the C-2 carbon of an acetate attached to carbons derived from the C-1 carbon of an acetate in a linear carbon chain, a feature rarely encountered in polyketide biosynthesis. The participation of dicarboxylic acid precursors had been proposed in the biosynthesis of brevetoxins, the polyether marine toxins isolated from red tide dinoflagellates (see Section 8.07.8.5.2), and it reasonably explained the formation of the "c-m-m-m" moiety (e.g., C-8/C-9/C-10/C-40 in Figures 9 and 10) from acetate-derived precursors (e.g., succinate) coming after two rounds through the tricarboxylic acid (TCA) cycle.

The C-37 and C-38 carbons of okadaic acid (**361**) or DTX-1 (**362**) were not derived from an intact acetate unit, and it has been suggested that these carbons come from an alternative C_2 source. Possible sources of C_2 units include alanine, glycerate, glycine, glycolate, lactate, pyruvate, and succinate. A stable isotope incorporation study was conducted to determine the biosynthetic origin of the C-37/C-38 position in (**361**) and (**362**).[327] While [1,2-$^{13}C_2$]glycine showed small enrichment on feeding to cultures of *P. lima*, [1,2-$^{13}C_2$]calcium glycolate (synthesized from [1,2-$^{13}C_2$]bromoacetic acid) showed enhanced resonances corresponding to C-37 and C-38, both appearing as doublets arising from ^{13}C–^{13}C coupling ($J_{C\text{-}37/C\text{-}38} = 35$ Hz). Thus, these findings implied that the carbons C-37 and C-38 of okadaic acid (**361**) and DTX-1 (**362**) were derived from glycolate (HO_2CCH_2OH).

The biosynthesis of the water-soluble toxin, DTX-4 (**370**), possessing a sulfated acyl ester chain and the diol ester (**368**) was also investigated by feeding experiments with ^{13}C- and ^{18}O-labeled



Scheme 13

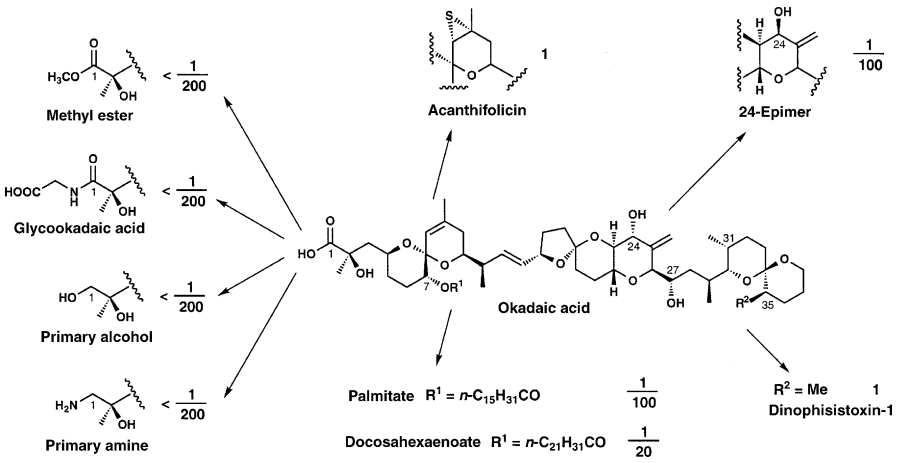


Figure 7 Structure and activity relationships of okadaic acid derivatives. (The activity of okadaic acid was normalized as 1.)

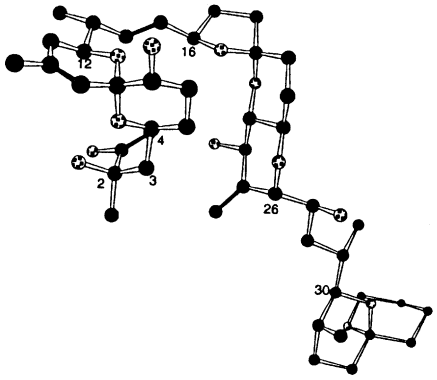


Figure 8 Stereostructure of okadaic acid in organic solvent. (Hydrogen atoms are omitted for clarity.)

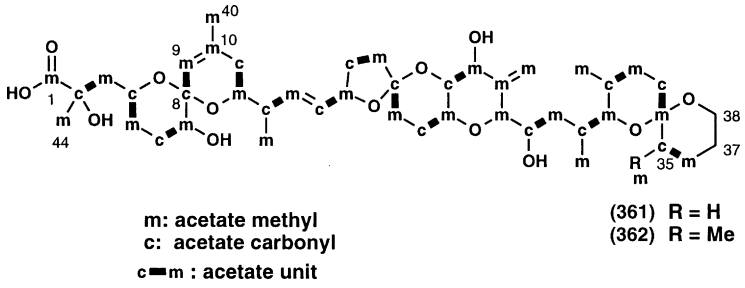


Figure 9 ^{13}C Labeling pattern of okadaic acid.

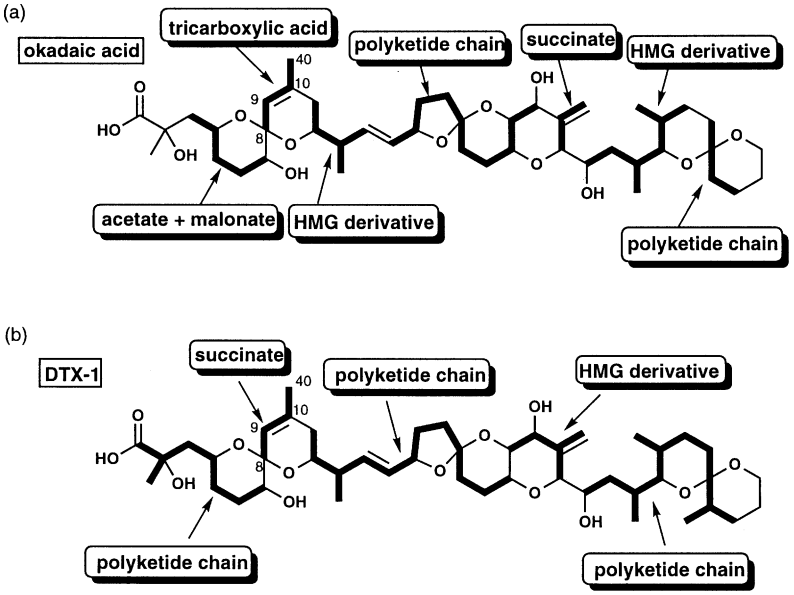


Figure 10 Possible biosynthetic precursors of okadaic acid or dinophysistoxin-1 (DTX-1).

precursors to *P. lima*.[328] Feeding experiment with [1,2-$^{13}C_2$]glycolate revealed that the C-45/C-46 starter unit of the ester chain of (**368**) or (**370**) was derived from glycolate. The ^{13}C NMR spectrum of the diol ester (**368**) labeled from [2-^{13}C, ^{18}O]glycolate displayed ^{18}O isotopically shifted peaks for C-38 $\Delta\delta = -0.023$ ppm) and C-45 $\Delta\delta = -0.031$ ppm), implying that the ^{13}C—^{18}O bonds in the glycolate precursor were retained in the C-38 and C-45 positions of (**368**). Feeding experiments with [1-^{13}C, $^{18}O_2$]acetate showed that in the okadaic acid skeleton moiety of (**368**) and (**370**) only two positions, C-4 and C-27, retained labeled oxygen while the ^{13}C NMR signals for C-2, C-8, C-19, or C-23, which could in principle bear ^{18}O from ^{18}O-labeled acetate, did not show isotope shifts. In the C_{14} sulfated side-chain of (**370**), ^{18}O isotopically shifted peaks were observed for all five of the oxygen-bearing carbon atoms derived from C-1 of the acetate (Figure 11).

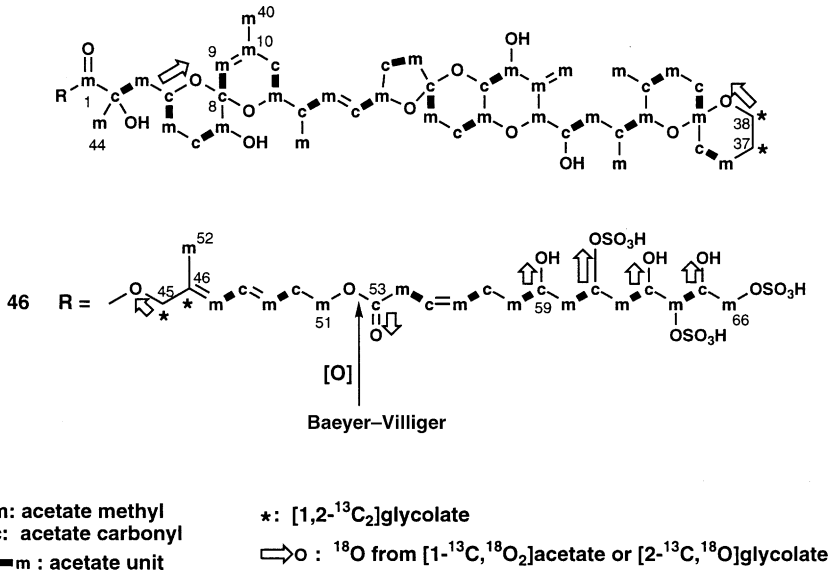
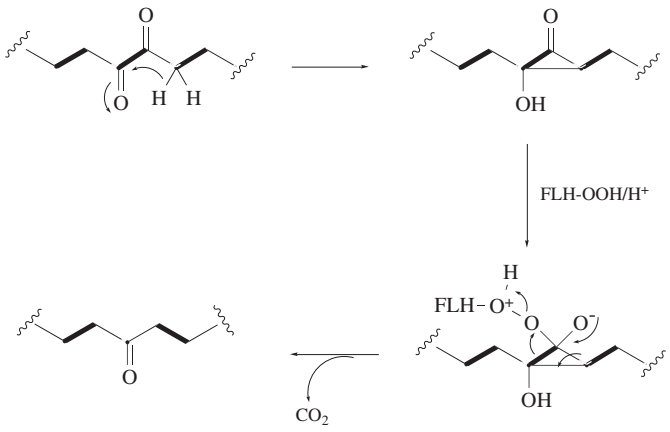


Figure 11 ^{13}C and ^{18}O Labeling patterns of DTX-3 (**370**).

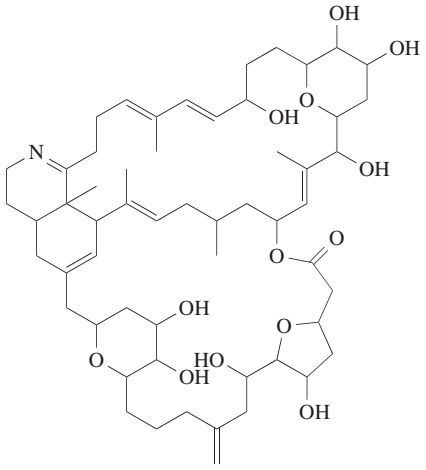
^{18}O was retained at the ester carbonyl C-53 and not at the diol oxygen (C-51), and feeding experiments with [2-$^{13}CD_3$]acetate revealed that the retention of deuterium at C-51 was substantially lower than that of C-66 derived from the methyl of an intact acetate unit in the sulfated ester chain. Although both C-51 and C-66 arise through cleavage of an acetate unit, it was suggested that the cleavage mechanism in each case is different and C-51 and C-53 were once part of an intact acetate unit that is cleaved in a Baeyer–Villiger oxidation step (Figure 11).[329] This hypothesis was confirmed by resolution enhancement of the ^{13}C NMR spectrum of (**370**) labeled from [1,2-$^{13}C_2$]acetate, which revealed narrow satellites corresponding to a $^2J_{COC}$ coupling of 2.6 Hz around the central resonances of both C-51 and C-53. This observation can only be explained if the two carbons arose from the same acetate unit. Baeyer–Villiger oxidation of a polyketide chain is rare and such reactions may be mediated by flavin monooxygenase. In addition, a similar monooxygenase-mediated reaction could be invoked in which a single carbon is ultimately eliminated from the polyketide chain as carbon dioxide. Thus, the generation of isolated carbons derived from the methyl group of an acetate in the polyketide chains of the okadaic acid skeleton was proposed, as shown in Scheme 14; oxidation of a methyl-derived carbon to yield an α diketide followed by a Favorski-type rearrangement, peroxide attack, and collapse of the cyclopropanone would yield a shortened polyketide chain containing an oxidized methyl-derived carbon. Such a process explains how the backbone carbons C-10, C-25, and C-26 in the polyketide chain of okadaic acid each arise from the methyl group of a cleaved acetate unit. This carbon-deletion step may account for the interrupted pattern of acetate units in the chain instead of the participation of dicarboxylic acid precursors (Figures 10(a) and 10(b)).

From the dinoflagellate *Prorocentrum lima* from which okadaic acid and its esters were isolated, prorocentrolide (**377**), a toxic macrocycle, formed from a C_{49} fatty acid incorporating a C_{27} macrolide

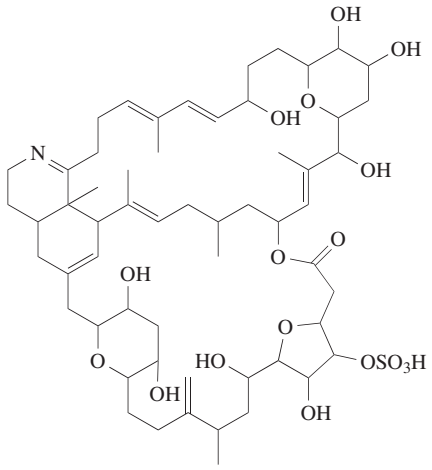


Scheme 14

and a hexahydroisoquinoline in its unique structure.[330] Prorocentrolide (**377**) had a mouse lethality of 0.4 mg kg^{-1} (i.p.), cytotoxicity against L1210 cell (IC_{50} 20 μg ml^{-1}), and negative antimicrobial activities against *Aspergillus niger*, *Candida rugosa*, and *Staphylococcus aureus* at a dose of 80 μg/disk. Prorocentrolide B (**378**) has been obtained from the dinoflagellate *Prorocentrum maculosum* as a fast-acting toxin, showing a type of activity not accounted for by other diarrhetic shellfish poisoning toxins.[331]



(377)



(378)

The co-occurrence of prorocentrolide with okadaic acid (**361**) in the dinoflagellate *P. lima* indicated that dinoflagellates are capable of producing polyethers of entirely different skeletons. Biosynthetic studies on prorocentrolide (**377**) were also carried out[325] and revealed ^{13}C labeling patterns as shown in Figure 12. Out of 56 carbons, 44 carbons were labeled by experiments with single ^{13}C-labeled acetates while 49 carbons were revealed to be derived from acetate from feeding experiments with doubly ^{13}C-labeled acetate. Two carbons at C-43 and C-50 were labeled with both methyl and carboxyl carbons of acetates. Three carbons constituting the isoquinoline ring could not be labeled. The probable biosynthetic precursors were proposed from the labeling patterns as shown in Figure 13.

8.07.8.3.2 Pectenotoxin and yessotoxin

Other than okadaic acid, dinophysistoxins, and their analogues, Yasumoto *et al.* identified two kinds of polyether toxins, pectenotoxin and yessotoxin, as DSP toxins. Pectenotoxins (PTX) were

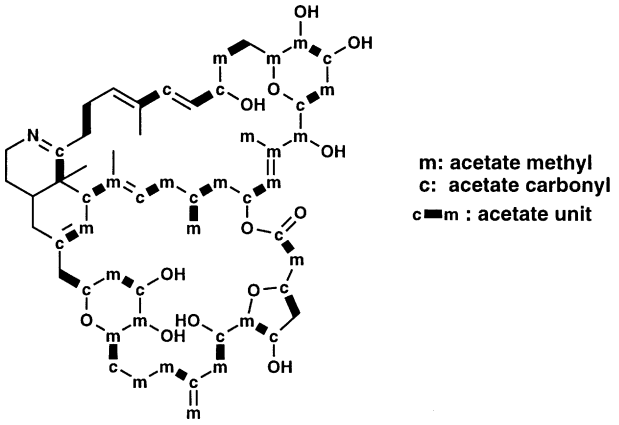


Figure 12 ^{13}C Labeling pattern of prorocentrolide.

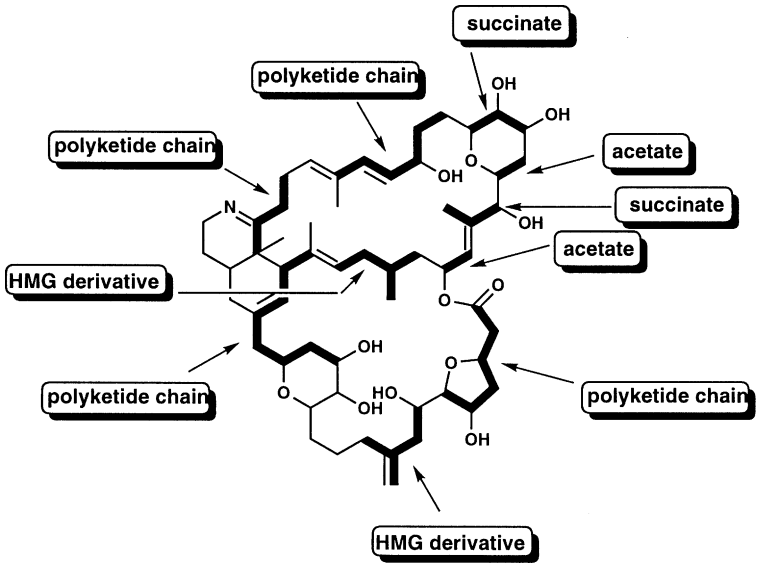
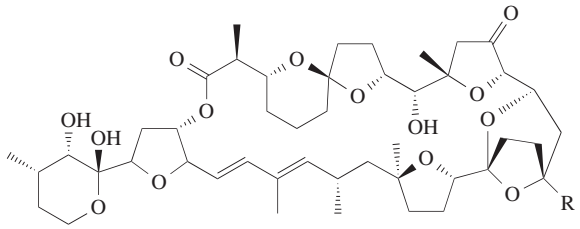


Figure 13 Probable biosynthetic precursors of prorocentrolide.

first isolated from toxic scallops along with DTXs.[312] The digestive glands (200 kg) of the scallop *Patinopecten yessoensis* collected in July 1982, at Mutsu Bay, Japan, were extracted with acetone, and the ether-soluble materials of the acetone extract were subjected to chromatographic separations using columns of silicic acid, Sephadex LH-20, reverse-phase C_8 (LiChroprep RP-8), alumina, Toyopearl HW-40, and HPLC (Develosil silicic acid) to afford five pure toxins, PTX1 (20 mg) as crystals, PTX2 (40 mg), PTX3 (10 mg), PTX4 (7 mg), and PTX5 (0.5 mg). The structure of PTX1 (**379**) was determined by X-ray diffraction methods, and PTX2 (**380**) was identified as 43-deoxy PTX1 on the basis of spectral data.[312] Spectral studies of PTX3 (**381**) showed PTX3 was a

43-aldehyde derivative of PTX2.[332] From a chemical point of view, it was notable that pectenotoxins were substantially different from other described dinoflagellate toxins. Specifically, they differed from others in having a long carbon backbone (C_{40}), a C_{33} lactone ring, and a unique dioxabicyclo moiety. The large oxygen-rich internal cavity was largely similar to the cavities found in the polyether ionophores from terrestrial microorganisms. Coexistence of DTXs and PTXs having such different skeletons is an interesting subject in the biosynthesis of polyether compounds in dinoflagellates.



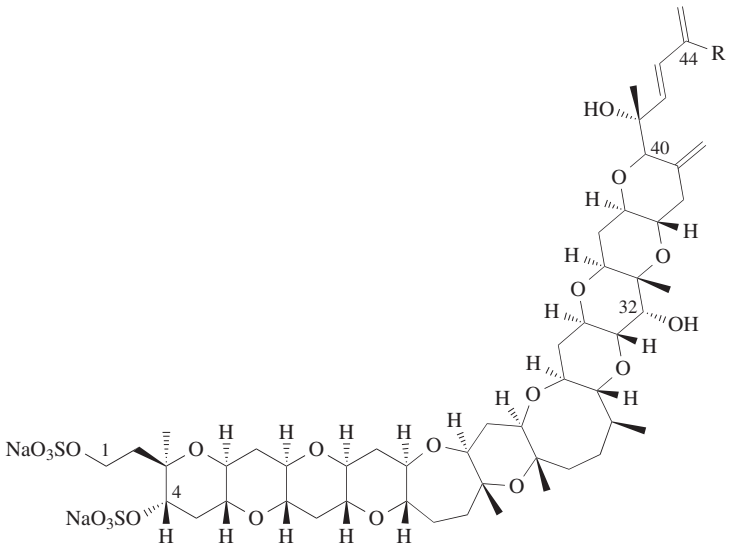
(**379**) R = CH_2OH
(**380**) R = Me
(**381**) R = CHO

Investigation of the cytotoxic constituents of a two-sponge association (*Poecillastra* sp. and *Jaspis* sp.) collected off Cheju and Komun Islands, Korea, led to the isolation of PTX2 (**380**) as an active compound. In an *in vitro* assay, (**380**) displayed very potent cytotoxic activities against human lung (A-549), colon (HT-29), and breast (MCF-7) cancer cell lines. PTX2 (**380**) also exhibited selective cytotoxicity against several cell lines representing ovarian, renal, lung, colon, CNS, melanoma, and breast cancer, with differences in LC_{50} values between sensitive and resistant cell lines of 100-fold or more.[333]

The effects of PTX1 (**379**) on liver cells were investigated by fluorescence microscopy. The *in vitro* application of PTX1 to these cells induced reduction in number and loss of the radial arrangement of microtubules. It also induced disruption of stress fibers and accumulation of actin at the cellular peripheries. Further studies were undertaken to explore the feasibility of using PTX1 as a tool for analyzing the properties of cytoskeletal proteins.[334] Sequential ultrastructural changes were studied in mouse digestive organs after i.p. injections of DSP toxins, DTX-1 (**362**) (see Section 8.07.8.3.1) and PTX1 (**379**). DTX-1 (**362**) produced severe mucosal injuries in the small intestine within 1 h after the administration of the toxin. The injuries were divided into three consecutive stages: extravasation of villi vessels, degeneration of absorptive epithelium, and desquamation of the degenerated epithelium from the lamina propria. In contrast to (**362**), PTX1 (**379**) resulted in no abnormalities in the small intestine, but did cause characteristic liver injuries. Within 1 h after the injection of PTX1 (**379**) numerous nonfatty vacuoles appeared in the hepatocytes around the periportal regions of the hepatic lobules. Electron microscopic observations with colloidal iron demonstrated that these vacuoles originated from invaginated plasma membranes of the hepatocytes.[335]

Yessotoxin is another class of DSP toxins, isolated from digestive glands of the scallop *Patinopecten yessoensis* (84 kg) collected in Mutsu Bay, Japan, in 1986. Their acetone extracts were partitioned between ether and aqueous MeOH, and the methanolic layer was suspended in water and extracted with 1-butanol. The toxic extract was separated by chromatographies using aluminum oxide, two kinds of reversed phase octadecyl silane (ODS) columns, and gel permeation on Toyopearl HW-40 to yield a purified toxin (60 mg), named yessotoxin (YTX (**382**)).[336] The toxin killed mice at a dose of 100 μg kg^{-1} (i.p.) but caused no fluid accumulation in suckling mice intestines even at a fatal dose. The planar structure of (**382**) was elucidated by detailed analyses of several kinds of 2D NMR techniques. The presence of sulfate ester was suggested by ion chromatography of sulfate ions liberated by solvolysis of (**382**) in pyridine–dioxane (1:1) at 120 °C for 3 h, which also afforded desulfated YTX. YTX (**382**) proved to be a polyether compound having a ladder-shape polycyclic skeleton with an unsaturated side-chain of nine carbons, two sulfate esters, and no carbonyl groups. The negative ion fast atom bombardment tandem mass spectrum of (**382**) showed a series of ions due to fragmentation at positions characteristic of cyclic ethers to demonstrate the applicability of tandem mass spectrometry to the structural elucidation of polyether compounds of this size.[337] The

mass spectrometric data alone allowed assignment of the number and location of 11 ether rings of (**382**), which agreed with those previously deduced by NMR studies. The relative configuration of YTX (**382**) and the ring conformation were determined by NMR experiments.[338] Additionally, two other new analogues, 45-hydroxyYTX (**383**) and 45,46,47-trinorYTX (**384**) were isolated from toxic scallops and their relative stereochemistries were also determined.



(**382**) R = 45 47

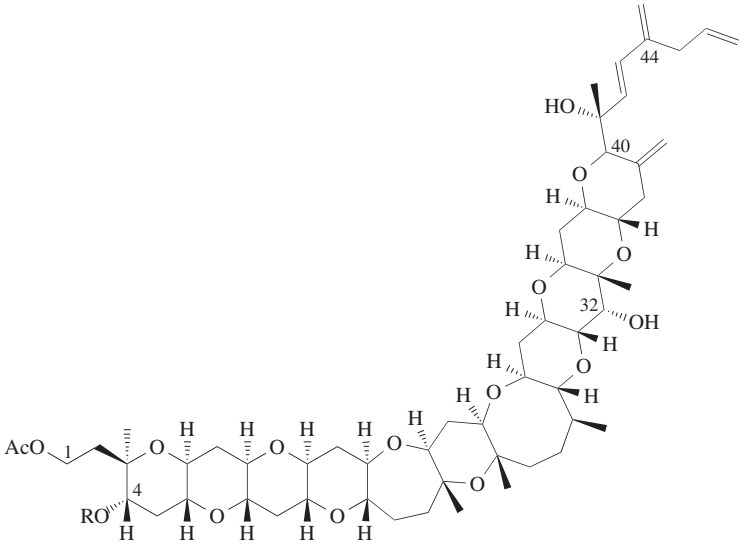
(**383**) R = OH

(**384**) R = H

Furthermore, the absolute configuration of YTX (**382**) was studied by applying a modified Mosher's method using the new chiral anisotropic reagent, 2NMA [methoxy-(2-naphthyl)acetic acid (**385**)].[339] Esterification of 32-OH of YTX (**382**) was initially attempted, but was unsuccessful because of its axial orientation. YTX (**382**) was then subjected to solvolysis as described above (pyridine–dioxane (1:1) at 120 °C for 3 h)[336] to give bisdesulfated YTX, which in turn was treated with an excess of acetic anhydride in pyridine at 0 °C for 45 min to give a mixture of recovered bisdesulfated YTX, 1-*O*-acetyl YTX, and 1,4-di-*O*-acetyl YTX, separable by HPLC. 1-*O*-Acetyl YTX (600 μg) thus obtained having free OH at C-4 was divided into two portions, and each portion was treated with (*R*)- and (*S*)-2NMA, EDC, DMAP, and Et_3N in chloroform for 43.5 h at room temperature to give, after HPLC purification, (*R*)- (**386**) and (*S*)-2NMA esters (**387**), respectively. By analyzing the COSY and HOHAHA spectra of (**386**) and (**387**) and the $\Delta\delta$ values [$\delta_R - \delta_S$] of proton chemical shifts, the absolute configuration of the hydroxyl group at C-4 was determined to be *S*, and, since the relative stereochemistry of the other asymmetric centers was established,[338] the absolute configuration of YTX was determined, as shown in (**382**).[340]

YTX (**382**) had a structural resemblance with brevetoxins (see Section 8.07.8.5.2) and ciguatoxin (see Section 8.07.8.4.1), which are known as potent activators of voltage-gated sodium channels but are not cytotoxins. However, YTX (**382**) did not potentiate those channels and showed cytotoxicity and different toxicological properties. The histopathological response of male mice to YTX (**382**) was compared with that to desulfated YTX. The target organ of the former was the heart and those of the latter were the liver and pancreas. Using electron microscopy, marked intracytoplasmic edema in cardiac muscle cells was seen within 3 h after the i.p. injection of over 300 μg kg^{-1} of YTX (**382**). In contrast, desulfated YTX at the same dose caused severe fatty degeneration and intracellular necrosis in the liver and pancreas, but not in the heart, within 24 h of i.p. injection. Biochemically, the content of triglycerides in the liver of mice treated with desulfated YTX increased about 60 times, and phospholipids twofold more than the control levels of those of mice treated with YTX (**382**).[341]

(385) (2NMA)



(386) R = (*R*)-2NMA
(387) R = (*S*)-2NMA

8.07.8.4 Ciguatera

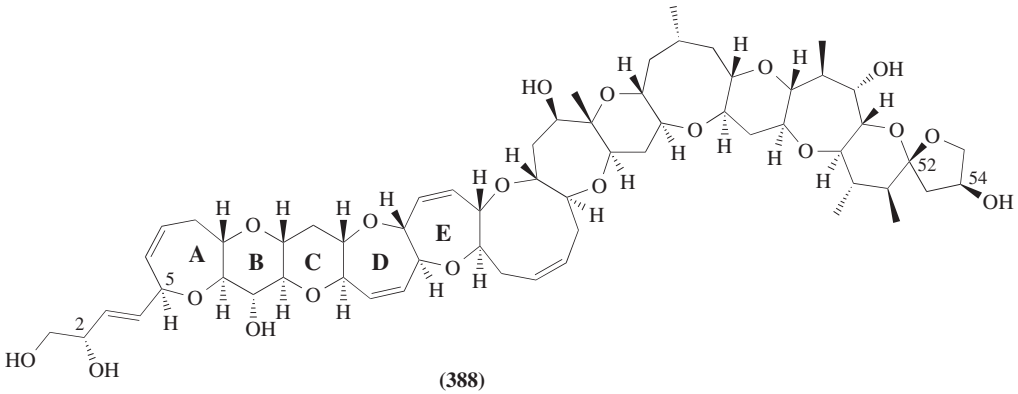
Ciguatera refers to a type of human intoxication resulting from ingestion of coral reef fish.[342] Globally over 20 000 people are estimated to suffer annually from the poisoning, making it one of the largest-scale food poisonings of nonbacterial origin. Though rarely fatal, the poisoning poses a serious threat to public health and tropical fisheries because of its unpredictable occurrence and the implication of numerous fish species. There are two groups of compounds implicated in the poisoning; the main responsible toxins are ciguatoxin (CTX) and its congeners, and the other is maitotoxin (MTX). The clinical symptoms of this poisoning are diverse. In addition to gastrointestinal malaise, vomiting, and diarrhea, which are common in food poisoning, there is a neurological component. Dizziness, tingling of the extremities, and the sensation of temperature reversal are among the salient characteristics of ciguatera poisoning. Other symptoms include joint pain, miosis, erethism, cyanosis, and prostration. Cardiovascular disturbances are low blood pressure and bradycardia. Virtually all victims recover, albeit painfully and slowly. The low fatality rate is due solely to the minute concentration of the toxin in fish flesh.

8.07.8.4.1 Ciguatoxin

Research efforts on ciguatera have been undertaken by institutions in Pacific countries. The historical story of ciguatoxin research at the University of Hawaii was reviewed by Scheuer.[343] His research group initiated the study in 1957, and by 1980 they had isolated 1.3 mg of HPLC-pure ciguatoxin from approximately 1100 kg of moray eels. The toxin was successfully crystallized in MeOH-d_4 solution and the molecular weight was determined to be 1111 Da.[344,345] Although the crystal was not suitable for single-crystal X-ray analysis, the toxin was characterized to be a polyether compound by NMR studies using an inadequate amount of material.

Meanwhile, Yasumoto *et al*. had identified an epiphytic dinoflagellate, *Gambierdiscus toxicus*, as a causative organism in 1977.[346] It was, however, extremely difficult to obtain enough ciguatoxin for structural elucidation. Although *G. toxicus* produced maitotoxin, a second important toxin in ciguatera, no ciguatoxin was isolated when it was cultured. The structure of ciguatoxin was finally elucidated by Yasumoto's group and the resident French public health workers in 1989.[347–349] For that study moray eels (*Gymnothorax javanicus*, ca. 4000 kg) collected in French Polynesian waters were extracted to yield no more than 0.35 mg of ciguatoxin (CTX (**388**)), and 0.75 mg of a congener, CTX4B (**389**), was isolated from the ciguatera causative dinoflagellates, *Gambierdiscus toxicus*,

collected in the Gambier Islands in French Polynesia. Using those samples, the structures of CTX (**388**) and CTX4B (**389**) including the relative stereochemistries were successfully elucidated as polycyclic ethers by NMR methods.



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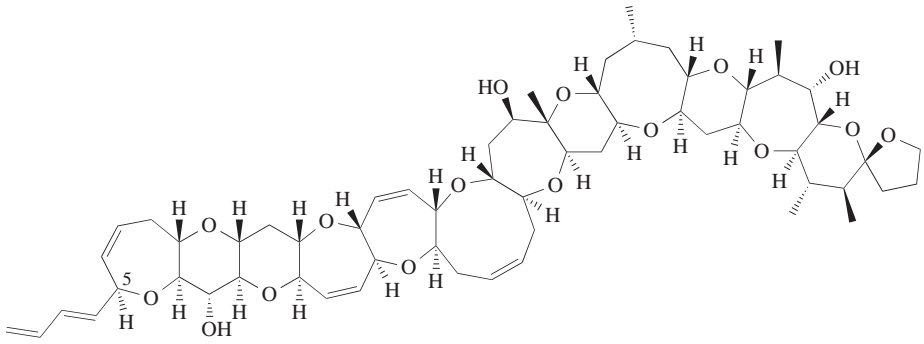
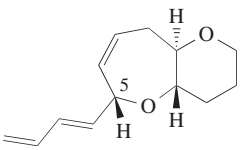


(389)

The molecular formulas of CTX (**388**) and CTX4B (**389**) were determined as $C_{60}H_{86}O_{19}$ and $C_{60}H_{84}O_{16}$, respectively, by high-resolution FABMS. The skeletal structure was established mainly on the basis of ^{1}H-^{1}H 2D NMR data. Since some parts of the molecules were unassignable due to extreme broadening or even disappearance of signals, measurement of ^{1}H NMR at low temperature was attempted so as to make the perturbation of the ring slow enough to detect these resonances. The stereochemistry of hydroxyls and methyls substituted on the ether rings was clarified by NOE difference experiments and by combined use of MM2 energy calculations and spectral simulations. The structural difference between CTX (**388**) and CTX4B (**389**) was elucidated by COSY analyses, showing that the latter was a less oxidized entity and had a *trans*-butadiene moiety at one terminus of the molecule and lacked a hydroxyl group at C-54 at the other end. The moray eel, which is placed near the top of the coral ecosystem, tends to contain more polar congeners, while the dinoflagellate produces less polar ones. It was thus suggested that less polar congeners produced by *G. toxicus* are precursors to the more polar toxins found in fish, the latter formed by oxidative enzyme systems in the fish. Interestingly, toxicity of oxidized metabolites was often increased; the lethal potency against mice (i.p.) of CTX (**388**) and CTX4B (**389**) was 0.35 μg kg^{-1} and ca. 4 μg kg^{-1}, respectively, the former being 11 times more toxic than the latter.

The absolute stereochemistry of the C-5 position of CTX4B (**389**) was suggested to be *R* by the CD spectral data of compound (**390**), which was synthesized stereoselectively and had the AB ring framework of (**389**).[350] The stereochemical assignment of C-54 of CTX (**388**) was confirmed by synthesis of compounds (**391**) and (**392**) corresponding to the KLM ring model of CTX (**388**) and its epimer, and comparison of their ^{1}H NMR data.[351] The ^{13}C NMR assignments of CTX (**388**) were achieved with 1.1 mg of the sample (1 μmol) obtained from carnivorous fish collected in French Polynesia, Kiribati, Micronesia, and Fiji.[352] ^{13}C-decoupled HMQC and HSQC spectra in CD_3CN/D_2O (2:1) and C_5D_5N/D_2O (20:1) revealed $^{1}J_{CH}$ arising from the flexible part of the molecule which yielded no sharp peaks in the 1D ^{13}C NMR spectrum.



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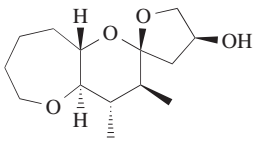

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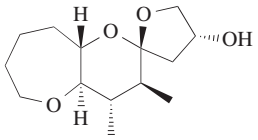
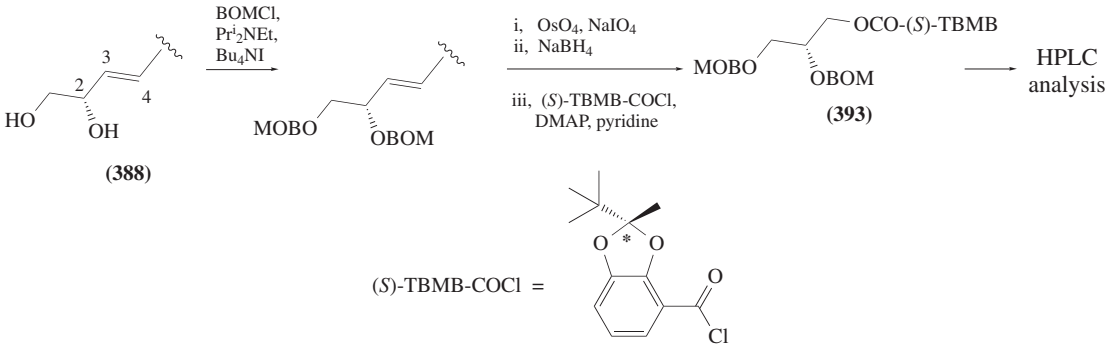


(392)

In order to determine the absolute configuration of the C-2 position of CTX (**388**), the 1,2-diol of (**388**) was protected as benzyloxymethyl ethers, and then the 3,4-double bond was oxidatively cleaved by treatment with OsO_4-$NaIO_4$ followed by $NaBH_4$ reduction. The resultant alcohol was esterified with a chiral fluorometric reagent, (*S*)-(+)-2-*t*-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [(*S*)-TBMB acid] to give (*S*)-TBMB (**393**), which compared with synthetic references on two HPLC systems (Scheme 15). Starting with only 5 μg of CTX (**388**) the absolute configuration at C-2 was successfully determined to be *S*.[353]

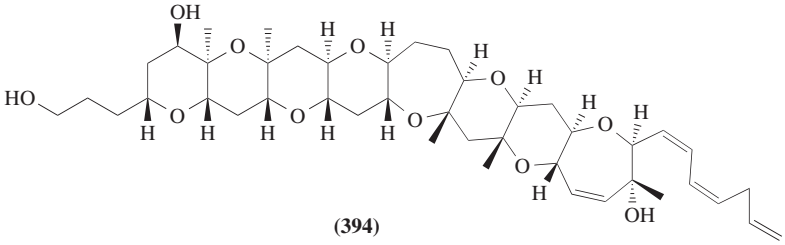


Scheme 15

A number of ciguatoxin analogues have also been structurally elucidated. One strain of the dinoflagellate *Gambierdiscus toxicus* collected at Rangiroa Atoll in French Polynesia was cultured, and from the harvested cells of 1100-L cultures three polyether toxic compounds, gambierol[354] (**394**) (1.2 mg, LD_{50} in mouse 50 μg kg^{-1} (i.p.), $C_{43}H_{64}O_{11}$), CTX3C[355] (**395**) (0.7 mg, 1.3 μg kg^{-1} (i.p.), $C_{57}H_{82}O_{16}$), and CTX4A[356] (**396**) (0.45 mg, 2 μg kg^{-1} (i.p.), $C_{60}H_{84}O_{16}$) were isolated. The ring system of gambierol (**394**) differed from that of CTX (**388**). Ring E in CTX3C (**395**) was an oxocene instead of the oxopene in CTX (**388**) and CTX3C (**395**) had no side-chain attached at C-5 of CTX (**388**). CTX4A (**396**) had the same molecular formula as CTX4B (**389**) and the difference in structure of (**396**) from (**389**) was found in the stereochemistry at the C-52 *spiro* carbon. Thus, these findings demonstrated the diversity of ciguatera toxins and the complex biosynthesis of polyether compounds in *G. toxicus*. Another ciguatoxin analogue, CTX2A1 (**397**) ($C_{57}H_{84}O_{18}$), was isolated from moray eels and its structure was elucidated to be a 2,3-diol derivative of CTX3C (**395**).[353] This was the first CTX analogue to be oxidized in the A ring and was an additional example showing the diverse metabolic modification of CTXs occurring in fish.

To confirm the absolute configuration of the C-5 position of CTX analogues, 100 μg of CTX4A (**396**) was reacted with *p*-bromobenzoyl chloride to yield the tris-*p*-bromobenzoyl derivative. The CD spectrum of CTX4A-tris-*p*-bromobenzoate was comparable with that of the *p*-bromobenzoate derived from a synthetic (5*R*)-AB-ring model compound, thus indicating that the absolute configuration at C-5 was *R*. Consequently, the whole stereostructure of CTX (**388**) was able to be assigned with (2*S*,5*R*)-configuration.[353]

The Caribbean ciguatoxin (< 100 μg) was isolated from a collection of barracuda and horse-eye jack (545 kg) from a ciguatera-endemic region of the Caribbean. Caribbean ciguatoxin was shown to have a molecular weight of 1123 by electrospray mass spectrum. In contrast, CTX (**388**) had a mass of 1111 Da. Structural characterization of the Caribbean ciguatoxin was carried out by the use of micro inverse-detection probes and micro cells for the HMQC and HMBC spectra to result in partial assignment of its structure.[357] Micro inverse-detection probes employed in conjunction with Shigemi micro NMR cells were shown to afford significant improvement in sensitivity. Caribbean ciguatoxin had only six alkenic protons while CTX (**388**) possessed ten. The segment of Caribbean ciguatoxin molecule from C-34 to C-42 positions was identical to (**388**), while Caribbean ciguatoxin lacks the C-54 hydroxyl group, appearing instead to have a hydroxyl at the 53-position.



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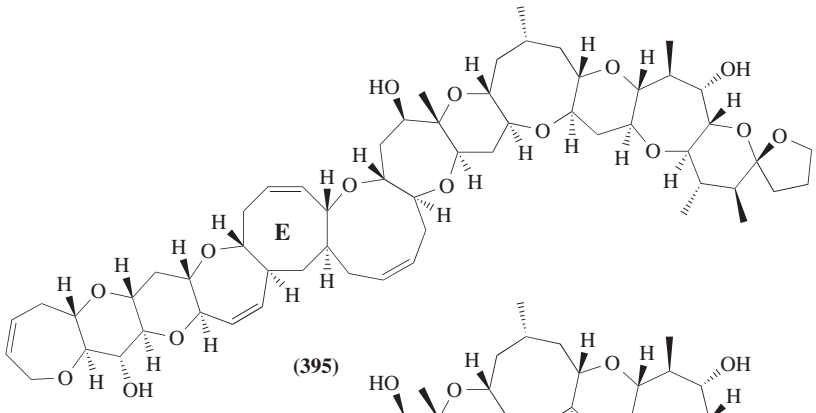

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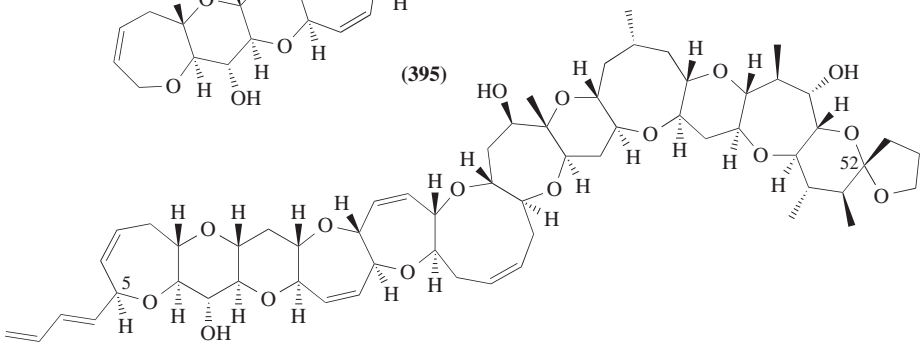

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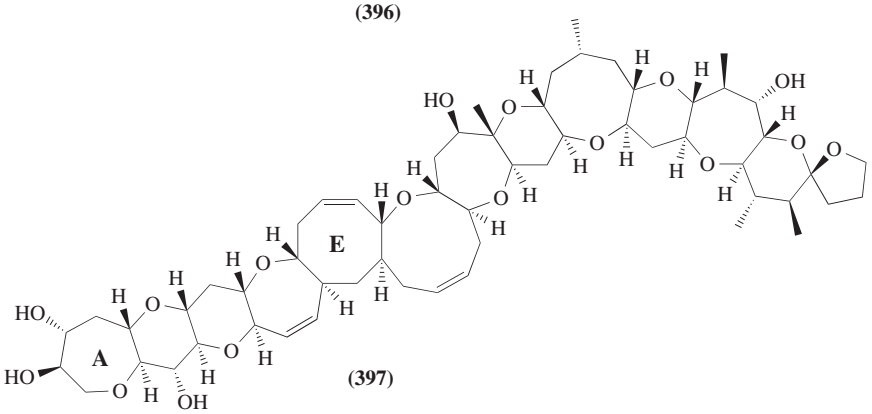
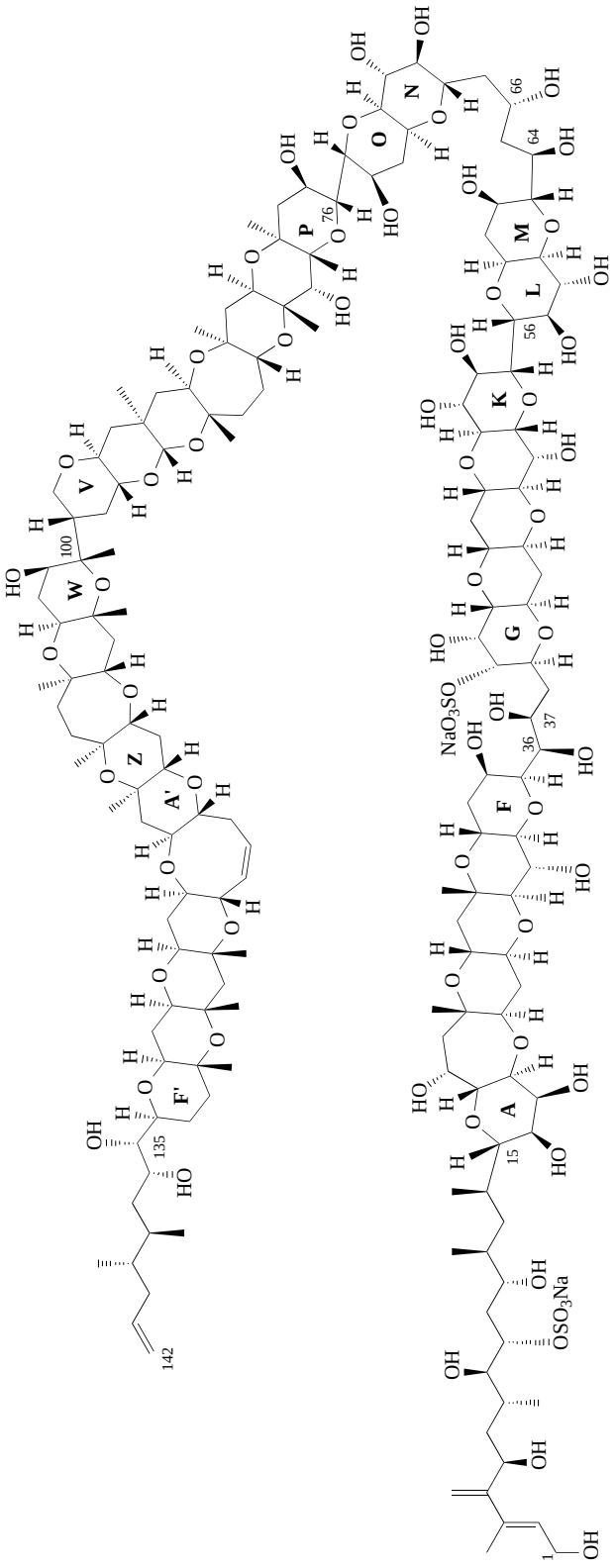


(397)

8.07.8.4.2 Maitotoxin

Maitotoxin (MTX (**398**)) was first discovered by Yasumoto and Satake from the surgeonfish *Ctenochaetus striatus* as one of the causative toxins of ciguatera and was named after the Tahitian fish "maito." The toxins accumulated in herbivorous fish are diverse, and MTX (**398**) was later confirmed to originate from the dinoflagellate *Gambierdiscus toxicus*.[342] MTX (**398**) is one of the most complex natural products having molecular weight 3422 Da (nominal mass) and molecular formula $C_{164}H_{256}O_{68}S_2Na_2$, and its chemical structure has been successfully determined. The lethality of maitotoxin to mice (0.05 μg kg^{-1}, i.p.) is only exceeded by a few proteins. The mechanism underlying its high potency is now explainable by the activation of voltage-independent calcium channels, thus producing a highly enhanced calcium influx of extracellular Ca^{2+} ion across the cell membrane.[358]



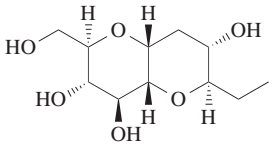
(398)

Isolation of MTX (**398**) was performed by Yasumoto's group by culturing the dinoflagellate *G. toxicus* from the Gambier Islands, French Polynesia in a nutrient-enriched seawater medium.[359] Fernback flasks, each containing 21 of the medium, were inoculated with seed cultures of the dinoflagellate and maintained at 25 °C for 38 days under illumination of 4000–8000 lux at an 18/6 light:dark photoperiod. After reaching a cell density of about 1000 cells ml^{-1}, the dinoflagellates were collected by filtration and extracted twice with MeOH and twice with $MeOH/H_2O$ (1:1) under reflux. After concentration of the combined extracts by evaporation, the residual suspension was partitioned between CH_2Cl_2 and aqueous MeOH, and the latter was extracted with butanol. The butanol layer was toxic and was subjected to separation by silica gel column, C18 column, Develosil C8, and Develosil TMS-5 column to afford MTX (**398**). The eluates were monitored by the use of mouse lethality (intraperitoneal injection) tests and by a UV flow detector at a wavelength of 225 nm. From 4000 l of culture of the dinoflagellate, 20 mg of MTX (**398**) was obtained as a colorless solid.

The presence of two sulfate ester groups in the molecule was apparent from the IR and mass spectra, and from analyses of solvolysis products (pyridine/dioxane (1:1), 120 °C, 4 h; HPLC analysis on a C18 column with an aqueous solution containing 1.0 mM tetrabutylammonium hydroxide and 0.8 mM 1,3,5-benzenetricarboxylic acid, monitored at 280 nm).[359] Despite its large size, MTX (**398**) had no known repeating units, such as amino acids and sugars, no carbonyl groups, no side-chains (apart from those at the termini) other than methyls or an exomethylene, and no carbocycles. Structural elucidation of MTX (**398**) was carried out by extensive 2D NMR measurements. A large portion of the structure was shown to be a brevetoxin-type polyether. Chiefly due to extensive overlapping of ^1H and ^{13}C NMR signals even in the 2D spectra, MTX (**398**) was treated with $NaIO_4$ followed by reduction with $NaBH_4$, resulting in cleavage of the molecule into two major parts, fragments A (C-1–C-36) and B (C-37–C-135) together with a small C_9 fragment (C-136–C-142).[360] Fragments A and B had molecular weights 964 and 2328, respectively. Their structures were elucidated by extensive 2D NMR and collisionally activated dissociation (CAD) MS/MS experiments. Reassembling the three fragments led to the complete structure of MTX ($C_{164}H_{256}O_{68}S_2Na_2$), encompassing two sulfate esters as described above, 28 hydroxyls, and 32 ether rings.[361] The relative stereostructure was partly assigned by spectral data and was revealed to comprise *trans*-fused polycyclic ethers except for rings L/M and N/O which are *cis*-fused. The stereochemical correlations for the acyclic linkages such as rings K/L, O/P, and V/W were revealed on the basis of NMR data with the aid of molecular mechanics calculations. The stereochemistry of the acyclic parts, C-5–C-14, C-36–C-37, C-64–C-66, and C-135–C-139 remained to be determined in 1994.[362] The structure of MTX (**398**) was confirmed by the complete ^{13}C NMR assignments and NOEs data obtained by a 3D pulsed field gradient NOESY-HMQC spectrum. The ^{13}C-enriched sample for the 3D spectrum was prepared by culturing the dinoflagellate *Gambierdiscus toxicus* in media containing ^{13}C-labeled carbonate and appeared to contain 4% of ^{13}C. The ether linkages and stereochemistry for cyclic ethers were verified on the basis of NOEs derived from the 3D NMR data. The complete ^{13}C NMR assignments of MTX (**398**) further supported the present structure.[363]

Structural studies of MTX (**398**) based on the combination of spectral studies and synthesis of the corresponding compounds were performed by Tachibana's research group collaborating with Yasumoto's group. To confirm the *cis*-fused L/M and N/O ring systems of MTX (**398**), a *cis*-fused 1,6-dioxadecalin system (**399**) was synthesized in a stereocontrolled manner, and the comparison of its ^1H and ^{13}C NMR data with those of natural toxin established the earlier stereochemical assignments.[364] The relative configuration of carbons within the C-63–C-68 acyclic linkage of MTX (**398**) was assigned by synthesis of stereodefined model compounds (**400**)–(**403**) and their comparison with MTX (**398**) in ^1H and ^{13}C NMR spectra. All four diastereomers (**400**)–(**403**) showed different NMR profiles and only (**400**) among these gave virtually identical ^1H and ^{13}C NMR data to those for the C-63–C-68 region of MTX (**398**).[365] The relative configuration of carbons within the C-35–C-39 acyclic linkage of MTX (**398**) was suggested from the NOESY and E.COSY spectra of MTX (**398**), and confirmation of the assignment was achieved by the synthesis of a stereodefined model compound (**404**) and its comparison with MTX (**398**) in ^1H and ^{13}C NMR spectra.[366] From these results stereochemical assignments of the whole molecule of MTX (**398**) except for side chains were completed. The model compounds (**399**)–(**404**) were all optically active, all being prepared starting from D- or L-glucose, and eventually all these model compounds were antipodes of the corresponding portion of MTX (**398**).

Long-range carbon–proton coupling constants ($^{2,3}J_{C,H}$) were measured for MTX (**398**) by hetero-half filter TOCSY (HETLOC) experiments and phase-sensitive HMBC with use of 9 mg of a 4% ^{13}C-enriched sample. The necessary coupling constants within the terminal acyclic portions of MTX (**398**), where NOE analysis was not successful owing to the presumed coexistence of multiple



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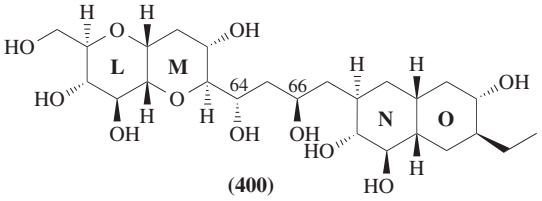

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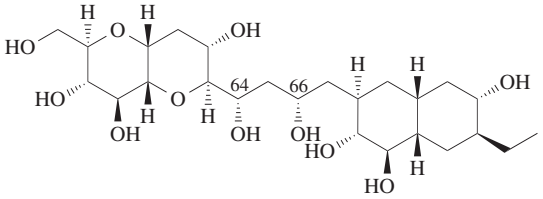

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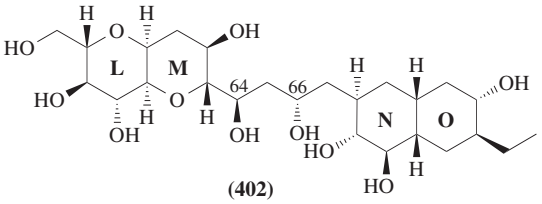

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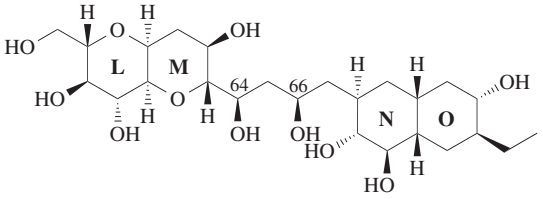

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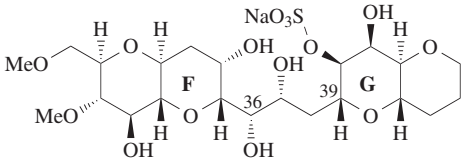
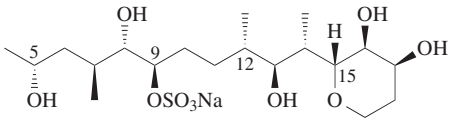


(404)

conformers, were thus obtained for the resultant elucidation of relative configurations for the acyclic C-5–C-9 stereogenic centers to be 5*R**, 7*R**, 8*R**, and 9*S**.[367] For the C-9–C-15 portion, from the analyses of conventional NOE, $^{3}J_{H,H}$-, and $^{2,3}J_{C,H}$-values determined by HETLOC and phase-sensitive HMBC experiments, the relative stereochemical relationships for H-9/H-12, H-12/H-13, and H-14/H-15 were established, leaving only the relationship between H-13 and H-14 unassigned. These findings suggested the configuration of the side chain (C-5–C-15) to be either (**405**) or (**406**) in which the diastereomeric relationship was inverted at the C-13–C-14 bond. Thus, in order to assign this diastereomeric relationship, and also to confirm the relative configurations at C-9, C-12, and C-13 as deduced from the NMR data, the diastereomeric fragments (**405**) and (**406**) were synthesized for comparison of their NMR data with those of MTX (**398**). These fragments were designed also to incorporate the asymmetric centers whose configuration had already been determined (C-5–C-8 and C-15–C-17) so as to reproduce the ^{1}H and ^{13}C chemical shifts of the natural product. In the comparison of the ^{1}H and ^{13}C data of diastereomers (**405**) and (**406**), which were prepared in a stereocontrolled manner, the observed chemical shifts of (**405**) clearly matched those of MTX (**398**) more closely. These results unambiguously established the relative configuration of the C-1–C-15 fragment of MTX (**398**) to be 5*R**, 7*R**, 8*R**, 9*S**, 12*R**, 13*R**, 14*R**, and 15*S**.[368]



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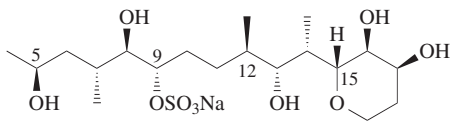
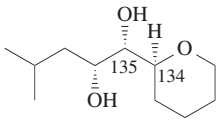


(406)

The relative stereochemistry between C-136 and C-138 in the other terminal acyclic chain was unambiguously assigned on the basis of the $^{3}J_{H,H}$ and $^{2,3}J_{C,H}$ data. The stereochemical relationships among C-134, C-135, and C-136 were also deduced from similar NMR analysis and confirmed by the comparison of the synthesized model compound (**407**) with MTX (**398**) in their ^{1}H NMR. In order to determine the absolute configurations along with the relative configuration between C-138 and C-139, which was not deduced by NMR due to signal broadening, the four stereoisomers (**408**)–(**411**) possible for 3,4-dimethyl-6-hepten-1-ol were synthesized and compared with a degraded fragment of the natural product by GC with a chiral column to identify the diastereomer (**408**) as the natural diastereo- and optical isomer, thus establishing the 138*R*, 139*S* configuration of MTX (**398**). From all of these results, the Tachibana–Yasumoto group established the complete absolute stereochemistry of MTX to be represented by structure (**398**).[369]



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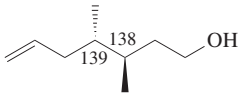

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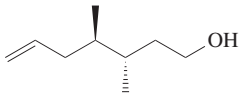

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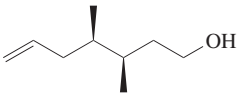

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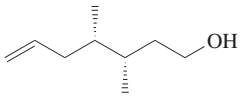


(411)

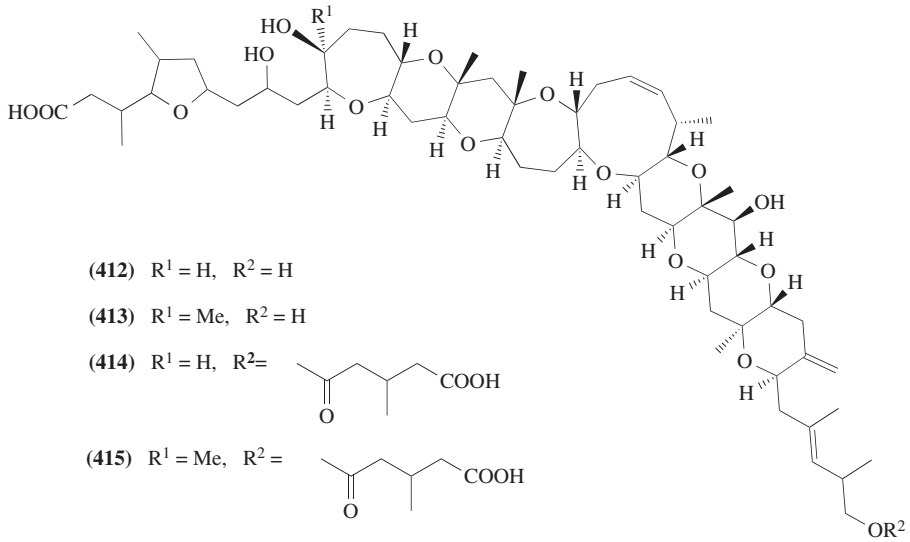
The relative stereochemistry of MTX (**398**) was also studied independently by Kishi and co-workers via organic synthesis.[370] The relative stereochemistry of the C-1–C-15 portion was elucidated via the synthesis of the eight diastereomers possible for the C-1–C-11 portion and the eight diastereomers possible for the C-11–C-15 portion and the comparison of their proton and carbon NMR characteristics with those of MTX (**398**), and the synthesis of two remote diastereomers and comparison of their proton and carbon NMR characteristics with those of MTX (**398**). The relative stereochemistry of the C-35–C-39, C-63–C-68, and C-134–C-142 acyclic portions was established via the synthesis of the diastereomers possible for models of these fragments and comparison of their proton and carbon NMR characteristics with those of MTX (**398**). By these studies, the complete relative stereochemistry of MTX (**398**) was established. Some biogenetic considerations were given to speculate on the absolute configuration of MTX (**398**). The preferred solution conformations of model compounds for the four acyclic portions of MTX (**398**) were elucidated on the basis of vicinal proton coupling constants, and by assembling them, the approximate global conformation of MTX (**398**) was suggested to be represented by the shape of a hook, with the C-35–C-39 portion being its curvature.

In addition to its lethal potency against mice, MTX (**398**) possesses multiple activities, such as hormone stimulation, neurotransmitter release, activation of phosphoinositide degradation, and potentiation of protein kinase, all of which appear to be linked to elevation of the intracellular Ca^{2+} concentration.[371] Thus the toxin, which is commercially available, serves as a tool for studies on cellular events associated with Ca^{2+} flux. As an example of these studies, Daly *et al.* reported that MTX (**398**) elicited a marked influx of $^{45}Ca^{2+}$ into NIH 3T3 fibroblast cells.[372] The influx was blocked by imidazoles (econazole, miconazole, SKF 96365, clotrimazole, calmidazolium) with IC_{50} values from 0.56 μM to 3 μM. The pattern of inhibition of MTX-elicited calcium influx did not correspond to the ability of the agents to block elevation of calcium that ensues through calcium-release activated calcium (CRAC) channels after activation of phosphoinositide breakdown by ATP in HL-60 cells. Tachibana and co-workers studied the blockade of MTX-induced Ca^{2+} influx in rat glioma C6 cells by alkylamines.[373] A series of alkylamines, namely, mono-, di-, and trihexylamine and tetrahexylammonium iodide, were tested for inhibition against Ca^{2+} influx by MTX (**398**) in rat glioma C6 cells. The Ca^{2+} entry was monitored by $^{45}Ca^{2+}$ influx and Ca^{2+}-sensitive fluorescent dye (fura-2) assays. While hexylamine showed no significant inhibition, the other amines exhibited potent inhibition upon $^{45}Ca^{2+}$ influx assays along the increasing number of alkylation; dihexylamine with IC_{50} of approximately 50 μM, trihexylamine with approximately 20 μM, and tetrahexylammonium iodide with approximately 5 μM. C6 cells reportedly lack ion channels other than two types of K^+ channels (delayed rectifier and Ca^{2+}-activated channels), both of which are blocked by charybdotoxin. The toxin did not affect the MTX action, hence suggesting that the inhibition by the amines was not via K^+ channels. Implication of membrane potential was also ruled out by experiments using a voltage-sensitive fluorescent dye, DiSC2(5), where these amines did not alter membrane potential in the presence of MTX (**398**). One of the possible explanations is that MTX (**398**) interacts with a non-voltage-gated and usually inactive channel presumably belonging to the ion channel superfamily, through which the alkylamines block ion entry by a mechanism similar to that proposed for K^+ channels.

8.07.8.4.3 Gambieric acid

Potent antifungal substances were found in one strain of the dinoflagellate *Gambierdiscus toxicus*, an epiphytic species implicated in ciguatera as the source of maitotoxin and ciguatoxins. While these

toxins were retained in the algal cells during culture, the antifungals were released into the medium. Activity-guided purification led to the discovery of four polyethers, designated gambieric acids A, B, C, and D (**412**)–(**415**).[374,375] Their property of inhibiting the growth of *Aspergillus niger* was of unprecedented potency, exceeding that of amphotericin B by a factor of 2000. Gambieric acids A (**412**) and B (**413**) and a mixture of gambieric acids C (**414**) and D (**415**) inhibited the growth of *A. niger* at 10, 20, and 10 ng/disk, respectively, by the paper disk method, while amphotericin B and okadaic acid were inhibitory at doses of 20 μg/disk and 10 μg/disk, respectively. Gambieric acid A (**412**) at a dose of 1 mg kg^{-1} showed no toxicity against mice upon an intraperitoneal injection. The cytotoxicity (IC_{50}) of the mixture of gambieric acids C (**414**) and D (**415**) against mouse lymphoma L5178Y cell was 1.1 μg ml^{-1} when monitored by [^{3}H]thymidine incorporation.

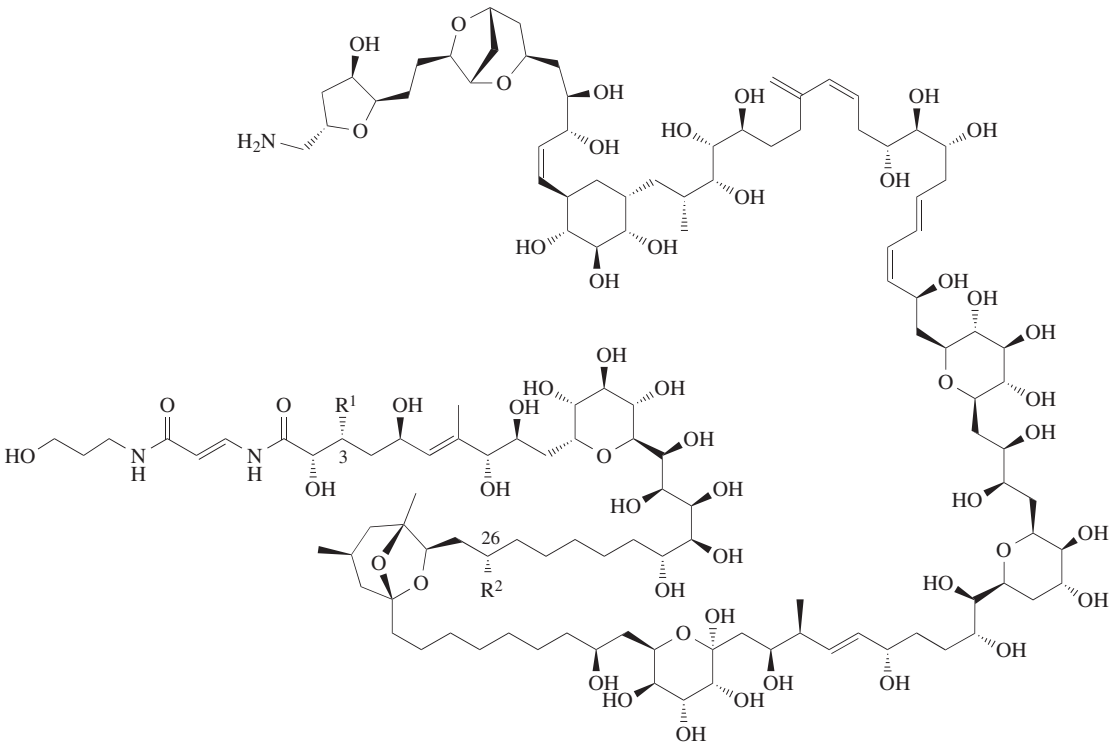


The dinoflagellate *G. toxicus* (GII1 strain) was isolated in the Gambier Islands, French Polynesia, and was cultured in a seawater medium (see Section 8.07.8.4.2). The medium (5000 l), free of algal cells, was passed through a column of Amberlite XAD-2. Antifungal compounds were retained on the column and eluted with methanol. Purification of the eluate by solvent partition and column chromatography afforded gambieric acids A (**412**) (0.6 mg) and B (**413**) (0.15 mg) and a mixture (5.8 mg) of gambieric acids C (**414**) and D (**415**). The structures of gambieric acids were elucidated by NMR and negative FABMS data as well as by hydrolysis to furnish 3-methylglutaric acid. They had novel brevetoxin-type structures consisting of nine contiguous ether rings (7/6/6/7/9/6/6/6/6) and one isolated tetrahydrofuran. Gambieric acids C (**414**) and D (**415**) were 3-methylhemiglutarates of gambieric acids A (**412**) and B (**413**), respectively. The strain GII1 did not produce ciguatoxins, indicating the biosynthetic versatility of this organism. From an ecological point of view, it is interesting to note that epiphytic dinoflagellates release antifungals from the cells, while retaining maitotoxin, which has no antimicrobial activity. Because of their solubility, gambieric acids may stay on the surface of the substrate near the dinoflagellates and exert an allelopathic function against other epiphytic organisms. Maitotoxin, on the other hand, may act as an antifeedant with its extreme toxicity toward higher animals.

8.07.8.5 Other Toxins

8.07.8.5.1 Palytoxin

Palytoxin (**416**) ($C_{129}H_{223}N_3O_{54}$) is one of the most potent and complex marine toxins, associated with marine coelenterates (zoanthids) of the genus *Palythoa*.[376] Its intravenous lethality (LD_{50}) ranged from 0.025 μg kg^{-1} in the rabbit to 0.45 μg kg^{-1} in the mouse. The structural elucidation of this complex toxin was achieved early in the 1980s by three chemistry laboratories, those of Moore and Bartolini,[377] Uemura *et al.*,[378] and Kishi and co-workers.[379] All aspects of stereochemistry of (**416**) were rigorously determined by comparison of synthetic fragments with the natural product.



(416) $R^1 = R^2 = Me$

(417) $R^1 = R^2 = H$; OH → H (position not located)

Though first isolated from the zoanthid *Palythoa toxica*, the biogenetic origin of palytoxin (**416**) was questioned because of marked seasonal and regional variations. Sporadic occurrence of palytoxin in an alga, crabs, and a herbivorous fish also suggested that the toxin might be produced by a microorganism and transmitted to the marine food chain, as is the case with ciguatera (see Section 8.07.8.4). A dinoflagellate *Ostereopsis siamensis* is taxonomically closely related to *Gambierdiscus toxicus* (see Section 8.07.8.4), and contains potent toxins, named ostereocins. Yasumoto and co-workers studied the toxins in *O. siamensis*, and identified the major ostereocin as a palytoxin analogue.[380] They collected *O. siamensis* at Aka Island, Okinawa, Japan, and the dinoflagellate was grown for 30 days under the same culture conditions as *G. toxicus*. The extracts of the harvested algal cells were separated by solvent partition and column chromatographies. Purification was monitored by mouse assay following characteristic UV maxima. From 9361 of culture, 3.8 mg of ostereocin D (**417**) ($C_{127}H_{220}N_3O_{53}$) was isolated as a major toxin constituent accounting for 70% of total toxicity. The lethality (LD_{50} value) of (**417**) in mice (i.p.) was 0.75 μg kg^{-1}. Extensive spectral studies revealed that ostereocin D (**417**) was a 3,26-bisdesmethyldeoxy analogue of palytoxin. Determination of the position of the missing hydroxyl group in (**417**) was hampered by heavily overlapping signals of methine protons and oxycarbons. The remainder of ostereocin D (**417**) was believed to be identical regiochemically to palytoxin (**416**), and was deduced to have the same stereochemistry as palytoxin (**416**) on the basis of the analysis of $^3J_{H,H}$ values. From the dinoflagellate *O. siamensis*, palytoxin (**416**) itself was not identified and minor ostreocin analogues were detected by electrospray ionization mass spectrometry. The small structural changes barely affected the mouse lethality; interestingly, the minor analogues had reduced cytotoxicity and hemolytic potency. From this study, though it was not clear whether *O. siamensis* was a symbiont of *Palythoa* spp., the dinoflagellate was strongly indicated to be a source of palytoxin (**416**).

In coral reefs of the Caribbean Sea (Colombia) palytoxin (**416**) was detected in zoanthid species of the genera *Palythoa* and *Zoanthus* by HPLC or assaying the delayed hemolysis in human erythrocytes produced by aqueous extracts (which was inhibited by ouabain pretreatment). The toxin content of the polyps and colonies was highly variable and was not related to their reproductive cycle or to the amount of symbiotic algae. Sequestration of palytoxin (**416**) was observed in crustaceans (*Platypodiella* sp.) living in close association with *Palythoa* colonies and in polychaete

worms (*Hermodice carunculata*) feeding on the zoanthids. Resistance of marine animals to the toxin may enable palytoxin to enter food chains.[381]

Extensive pharmacological and biochemical researches have been carried out on palytoxin (**416**). Palytoxin (**416**) was found to exhibit unique biochemical and pharmacological effects such as membrane depolarization, stimulation of arachidonic acid release, stimulation of neurotransmitter release, inhibition of Na^+/K^+-ATPase, induction of contraction of smooth muscle, and tumor-promoting activity.[382] For example, it has been reported that palytoxin (**416**), which at nanomolar concentrations enhances the permeability of mammalian cell membranes to both Na^+ and Ca^{2+}, modulated cytosolic pH in human osteoblast-like Saos-2 cells via an interaction with Na^+/K^+-ATPase.[383] The detailed mechanism of its action accounting for its variable biological effects is still largely unknown.

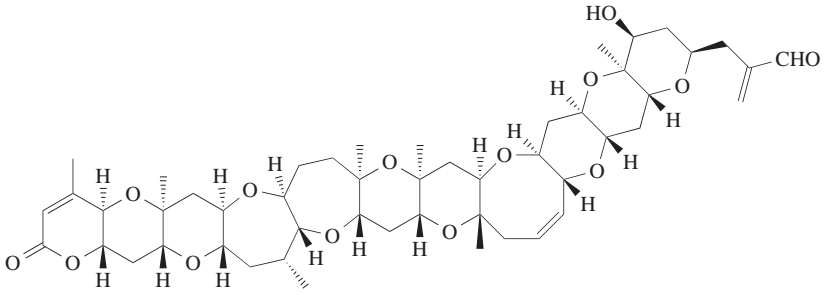
8.07.8.5.2 Brevetoxin

Brevetoxins are the major toxins in the Florida red tide organism, *Gymnodinium breve*.[384] A dense growth or bloom of dinoflagellates under certain favorable conditions has caused a phenomenon known as "red tide" and the blooms of the dinoflagellate *Gymnodinium breve* have led to massive fish kills, mollusk poisoning, and human food-poisoning along the Florida coast, in the Gulf of Mexico, and in many other regions of the world. In addition, many mass poisonings of humans have also been caused by consumption of affected seafood. The symptoms in human victims included diarrhea, dizziness, respiratory problems, eye irritation, and others.

Lin *et al*. demonstrated the structure of brevetoxin B (**418**), the major toxin responsible for the red-tide poisonings, by X-ray crystallography in 1981.[385] Brevetoxin B (**418**) was isolated from the cultures of *G. breve*, incubated at 25 °C for 21 days under constant illumination with standard fluorescent light. From a medium containing 5×10^8 cells, 5.0 mg of brevetoxin B (**418**) was isolated. Brevetoxin B (**418**) was made up of a single carbon chain locked into a rigid ladder-like structure consisting of 11 contiguous *trans*-fused ether rings, and there had been no precedent for this class of linear-condensed polycyclic ether compounds. Other marine toxins such as ciguatoxin, maitotoxin, and others (see Sections 8.07.8.3 and 8.07.8.4) proved to be included in this class of ladder polyether compounds. The structure of brevetoxin A (**419**), the most toxic component (LC_{100} 4 ng ml^{-1} to guppies), was elucidated by X-ray analysis of the crystals of a dimethyl acetal derivative in 1986 by Shimizu *et al*.[386] In the NMR spectrum of (**419**) certain signals were not observed and some peaks were unusually broadened, which was explained by the speculation that (**419**) undergoes a rather slow conformational change between the boat-chair and crown form in solution. Nakanishi and co-workers also described the structure of (**419**) based on NMR and MS data.[387] Several other toxins were also isolated from the organism *G. breve*, and they were divided into the brevetoxin A and B series by their skeletons.[295] Hemibrevetoxin B (**420**) with a skeleton about half the size of those of brevetoxins was also isolated from *G. breve*.[388]

The biosynthesis of these unprecedented polycyclic ethers was of considerable interest and it was postulated that the all-*trans* cyclic structure could be formed by a cascade of opening of all-*trans* epoxides, which are probably formed by epoxidation of *trans* double bonds. The biosynthetic origins and assignments of ^{13}C NMR signals of brevetoxin B (**418**) were studied by feeding experiments with [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates and methyl-^{13}C-methionine.[389] A 2D INADEQUATE experiment was carried out on a sample incorporating [1,2-$^{13}C_2$]acetate to reveal the contiguous carbon pairs derived from one acetate unit. The labeling patterns thus obtained (Figure 14), however, could not be interpreted by the simple acetogenin pathway, and it was proposed that labeled acetate was metabolized through the TCA cycle and incorporated into dicarboxylic acids before being utilized for the toxin biosynthesis (Figure 14).[390,391]

Pharmacological studies have revealed that brevetoxins (BTX) exhibit their damaging effects by acting on the voltage-sensitive domains of sodium channels[392] (specific receptor site 5), which are situated in the membranes of the cells. Nicolaou, who achieved the total synthesis of BTX B (**418**),[393] examined several synthetically prepared structural analogues of BTX B (**418**) in synaptosome receptor binding assays and by functional electrophysiological measurements. A truncated analogue of BTX was not ichthyotoxic at micromolar concentrations, showed decreased receptor-binding affinity, and caused only a shift of activation potential without affecting mean open times or channel inactivation. An analogue with the A-ring carbonyl removed bound to the receptor with nanomolar affinity, produced a shift of activation potential and inhibited inactivation, but did not induce longer mean open times. An analogue in which the A-ring diol was reduced showed low binding affinity,



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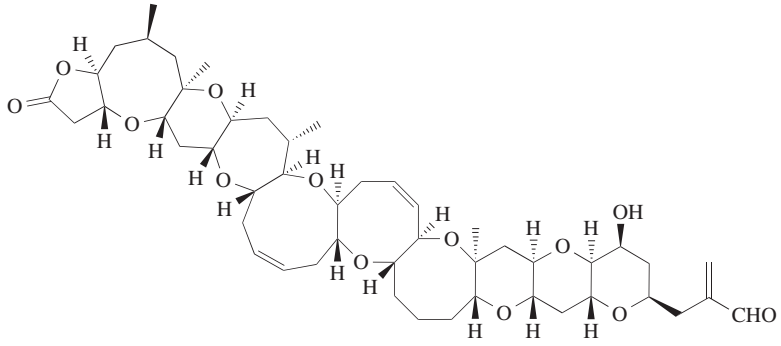

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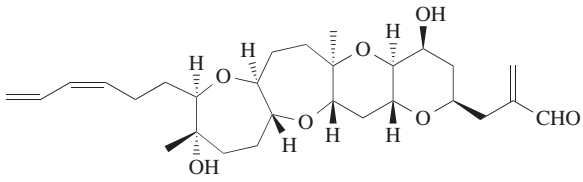


(420)

yet populated five subconductance states. These data were consistent with the hypothesis that binding to sodium channels requires an elongated cigar-shaped molecule, approximately 30 Å long. A detailed model was proposed for the binding of brevetoxins to the channel which explained the differences in the effects of the BTX analogues.[394] Nakanishi and co-workers found that BTX also induces selective ion movements across lipid bilayers through transmembrane BTX self-assemblies. They examined the self-assembly of several BTX derivatives in the presence and absence of cations and lipid bilayers using powerful porphyrin chromophores as CD labels. BTX derivatives self-assemble into tubes, which can bind to metals when inserted into the bilayer to form transmembrane pores. Depending on the tendency of the BTX derivative to self-aggregate, it may aggregate in solution before membrane insertion, or may insert itself into the membrane as a monomer before assembling the pore. The active BTX B complex in lipid bilayers is a cyclic, transmembrane self-assembly consisting of antiparallel aligned BTX molecules that can mediate selective ion movement through membranes. The differences in pore formation mechanisms between BTX derivatives may be reflected in differences in pore formation by natural BTX variants, perhaps explaining their varying levels of toxicity.[395] Nakanishi's group also investigated ion movement across large unilamellar egg phosphatidylcholine (PC) vesicles containing 0.3% BTX B (**418**). The transport rates of the ions were found to be sensitive to temperature of vesicle formation, cholesterol concentration, and the ion size. The most dramatic rate increase was achieved with vesicles containing 50% cholesterol. The BTX B skeleton was shown to be oriented perpendicular to the lipid surface. The sensitivity of BTX B channel formation to temperature, presence or absence of cholesterol, etc., suggested that *in vivo* penetration into specific cell membranes and self-assembly stabilization may also be governed by the presence or absence of membrane proteins.[396] BTX B (**418**) was utilized as a model for the study of the scope and limitations of porphyrin chromophores for structural studies

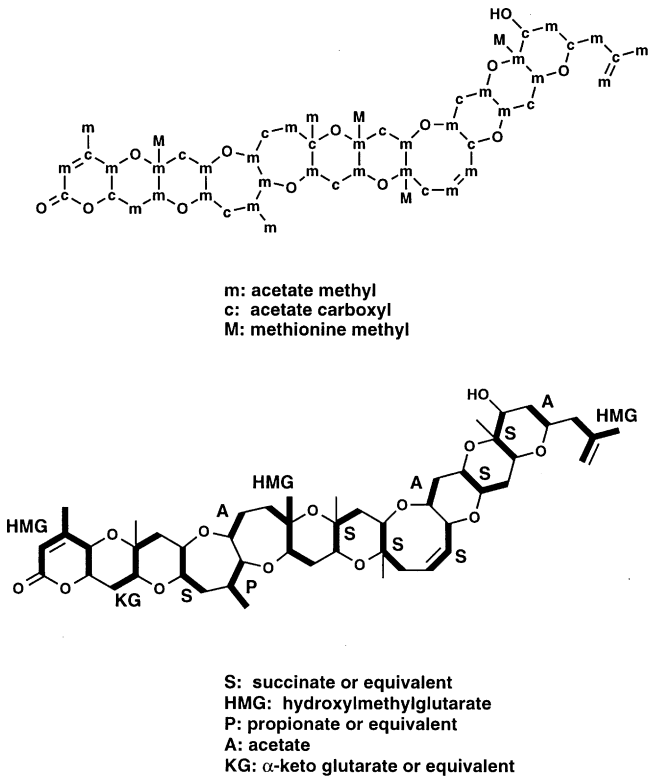


Figure 14 Incorporation patterns of labeled acetate into brevetoxin B and hypothetical building blocks of the molecule.

by the exciton coupled circular dichroic (CD) method. Porphyrins at the termini of dimeric steroids and BTX B (**418**) exhibited exciton coupling over interchromophoric distances up to 50 Å (Figure 15). As a result, porphyrin chromophores were found to be promising reporter chromophores for extending the exciton coupled CD method to structural studies of biopolymers.[397]

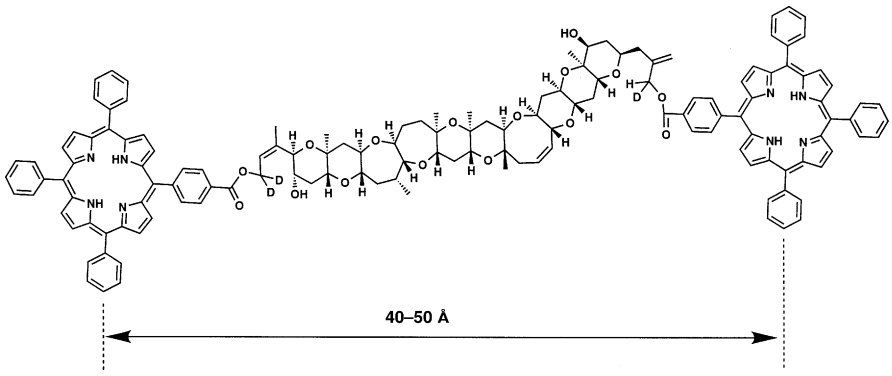
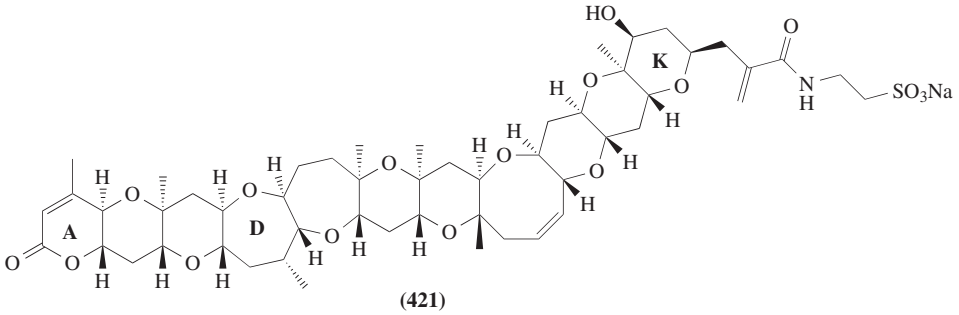
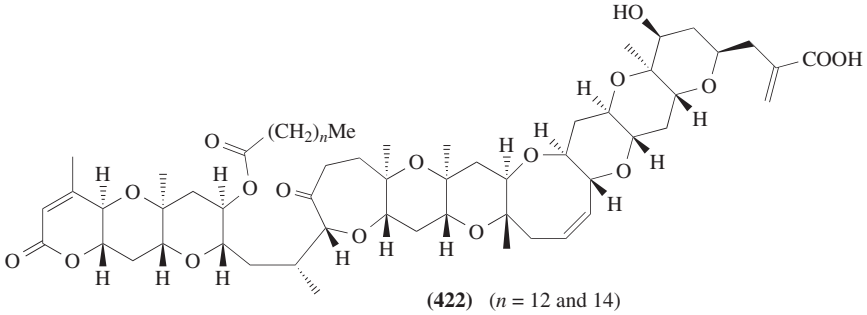


Figure 15 A brevetoxin B-bridged porphyrin dimer for the study of extending the exciton coupled CD method.

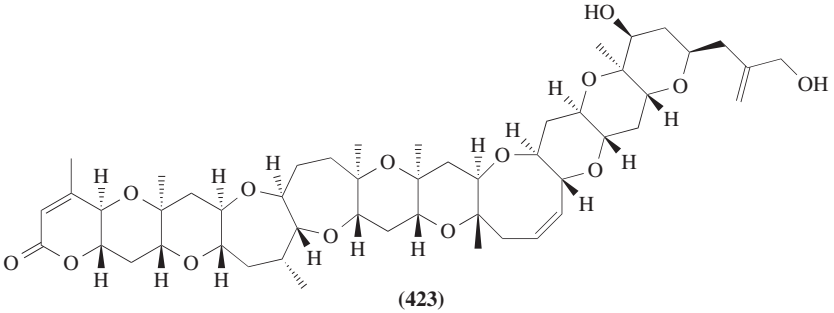
Other BTX-related compounds have been isolated and their structures reported. In December 1992 toxicated bivalves were noted for the first time in New Zealand. The toxicated shellfish *Austrovenus stutchburyi* (80 kg) collected at the Bay of Plenty, New Zealand in January 1993, were extracted with 80% MeOH under reflux. The extract was separated by solvent partition and chromatography on columns of SiO_2, ODS, and Sephadex LH-20, followed by reverse-phase HPLC to give 5.4 mg of brevetoxin B_1 (BTX-B_1 (**421**))).[398] The structure was elucidated by comparison of its spectral data with those of BTX B (**418**). NMR techniques revealed that the structures of BTX-B_1 (**421**) and BTX B (**418**) differed only in the functional group of the ring K side-chain; (**421**) had a carboxyl group at C-42 in place of the aldehyde group in (**418**), and (**421**) bore a taurine attached to the C-42 carboxyl group via an amide linkage. The minimum lethal dose of BTX-B_1 (**421**) was 0.05 mg kg^{-1} (i.p.) in mice. The animals exhibited irritability immediately after injection, followed by hind and/or hind-quarter paralysis, severe dyspnea and convolutions prior to death due to respiratory paralysis; these symptoms were very similar to those caused by brevetoxins. However, mice which did not develop respiratory difficulty recovered slowly. Interestingly, BTX-B_1 (**421**) at 100 ng ml^{-1} was not toxic to freshwater "zebra fish" (1.0–1.5 g) in 1 h, unlike BTX-A (**419**), BTX-B (**418**), and other brevetoxin analogues. Although it is well known that several ichthyotoxic brevetoxins, such as BTX-A (**419**) and BTX-B (**418**), are produced by the dinoflagellate *G. breve*, this was the first isolation of a BTX derivative from shellfish. Another brevetoxin analogue, brevetoxin B_3 ($BTXB_3$ (**422**)), was isolated from the greenshell mussel *Perna canaliculus* collected in January 1993 at the Coromandel Peninsula, North Island of New Zealand.[399] From 30 kg of hepatopancreas 1.2 mg of $BTXB_3$ (**422**) was obtained as a mixture of two homologues differing in their acyl moieties; the acyl groups were identified as palmitoyl and myristoyl groups by fluorometric HPLC analysis after hydrolysis with 0.1N NaOH in 95% MeOH. Detailed analysis of NMR spectral data showed that in $BTXB_3$ (**422**) the brevetoxin B skeleton was modified by cleavage of ring D, esterification of the resulting alcohol, and oxidation of the aldehydic terminus. The proposed structure was further supported by negative ion FAB MS/MS experiments. Interestingly, $BTXB_3$ (**422**) did not kill mice



(421)



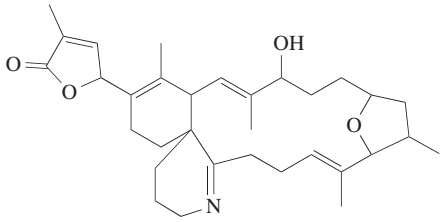
(422) (n = 12 and 14)



(423)

by i.p. injection at a dose of 300 μg kg^{-1}. It was highly likely that mussels detoxified BTX-B (**418**) to $BTXB_3$ (**422**), as $BTXB_3$ (**422**) had never been detected in dinoflagellates. The ^{1}H and ^{13}C NMR spectra of another polyether toxin, brevetoxin-3 (**423**), were totally assigned using a series of 2D NMR experiments, which included TOCSY, ROESY, HMQC, HMBC, and IDR (Inverted Direct Response)–HMQC–TOCSY. All work was performed on a sample consisting of 800 μg (0.95 μmol) at 500 MHz.[400]

A different type of marine toxin, gymnodimine (**424**),[401] was isolated from New Zealand oysters *Tiostrea chilensis* collected at Foveaux Strait, South Island. From 3 kg of oysters, 2.0 mg of (**424**) was obtained. Its unprecedented structure was elucidated by spectroscopic methods. When the oysters were found to be toxic at high levels, concurrent blooms of a dinoflagellate *Gymnodinium* cf. *mikimotoi* was also observed. It was unambiguously established that gymnodimine (**424**) was produced by the dinoflagellate *G.* cf. *mikimotoi*, on the basis of large-scale culture of the dinoflagellate, also collected at Foveaux Strait, and HPLC, ^{1}H NMR, and LC/MS analyses. Mouse lethality (i.p.) of gymnodimine (**424**) was 0.45 mg kg^{-1}. Gymnodimine (**424**) also showed potent ichthyotoxicity against a small freshwater fish *Tanichthys albonubes* at 0.1 ppm at pH 8. Gymnodimine (**424**) was isolated not only from cells but also from the culture medium, suggesting the possibility that it may cause massive fish kills like its related species *Gymnodinium mikimotoi* (formally *G. nagasakiense*) during seasonal blooms of the dinoflagellate.



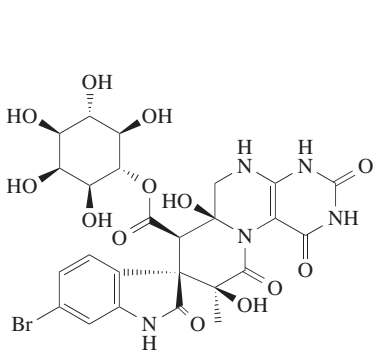
(424)

8.07.8.5.3 Surugatoxin

Surugatoxin (**425**) was first isolated from the toxic ivory shell *Babylonia japonica* by Kosuge *et al.* as a causative toxin of a food-poisoning outbreak in 1965 at Suruga Bay, Japan. Its structure was determined by X-ray analysis in 1972,[402] corrected to (**425**) in 1981,[403] and confirmed by its total synthesis.[404] However, surugatoxin (**425**) was later found to be nontoxic and the real causative agents of intoxication resulting from ingestion of the toxic shell proved to be neosurugatoxin (**426**) and prosurugatoxin (**427**).[405] Pharmacological studies suggested that the toxins had a selective affinity for ganglionic nicotinic receptors, its affinity constant for these receptors being more than three orders of magnitude greater than that of hexamethonium.[406] A Gram-positive bacterium (Aalxll strain), which was associated with the Japanese ivory shell *B. japonica*, was shown to be responsible for the production of surugatoxins.[407]

8.07.8.5.4 Polycavernoside

Fatal human intoxication occurred in Guam in late April 1991, due to ingestion of the red alga *Polycavernosa tsudai* (formerly *Gracilaria edulis*). The responsible toxins were investigated by Yasumoto and co-workers resulting in isolation of two toxins, polycavernoside A (**428**) and B (**429**).[408] The alga *P. tsudai* (2.6 kg) was collected in June 1991, at Tanguisson Beach, Guam, and was extracted with acetone. Solvent partition and column chromatography of the extract, guided by mouse bioassays, yielded polycavernosides A (**428**) (400 μg) and B (**429**) (200 μg). LD_{99} in mice (i.p.) was 200–400 μg kg^{-1} for both. The structure of polycavernoside A (**428**) was elucidated by spectral data, consisting of a 13-membered lactone with a triene side-chain and *O*-α-2,3-di-*O*-methylfucopyranosyl-(1″-3′)-*O*-β-2,4-di-*O*-methylxylopyranoside. The relative stereostructure was deduced mainly from NOE data and the stable conformations were suggested by a force-field calculation (program ''Dreiding'') for structural confirmation.[409]



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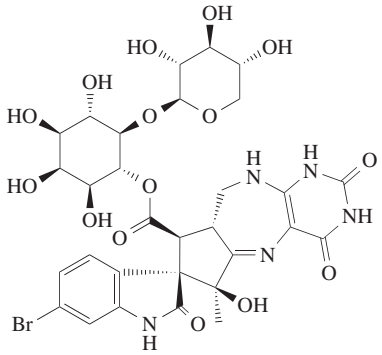

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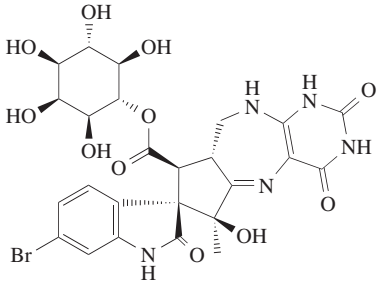

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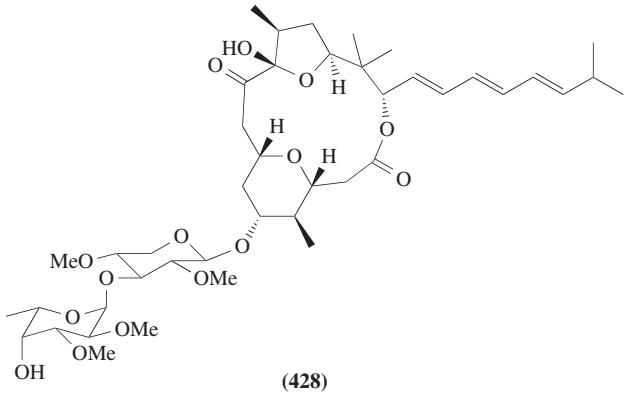

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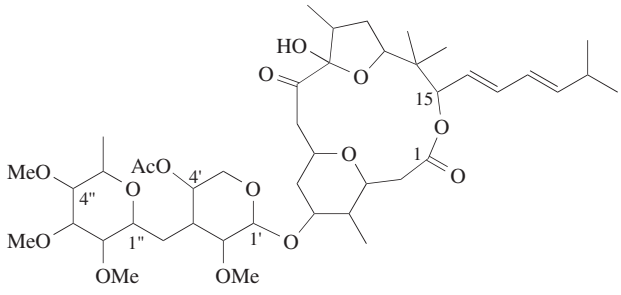
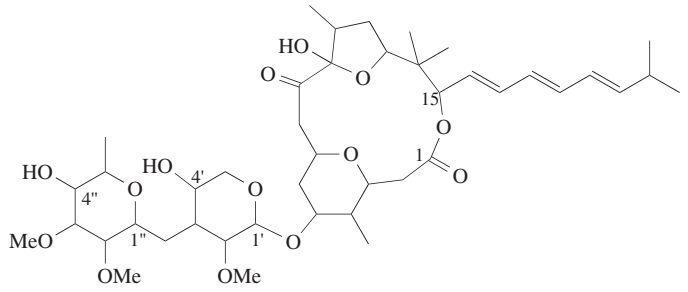


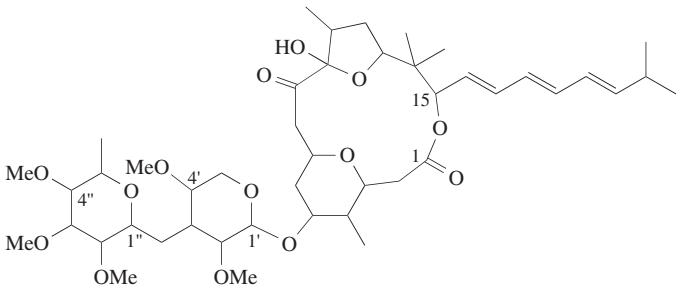
(429)

The algal toxicity rapidly decreased after the incident but rose again, though at lower levels, in the same season of the following year. Three other analogues, polycavernosides A2 (**430**), A3 (**431**), and B2 (**432**), were isolated together with (**428**) from the alga collected on the same beach in June 1992.[410] NMR spectral data of polycavernosides B (**429**) and B2 (**432**) showed that the conjugated

triene in the side-chain portion of (**428**) was replaced by a conjugated diene in (**429**) and (**432**). The fucopyranosyl-xylopyranosyl backbone was deduced as a common structure of the glycosidic residue of polycavernosides. Compound (**430**) was the 4′-*O*-demethyl analogue of (**428**), and (**431**) was the *O*-methylated analogue of (**428**) at the 4″-OH. The OMe group on C-4′ in (**428**) was replaced by an OAc group in (**429**), and (**432**) was the 4′-*O*-deacetyl analogue of (**429**). These structures were further supported by FAB/MS/MS by showing prominent ions corresponding to sequential loss of each residue.



(430)



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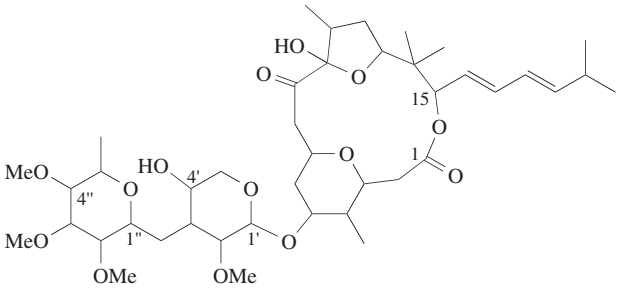


(432)

The synthesis of some partial structures of polycavernoside A (**428**) was studied by Murai and co-workers, and they revised the whole relative stereochemistry of polycavernoside A, as shown in Figure 16, by combination of the synthesized sugar and tetrahydropyran parts.[411]

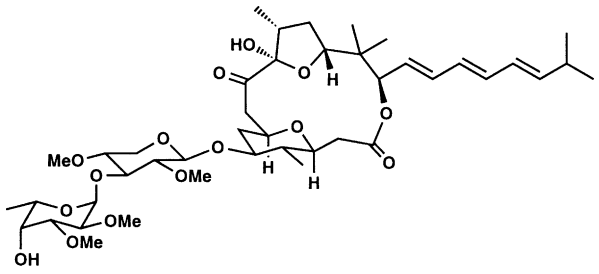


Figure 16 Relative stereochemistry of polycavernoside A, proposed by Murai and co-workers.

A smaller macrocycle, a trioxatridecane, of polycavernosides was reminiscent of the aplysiatoxins, which contained trioxadodecane. The sudden and transient occurrence of the toxins in the alga remained unexplained, but may provide a clue to previous outbreaks of fatal food poisoning caused by algae of the genus *Gracilaria*.

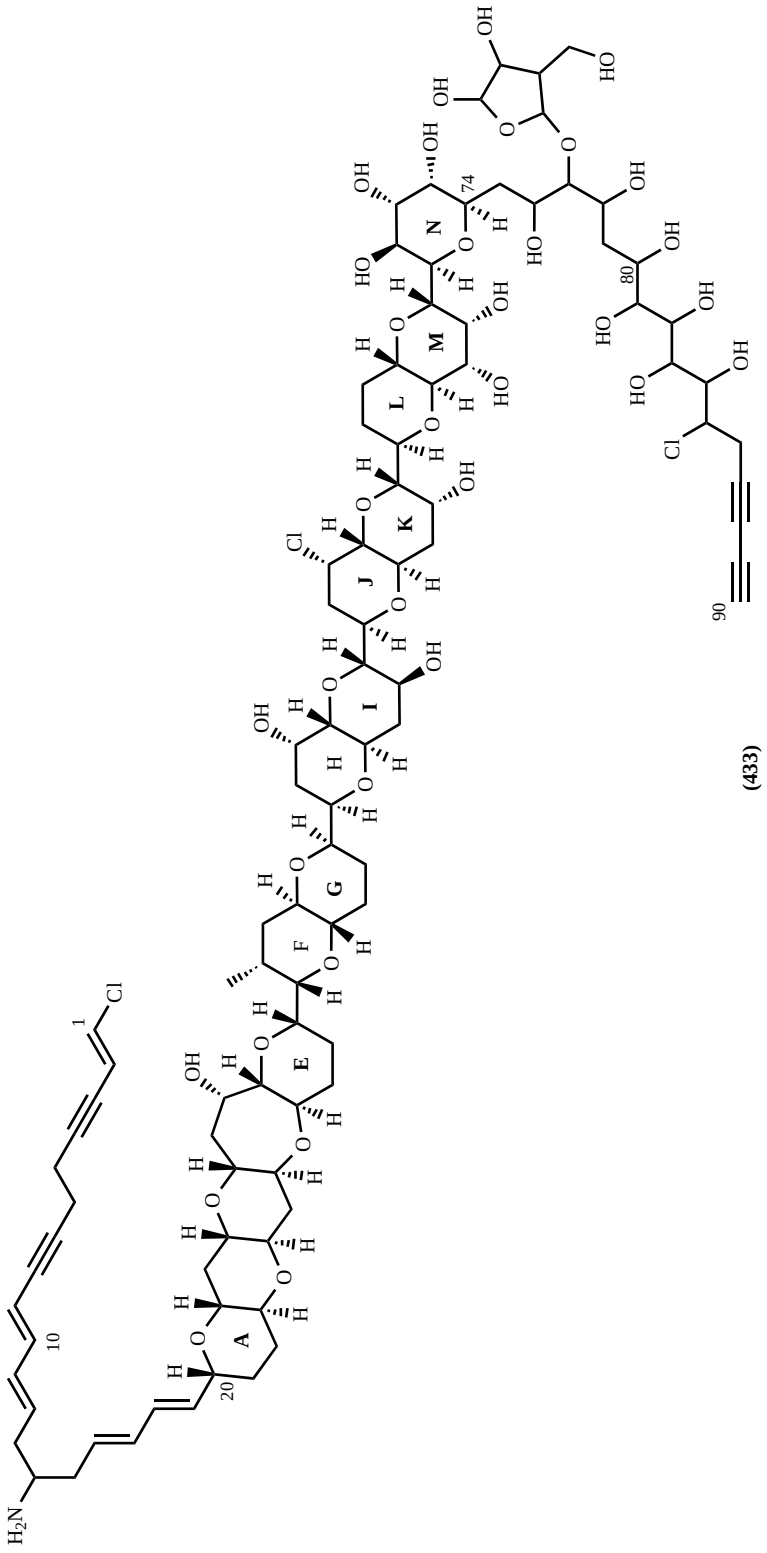
8.07.8.5.5 Prymnesin

Prymnesium parvum was a notorious red-tide organism belonging to Haptophyceae. The flagellate causes serious damage to aquaculture and marine ecology in many parts of the world, the greatest threat being to salmon farmed in Norway. Despite efforts by many research groups, the causative ichthyotoxin named prymnesin evaded purification for a long time and hence chemical and toxicological studies of the toxin have been hampered. In 1994 Yasumoto and co-workers succeeded for the first time in isolating two hemolytic-ichthyotoxic substances, named prymnesin-1 (PRM1) and prymnesin-2 (PRM2 (**433**)).[412] They obtained 10 mg of PRM1 and 15 mg of PRM2 from 400 l of cultures of *P. parvum*. Their hemolytic potencies exceeded that of plant saponins by more than 1000 times. The minimum concentration to cause hemolysis of a 1% mouse blood cell suspension and to kill freshwater fish *Tanichthys albonubes* was ca. 3 nM. FAB MS data on molecular ion species and their isotope distribution pattern containing ^{35}Cl and ^{37}Cl suggested a molecular formula $C_{107}H_{154}O_{44}NCl_3$ for PRM1 and $C_{96}H_{136}O_{35}NCl_3$ for PRM2 (**433**). The presence of chlorine atoms in the molecule was indicated by energy dispersive X-ray analysis. ^{13}C NMR measurement on ^{13}C enriched PRM2 *N*-acetate indicated 96 carbon signals (2 methyls, 24 methylenes, 10 alkenic methines, 53 other methines, and 7 quaternary carbons). Further detailed analyses of both ^{1}H NMR (COSY, HOHAHA, and NOESY) and ^{1}H observed ^{1}H–^{13}C correlation NMR (HSQC and HMBC) data disclosed the unprecedentedly unique structure of PRM2 (**433**).[413] The molecule was characterized by the 14 ether rings (6/6/6/7/6, 6/6, 6/6, 6/6, 6/6, and 6), polyene–polyyne bonds, three chlorine and one nitrogen atoms, and one pentose. Structural confirmation was enhanced by measuring NMR spectra of PRM2 (**433**), the *N*-acetate, and peracetate in two different solvents. The sugar moiety was analyzed by chiral GC after hydrolysis of (**433**) to confirm that the sugar was L-xylose. Perhydro-PRM2 was prepared by hydrogenation of (**433**) (Pd/C, H_2) to confirm the degree of unsaturation. In the ESIMS spectrum of perhydro-PRM2, molecular-related ions were observed at m/z 1963, revealing that the chlorine atom on C-1 had been substituted by a hydrogen atom during hydrogenation. The nature of unsaturation including five double and four triple bonds in (**433**) was thus verified. PRM1 probably had one pentose and one hexose added to the PRM2 skeleton.

The relative stereochemistry of the polyether moiety, C-20–C-74, of (**433**) was determined from the NOE data. The molecular structure of PRM2 (**433**) is conformationally rich with a number of key torsional bonds which were probed with contemporary conformational searching techniques in order to describe the three-dimensional structure of the prymnesin molecule. Important topological features include a backbone twist of 60° and an elongation length of 39.5 Å from ring "A" to ring "N" for the PRM2-molecule in the NOE preferred conformation. These and other molecular structural and energetic features determined by computational techniques were examined with the aim of understanding how PRM2 (**433**) exhibits its potent biological activity in relation to its inherent structural traits.[414]

8.07.8.5.6 Pinnatoxin

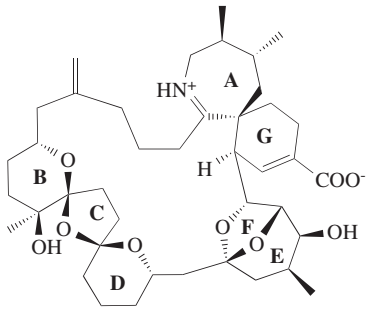
Shellfish of the genus *Pinna*, living mainly in shallow areas of the temperate and tropical zones of the Indian and Pacific Oceans, are commonly eaten in China and Japan, and food poisoning resulting from their ingestion occurs frequently. In Japan, six outbreaks involving 2766 people were reported between 1975 and 1991. In 1990 a water-soluble nonexogenous substance which showed a strong nerve toxicity, was isolated from *Pinna attenuate* collected in the South China Sea by Chinese investigators, and the toxin was named pinnatoxin.[415] Chinese investigators conjectured that this toxin was responsible for the human intoxication resulting from *P. attenuata*, which occurred at Guangdong, China, in 1980 and 1989, based on its predominant symptoms, i.e., diarrhea, paralysis, and convulsions. Pinnatoxin significantly increased the contractility of the aortic strips of rabbit and the ileums of guinea pig. The preliminary tests suggested that the actions of pinnatoxin were related to the α-receptor and the M-receptor, and that pinnatoxin is probably a Ca^{2+} channel



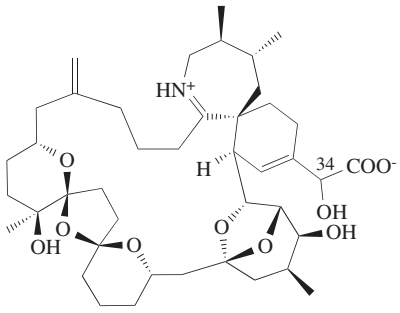
(433)

activator. Thereafter, identification of the specific toxins and clarification of their physiological activity proved to be challenging.

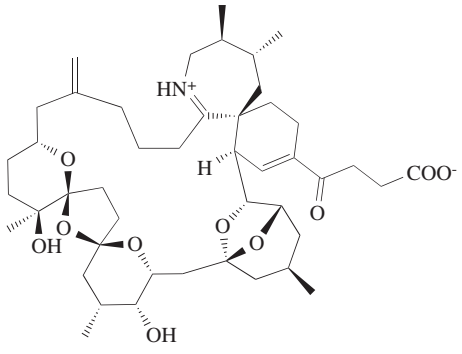
Uemura and co-workers succeeded in isolating four toxins, pinnatoxins A–D (**434**)–(**437**)[416] from *Pinna muricata* and elucidated their structures as an unprecedented class of polyether macrocycle with carboxylate and iminium functionalities.[417] From 45 kg of viscera of *P. muricata* collected in Okinawa, Japan, a bioassay-guided fractionation based on intraperitoneal mouse lethality tests afforded 3.5 mg of pinnatoxin A (**434**) (LD_{99} 180 μg kg^{-1}), 1.2 mg of a mixture of pinnatoxins B (**435**) and C (**436**), and 2 mg of pinnatoxin D (**437**). The mixture of (**435**) and (**436**) was the most toxic (LD_{99} 22 μg kg^{-1}), while pinnatoxin D (**437**) was found to be less toxic (LD_{99} 0.4 mg kg^{-1}) but exhibits cytotoxicity against P388 leukemia cells (IC_{50} 2.5 μg ml^{-1}). Their planar structures were determined mainly by interpretation of 2D NMR (DQF–COSY, HOHAHA, HSQC–HOHAHA, and HMBC) spectra. Pinnatoxin A (**434**) was revealed to be an amphoteric macrocyclic compound composed of a 6,7-spiro ring, a 5,6-bicyclo ring, and a 6,5,6-trispiroketal ring involving 14 chiral centers. Pinnatoxins B (**435**) and C (**436**) were obtained as a 1:1 mixture and deduced to be diastereomers at the C-34 position, both possessing one more carbon than pinnatoxin A (**434**).[416] Pinnatoxin D (**437**) had three more carbon units (C-34–C-36) than (**434**) and different functionalities at C-21, C-22, and C-28.[418] The biogenesis of the unique structure containing the 6,7-spiro ring (A and G rings) was proposed to be generated through a Diels–Alder reaction, and a relationship to the carbon skeleton of prorocentrolide (**377**) (see Section 8.07.8.3.1) was suggested. The relative stereochemistry of pinnatoxin A (**434**) was deduced, based on detailed analysis of NOESY and ROESY data, and $^{3}J_{H\text{-}H}$ coupling constants.[419]



(**434**)



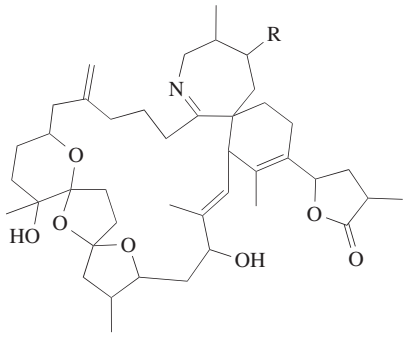
(**435, 436**): Diastereomers at C-34



(**437**)

Wright and co-workers studied the digestive glands of mussels *Mytilus edulis* and scallops *Placopecten magellanicus* collected from sites along the eastern shore of Nova Scotia, and isolated a family of macrocylic compounds named spirolides A–D.[420] The structures of two major components of them, spirolides B (**438**) and D (**439**), were elucidated by spectral studies as those containing a spiro-linked tricyclic ether ring system and an unusual seven-membered spiro-linked cyclic iminium moiety. The macrocycle skeleton of spirolides resembled that of pinnatoxins; in contrast with pinnatoxins, spirolides had four less carbons in the macrocycle moiety with no dioxabicyclo-[3.2.1]octane ring moiety (E and F rings). The fact that the spirolides were found in the digestive glands of shellfish, coupled with the observation that they occur on a seasonal basis, usually in

June–July, indicated a microalgal origin of these compounds and their spiro-linked polyether structure might suggest a dinoflagellate source. The spirolides caused potent and characteristic symptoms in the mouse bioassay (LD_{100} 250 μg kg^{-1}, i.p.).



(438) R = H
(439) R = Me

8.07.9 BIOACTIVE MARINE NATURAL PRODUCTS

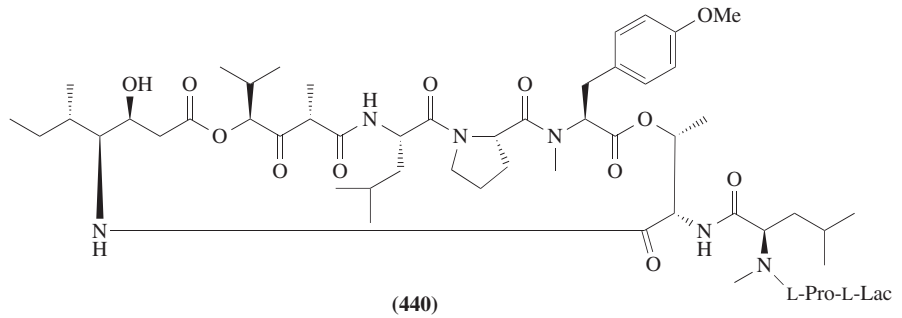
8.07.9.1 Drug Candidates

Marine natural products are regarded as potentially useful therapeutic agents. Didemnin B (**440**),[421] a cyclic peptide isolated from tunicate *Tridemnum solidum*, has been in clinical trial for some time as a potential anti-cancer drug; some complete or partial responses were observed in CNS-astrocytoma/glioblastoma and non-Hodgkin's lymphoma. Bryostatin 1 (**441**) is a marine-derived compound with anticancer potential, produced by the common fouling marine bryozoan (sea-mat) *Bugula neritina*.[422] Bryostatin 1 (**441**) showed promising Phase I clinical results, and was tested in Phase II human clinical trials by the National Cancer Institute. A potent antitumor compound, aplyronine A (**442**), was isolated from the sea hare *Aplysia kurodai*.[423] Aplyronine A (**442**) exhibited exceedingly potent antitumor activities *in vivo* against P388 murine leukemia (T/C = 545%, 0.08 mg kg^{-1}), Lewis lung carcinoma (T/C = 556%, 0.04 mg kg^{-1}), Ehrlich carcinoma (T/C = 398%, 0.04 mg kg^{-1}), colon 26 carcinoma (T/C = 255%, 0.08 mg kg^{-1}), and B16 melanoma (T/C = 201%, 0.04 mg kg^{-1}). The enantioselective total synthesis of aplyronine A (**442**) was also achieved.[424–426] Other promising antitumor compounds are the ecteinasicidins (see Section 8.07.9.2.3), which showed *in vivo* activity against leukemia, melanoma, ovarian sarcoma, and lung and mammary tumors, including human tumor xenografts. Manoalide (**443**), which was isolated from a marine sponge *Luffariella variabilis*,[427] was found to strongly inactivate phospholipase A_2 by irreversible modification and to show anti-inflammatory properties *in vivo*.[428] Manoalide (**443**) is under clinical trials in the United States as an anti-inflammatory agent. The potent anti-inflammatory diterpene glycoside, pseudopterosin C (**444**),[429] was isolated from the extract of sea whips *Pseudopterogorgia elisabethae*. The extract is a key ingredient in a skin care product that has been commercialized by a cosmetic company.

8.07.9.2 Topics on Tunicates

8.07.9.2.1 Vanadium accumulation by tunicates[430]

Henze discovered high levels of vanadium in blood cells of ascidians, known as sea squirts.[431] After Henze's discovery, many researchers tried to characterize the vanadium in ascidians, with interest in its presence in ascidians or in its possible involvement as an oxygen-carrier other than



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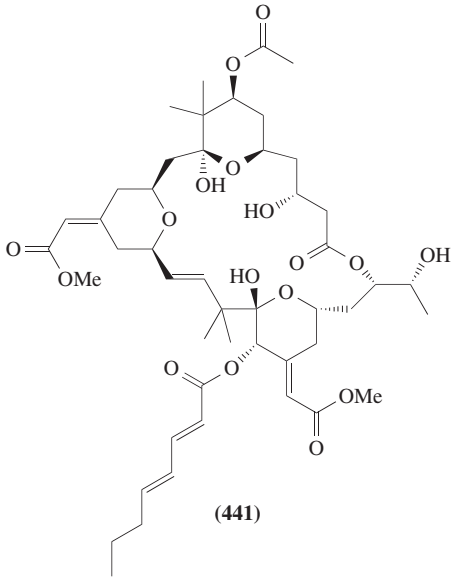

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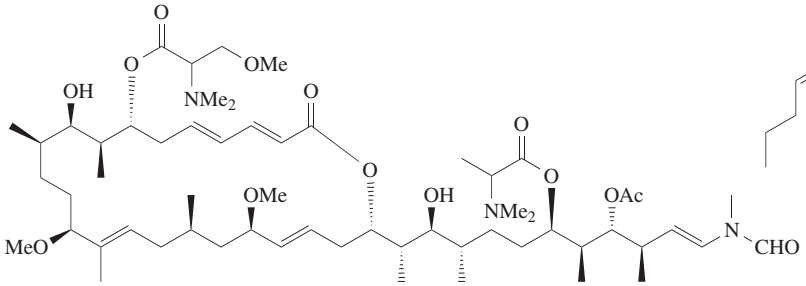

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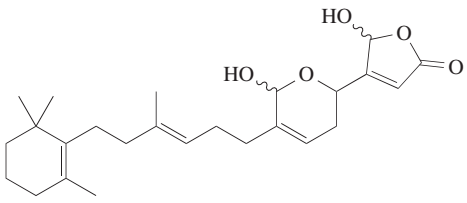

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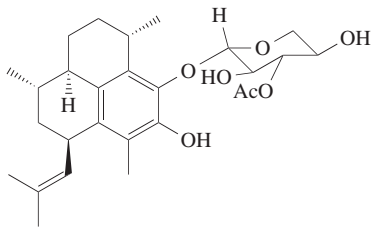


(444)

iron and copper. However, the mechanism of accumulation and the physiological role of vanadium have not been clarified.

The vanadium in ascidians has been analyzed by a variety of analytical methods, such as colorimetry, emission spectrometry, and atomic absorption spectrometry. These techniques vary widely in sensitivity and precision. Moreover, data were reported in terms of dry weight, ash weight, inorganic dry weight, or amount of protein. Thus, data could not be easily compared.

Michibata *et al.* reexamined the contents of vanadium in several tissues of ascidians by neutron-activation analysis, which is an extremely sensitive method for quantification of vanadium.[432,433] They collected many species of ascidians belonging to two of three suborders, Phlebobranchia and

Stolidobranchia, mainly from the waters around Japan and the Mediterranean. Specimens were dissected into eight samples for analysis, namely, blood cells, plasma, tunic, mantle (muscle), branchial basket, stomach, hepatopancreas, and gonad. The samples were dried and weighed and then they were mineralized at 500 °C and submitted to neutron-activation analysis in a nuclear reactor. Some of the stable vanadium in each sample was converted into the radioactive nucleide, ^{52}V, which emits γ-rays at 1434 keV. Since the frequency of γ-rays emitted depends on the level of stable vanadium in the sample, the original amount of vanadium in the tissues could be calculated. As a result (Table 20), the ascidian species belonging to Phlebobranchia apparently contained higher levels of vanadium than those belonging to Stolidobranchia. Furthermore, blood cells especially contained the highest amounts of vanadium among the tissues examined. Levels of iron and manganese determined simultaneously did not vary much among the members of the two suborders. The highest concentration of 350 mM vanadium was found in the blood cells of *Ascidia gemmata* belonging to Phlebobranchia,[434] 10^7 times higher than in seawater.[435]

Table 20 Concentrations of vanadium in tissues of several ascidians (nM).[430]

	Tunic	*Mantle*	*Branchial basket*	*Serum*	*Blood cells*
Phlebobranchia					
Ascidia gemmata	N.D.	N.D.	N.D.	N.D.	347.2
A. ahodori	2.4	11.2	12.9	1.0	59.9
A. sydneiensis	0.06	0.7	1.4	0.05	12.8
Phallusia mammillata	0.03	0.9	2.9	N.D.	19.3
Ciona intestinalis	0.003	0.7	0.7	0.008	0.6
Stolidobranchia					
Styela plicata	0.005	0.001	0.001	0.003	0.007
Halocynthia roretzi	0.01	0.001	0.004	0.001	0.007
H. aurantium	0.002	0.002	0.002	N.D.	0.004

N.D.: not determined.

Generally, ascidians have 9 to 11 different types of blood cells, which can be grouped into six categories on the basis of their morphology: hemoblasts, lymphocytes, leucocytes, vacuolated cells, pigment cells, and nephrocytes.[436] The vacuolated cells can be further divided into at least four different types: morula cells, signet ring cells, compartment cells, and small compartment cells. The morula cells have been regarded as vanadocytes for many years.[437] An Italian group first demonstrated that the characteristic X ray due to vanadium was not detected from morula cells but from granular amoebocytes, signet ring cells, and compartment cells and, moreover, that vanadium was selectively concentrated in the vacuolar membranes of these cells.[438]

Hirata and Michibata employed density gradient centrifugation for isolation of specific types of blood cells and neutron-activation analysis for quantification of vanadium contents. Vanadium-rich ascidians, *Ascidia ahodori*, *A. sydneiensis samea*, and *A. gemmata*, were used for this analysis. The pattern of distribution of vanadium coincided with that of signet ring cells but not that of morula cells or compartment cells. These results suggested that the signet ring cells were the true vanadocytes.[439]

Vanadium is a multivalent transition metal. Vanadium ions under ordinary aqueous conditions are limited to the oxidation states, +2, +3, +4, and +5, and to only the +3, +4, and +5 oxidation states under physiological conditions.[440] Vanadium ions in the +3 oxidation state [V^{III}] are usually unstable toward air oxidation, and V^{III} ions are hydrolyzed to $V(OH)^{2+}$ which tends to dimerize to $(VOV)^{4+}$ over pH 2.2. Under neutral and alkaline conditions, simple soluble V^{III} compounds without any strong ligands have never been reported. As described below, vanadium ions in the blood cells of ascidians are stable under strongly acidic conditions. Vanadium ions in the +4 oxidation state [V^{IV}] are paramagnetic and give a blue solution of oxo-ions, VO^{2+} (vanadyl ions), under moderately acidic conditions.

It is believed that vanadium in the +5 oxidation state [V^{V}] is dissolved in seawater but this possibility remains to be confirmed. With regard to the oxidation state of vanadium in ascidian blood cells, Henze[431] first suggested the existence of V^{V}, while later many groups reported V^{III}. Noninvasive physical methods including ESR, NMR, and superconducting quantum interference device (SQUID), have since been used to determine the intracellular oxidation state of vanadium.

Such studies indicated that the vanadium ions in ascidian blood cells were predominantly +3, with a small amount of V^{IV}.[441] After separation of the various types of blood cells of *A. gemmata*, Hirata and Michibata made noninvasive ESR measurements of the oxidation state of vanadium in the fractionated blood cells under a reducing atmosphere.[439] Generally only V^{IV} is detectable by ESR spectrometry. Weak signals due to V^{IV} were recorded when the subpopulation of vanadocytes was submitted to ESR spectrometry at 77 K under nitrogen and subsequent bubbling of oxygen gas into the lysate dramatically increased peak height. These results indicate that the oxidation state of vanadium in vanadocytes is predominantly +3, with a small amount of V^{IV}. The ratio of vanadium in the two states is 97.6:2.4. A marine polychaeta *Pseudopotamilla occelata* is reported to contain high levels of V^{III}.[442]

Henze[431] first reported the coexistence of sulfate with vanadium in ascidian blood cells. Vanadium was considered to bind to nitrogenous compound and sulfate to form a complex, designated heamovanadin, which acted as a respiration pigment in ascidian blood cells.[443] Bielig *et al*. suggested that heamovanadin could reduce vanadium.[444] However, the chemical structure of heamovanadin was not determined, even though model compounds were proposed. Kustin and co-workers proposed that heamovanadin was actually an artifact formed by air-oxidation and disputed the possibility that it might be involved in respiration.[445] They isolated a low-molecular compound consisting of three pyrogallol moieties, named tunichrome, from the blood cells of *Ascidia nigra* and *Ciona intestinalis*, which was considered to be involved in the reduction of vanadium.[446] However, tunichrome was absent in the vanadium-containing signet ring cells.[447] Ryan *et al*. observed the reduction of vanadium in the +5 and +4 oxidation states by a tunichrome, designated Mm-1, in buffer solution at pH 7 *in vitro*.[448] It is, however, unclear whether such a reaction could occur in ascidian blood cells.

A considerable amount of sulfate has always been found in ascidian blood cells,[449] suggesting that sulfate might be involved in the biological function and/or the accumulation and reduction of vanadium. However, Frank *et al*. suggested the existence of a nonsulfate sulfur compound such as an aliphatic sulfonic acid in ascidian blood cells.[450] Kanamori and Michibata found that the ratio of the level of sulfate to that of vanadium in the blood cells of the ascidian *Ascidia gemmata* was ca. 1.5 by using Raman spectroscopy, suggesting that sulfate ions were present as counter ions against V^{III}. They also found evidence that an aliphatic sulfonic acid was present in the blood cells.[451]

Henze[431] first reported that a homogenate of ascidian blood cells is extremely acidic and almost all subsequent investigations have supported his observation. However, Kustin and co-workers disputed the earlier reports and reported that the intracellular pH was neutral from measurements of transmembrane equilibrium of ^{14}C-labeled methylamine.[452] Hawkins *et al*.[453] and Brand *et al*.[454] also reported nearly neutral values for the pH of the interior of ascidian blood cells, from chemical shifts in ^{31}P-NMR. However, Frank *et al*.[455] demonstrated that the interior of the blood cells of *Ascidia ceratodes* has a pH of 1.8, based on the finding that the ESR line width accurately reflects the intracellular pH. Michibata *et al*.[434] measured the pH with a microelectrode under anaerobic conditions, and ESR spectrometry for each type of blood cells from vanadium-rich ascidians, *Ascidia gemmata*, *A. ahodori*, and *A. sydeneiensis samea*. Blood cells drawn from each species were fractionated by density-gradient centrifugation. The pH values after conversion to concentrations of protons ($[H^+]$) and levels of vanadium in each layer of cells are compared, showing that the patterns of distribution of protons and vanadium were similar. The signet ring cells contained high levels of vanadium and showed a low intracellular pH in all three species.

Next, Michibata and co-workers examined the presence of H^+-ATPase in the signet ring cells of the ascidian *Ascidia sydneiensis samea*.[456] The vacuolar-type H^+-ATPase is composed of subunits of 72 kDa and 57 kDa. Antibodies prepared against the 72 kDa and 57 kDa subunits of a vacuolar-type H^+-ATPase from bovine chromaffin granules indeed reacted with the vacuolar membranes of signet ring cells. Immunoblotting analysis confirmed that the antibodies reacted with specific antigens in ascidian blood cells. Furthermore, addition of bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibited the pumping function of the vacuoles of signet ring cells, and resulted in neutralization of the contents of the vacuoles.

Using neutron-activation analysis and an immunofluorescence method, Michibata *et al*. found that the amount of vanadium per individual increased dramatically two weeks after fertilization. Within two months, the amount accumulated in larvae was about 600 000 times higher than that in the unfertilized eggs of *A. sydneiensis samea*.[457] A vanadocyte-specific antigen, recognized by a monoclonal antibody specific to the signet ring cells, first appeared in the body wall at the same time as the first significant accumulation of vanadium.[458]

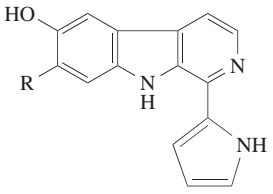
In general, heavy metal ions incorporated into the tissues of living organisms are known to bind to macromolecules such as proteins. Wuchiyama and Michibata searched for vanadium-binding

proteins in the blood cells of ascidians. Using a combination of SDS–PAGE and flameless atomic absorption spectrometry, they succeeded in isolating at least four different types of vanadium-binding proteins.[459]

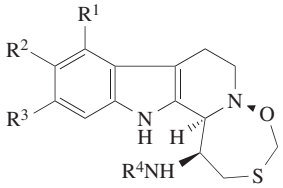
Although the unusual phenomenon that some ascidians accumulate vanadium to levels 10 million times higher than that in seawater has attracted researchers in various fields, the physiological roles of vanadium remain to be resolved. Endean[460] and Smith[461] proposed that the cellulose of the tunic might be produced by vanadocytes, while Carlisle[462] suggested that vanadium-containing vanadocytes might reversibly trap oxygen under conditions of low oxygen tension. The hypothesis has also been proposed that vanadium in ascidians acts to protect them against fouling or as an antimicrobial agent.[463] However, these proposals are still conjectural.

8.07.9.2.2 Eudistomins and related alkaloids

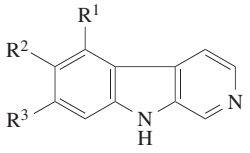
Several halogenated β-carbolines have been found in marine organisms, especially tunicates (ascidians or sea squirts). Eudistomin refers to a series of β-carboline alkaloids isolated from marine tunicates. Eudistomins A–Q (**445**)–(**461**) were extracted from the Caribbean colonial tunicate *Eudistoma olivaceum*.[464–466] Four groups of eudistomins were isolated, including simple β-carbolines (eudistomins D (**448**), J (**454**), N (**458**), and O (**459**)), pyrrolyl-β-carbolines (eudistomins A (**445**), B (**446**), and M (**457**)), pyrrolinyl-β-carbolines (eudistomins G (**451**), H (**452**), I (**453**), P (**460**), and Q (**461**)), and tetrahydro-β-carbolines with an oxathiazepine ring (eudistomins C (**447**), E (**449**), F (**450**), K (**455**), and L (**456**)). The structures were elucidated by spectroscopic data including high-resolution FABMS and EIMS and ^{1}H and ^{13}C NMR, and confirmed by syntheses. The stereochemistry of the eudistomins was studied by ^{1}H- and ^{13}C-homonuclear and heteronuclear correlation NMR spectra and analysis of the proton–proton spatial relationships (NOE). The isolated eudistomins were assayed against *Herpes simplex* virus type-1 (HSV-1) and showed antiviral activity. The most active compounds by far are ones containing the oxathiazepine ring (eudistomins C (**447**), E (**449**), K (**455**), and L (**456**)). The eudistomins also exhibited antimicrobial activity to widely different degrees, with the oxathiazepines being generally the most active. Interestingly, a mixture of eudistomins N (**458**) and O (**459**) displayed a remarkable degree of synergism. Synthetic eudistomin N (**458**) or O (**459**) alone is inactive, but a mixture exhibited antimicrobial activity.



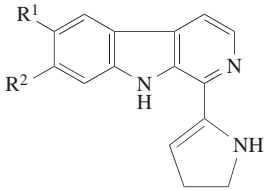
(**445**) R = Br
(**457**) R = H



(**447**) $R^1 = R^4 = H$, $R^2 = OH$, $R^3 = Br$
(**449**) $R^1 = Br$, $R^2 = OH$, $R^3 = R^4 = H$
(**450**) $R^1 = H$, $R^2 = OH$, $R^3 = Br$, $R^4 = C_2H_3O_2$
(**455**) $R^1 = R^2 = R^4 = H$, $R^3 = Br$
(**456**) $R^1 = R^3 = R^4 = H$, $R^2 = Br$

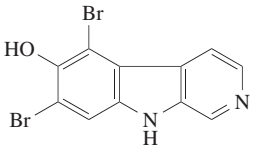


(**448**) $R^1 = Br$, $R^2 = OH$, $R^3 = H$
(**454**) $R^1 = H$, $R^2 = OH$, $R^3 = Br$
(**458**) $R^1 = H$, $R^2 = Br$, $R^3 = H$
(**459**) $R^1 = R^2 = H$, $R^3 = Br$



(**451**) $R^1 = H$, $R^2 = Br$
(**452**) $R^1 = Br$, $R^2 = H$
(**453**) $R^1 = R^2 = H$
(**460**) $R^1 = OH$, $R^2 = Br$
(**461**) $R^1 = OH$, $R^2 = H$

Several eudistomins have been proved to induce calcium release from sarcoplasmic reticulum (SR) in mammalian cells. The application of specific drugs which affect the Ca^{2+}-releasing action from SR is an effective approach to the resolution of an important problem in muscle biology concerning the mechanism in the excitation–contraction coupling between nerve and muscle. The Ca^{2+}-releasing effect is especially pronounced with 7-bromoeudistomin D[467] (BED (**462**)) and 9-methyl-7-bromoeudistomin D[468] (MBED (**463**)). MBED (**463**) was synthesized based on the structure–activity relationship between BED (**462**) and caffeine, a well-known inducer of Ca^{2+} release from SR. MBED (**463**) was found to be ca. 1000 times more potent than caffeine in its Ca^{2+}-releasing action. Ca^{2+} release induced by MBED (**463**) or caffeine was blocked by ruthenium red and high concentration of Mg^{2+}. This result suggests that in this pharmacological property, MBED is similar to caffeine. Thus, MBED can be a valuable tool for elucidating the molecular mechanism of Ca^{2+} release from SR.



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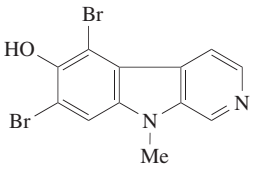


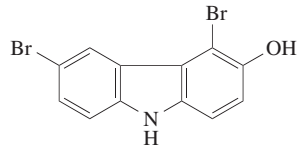
(463)

^{3}H-Labeled 9-methyl-7-bromoeudistomin D ([^{3}H]-MBED) was prepared, which binds to the caffeine binding site of terminal cisternae of the skeletal muscle sarcoplasmic reticulum and activates Ca^{2+}-induced Ca^{2+} release (CICR).[469–471] The hepatic microsomal [^{3}H]-MBED binding site distinguishes it from that of skeletal muscle SR. Properties of the binding site of [^{3}H]-MBED were investigated in aortic smooth muscle.[472] The specific activity was higher in microsomes than in other fractions. [^{3}H]-MBED binding sites in smooth muscle microsomes were of a single class with a high affinity, comparable with that in skeletal muscle SR. Caffeine competitively inhibited [^{3}H]-MBED binding, indicating MBED shares the same binding site with caffeine. Solubilization and fractionation of the microsomes gave two fractions of [^{3}H]-MBED binding activities. These results suggest that, in smooth muscle, there are multiple binding sites of [^{3}H]-MBED and caffeine, which might correspond to different pharmacological actions of caffeine on smooth muscle.

The tissue and subcellular distribution of the binding site of [^{3}H]-MBED were investigated in rabbits.[473] The order of specific activities of total homogenates was liver > brain > other tissues. All binding was completely suppressed by 10 mM caffeine, indicating that all [^{3}H]-MBED binding sites are modulated by caffeine. [^{3}H]-MBED binding sites distributed mainly in membrane fractions rather than soluble fractions in most tissues. In lung and liver, [^{3}H]-MBED binding was enriched in microsomes. [^{3}H]-MBED may be useful as a probe to investigate the actions of caffeine at the molecular level not only in muscles but also in a variety of tissues including liver, kidney, and lung.

Among bromoeudistomin D analogues, the Ca^{2+}-releasing activities of carboline derivatives were higher than those of carbazole derivatives, suggesting that a carboline skeleton is significantly important for the manifestation of Ca^{2+}-releasing activity and Ca^{2+} sensitivity of Ca^{2+}-induced Ca^{2+} release.[474] On the contrary, the analogues which have a carbazole skeleton and bromine at C-6 inhibit both Ca^{2+}- and caffeine-induced Ca^{2+} release. 9-Methyl-substitution of the analogue elevated its Ca^{2+}-releasing activity. Moreover, there is a close correlation between the enhancement of [^{3}H]-ryanodine binding to SR by the analogues and the activation of Ca^{2+} release by them.

4,6-Dibromo-3-hydroxycarbazole (DBHC (**464**)) was synthesized as an analogue of BED (**462**) and its pharmacological properties were examined.[475] In Ca^{2+} electrode experiments, DBHC (**464**) markedly inhibited Ca^{2+} release from SR, induced by caffeine and BED (**462**). [^{3}H]-Ryanodine binding to SR was suppressed by ruthenium red, Mg^{2+} and procaine, but was not affected by DBHC (**464**). [^{3}H]-Ryanodine binding to SR was enhanced by caffeine and BED (**462**). DBHC (**464**) antagonized the enhancement in a concentration-dependent manner. These results suggest that DBHC (**464**) binds to the caffeine-binding site to block Ca^{2+} release from SR. This drug is a novel type of inhibitor for the CICR channels in SR and may provide a useful tool for clarifying the Ca^{2+}-releasing mechanisms in SR.



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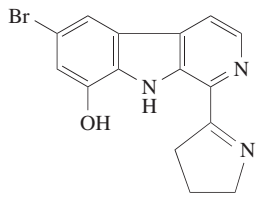
Eudistomins D (**448**), N (**458**), and O (**459**), and several synthetic β-carbolines with halogeno (Br, Cl, or I) and alkyloxy (RO-, with R = H, Me, or Ac) groups on the benzenoid ring proved to be novel inhibitors of cAMP phosphodiesterase.[476] Moreover, several other synthetic eudistomin D analogues are more active than eudistomin D (**448**) as phosphodiesterase inhibitors.

Eudistomidin A (**465**) was isolated from the Okinawan tunicate *Eudistoma glaucus* and exhibited strong calmodulin-antagonistic activity.[477] Eudistomidin A (**465**) was the first calmodulin antagonist from marine origin and is about 15 times more potent than W-7, a well-known calmodulin antagonist. Eudistomidins B (**466**), C (**467**), and D (**468**) were obtained form the same tunicate.[478] The absolute stereostructure of eudistomidin B (**466**) was elucidated from NMR and CD data, whereas that of eudistomidin C (**467**) was established by synthesis of 6-*O*-methyl-10(*R*)-eudistomidin C. Eudistomidins B, C, and D (**466**)–(**468**) showed potent cytotoxicity against murine leukemia L1210 (IC_{50} = 3.4, 0.36, and 2.4 μg ml^{-1}) and L5178Y (IC_{50} = 3.1, 0.42, and 1.8 μg ml^{-1}) cells, respectively. In addition, eudistomidin B (**466**) activated rabbit heart muscle actomyosin ATPase by 93% at 3×10^{-5} M, while eudistomidin C (**467**) exhibited calmodulin-antagonistic activity ($IC_{50} = 3 \times 10^{-5}$ M). Eudistomidin D (**468**) induced Ca^{2+} release from SR, about 10 times more potent than caffeine. Two new β-carboline alkaloids, eudistomidins E (**469**) and F (**470**), have also been isolated from the same tunicate.[479] Eudistomidins E (**469**) and F (**470**) are structurally unique with the tetrahydropyrimidine ring fused to the β-carboline ring. Since these new β-carboline alkaloids appear to be biogenetically related to eudistomin E (**449**) or eudistomidin C (**467**), the absolute configuration of C-10 in eudistomidins E (**469**) and F (**470**) is assumed to be *S*, the same as that of eudistomin E (**449**) or eudistomidin C (**467**).



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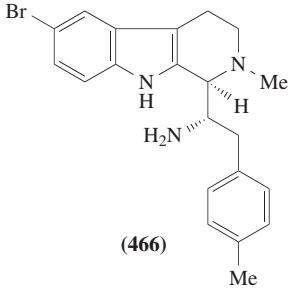

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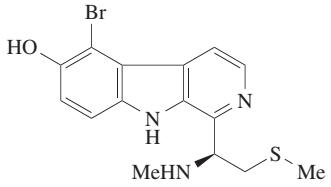

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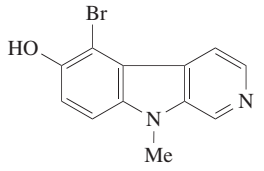

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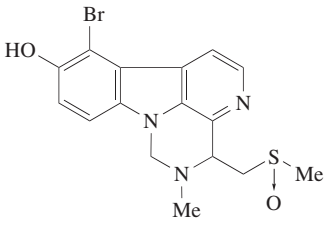

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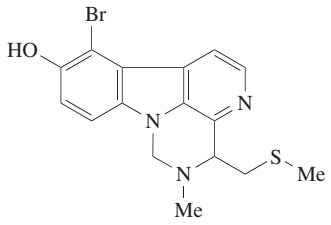

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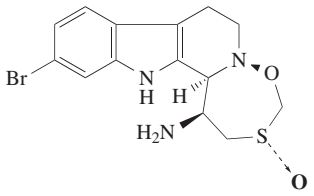
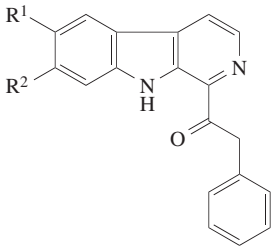
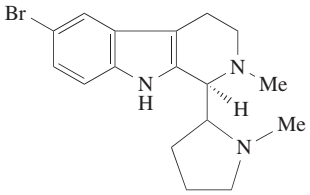
Eudistomin K (**455**) and its sulfoxide (**471**) were isolated from the New Zealand ascidian *Ritterella sigillinoides*.[480,481] The sulfoxide (**471**) also shows antiviral activity against *Polio* and *Herpes simplex*. The structure of eudistomin K (**455**) was determined by X-ray analysis,[482] in which the stereochemistry of the favored invertomer was established, and that of eudistomin K sulfoxide (**471**) by semisynthesis from eudistomin K (**455**). Three other β-carbolines, named eudistomins R, S, and T (**472**)–(**474**), were obtained from a Bermudan tunicate *Eudistoma olivaceum* by using an amino-bonded HPLC column.[483] A 2-methyl-1,2,3,4-tetrahydro-β-carboline with an *N*-methylpyrrolidine at C-1, named woodinine (**475**), was isolated from a New Caledonian ascidian *Eudistoma fragum*,[484] extracts of which exhibited antimicrobial activity.



(471)

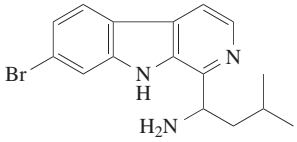


(472) $R^1 = H$, $R^2 = Br$
(473) $R^1 = Br$, $R^2 = H$
(474) $R^1 = R^2 = H$



(475)

The New Caledonian tunicate *Eudistoma album* contained the new eudistalbins[485] A (**476**) and B (**477**) in addition to eudistomin E (**449**). Eudistalbin A (**476**) showed some cytotoxicity against KB cells ($IC_{50} = 3.2$ μg ml^{-1}), whereas eudistalbin B (**477**) was inactive. The New Caledonian tunicate *Pseudodistoma arborescens* has yielded arborescidines A, B, C, and D (**478**)–(**481**), of which only arborescidine D (**481**) exhibited cytotoxicity against KB cells ($IC_{50} = 3$ μg ml^{-1}).[486]



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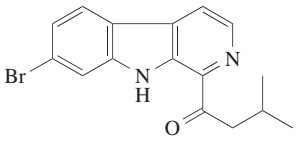

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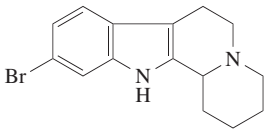
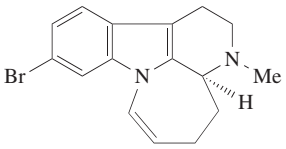


(478)



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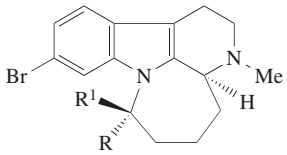
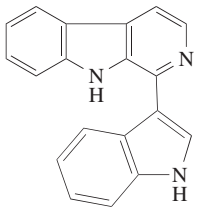
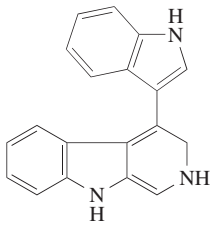


(480) $R^1 = OH$, $R^2 = H$
(481) $R^1 = H$, $R^2 = OH$

Eudistomin U (**482**), isolated from the Caribbean ascidian *Lissoclinum fragile*, is the first bisindole among these β-carbolines.[487] Spectral data, especially ^{1}H- and ^{13}C NMR data, were in good accordance with those reported for β-carboline and indole, thus supporting the proposed structure. Isoeudistomin U (**483**), from the same ascidian, comprises indole and dihydro-α-carboline moieties.[487] These compounds showed a strong antibacterial activity. The structure of eudistomin U (**482**) was confirmed by its syntheses by two groups.



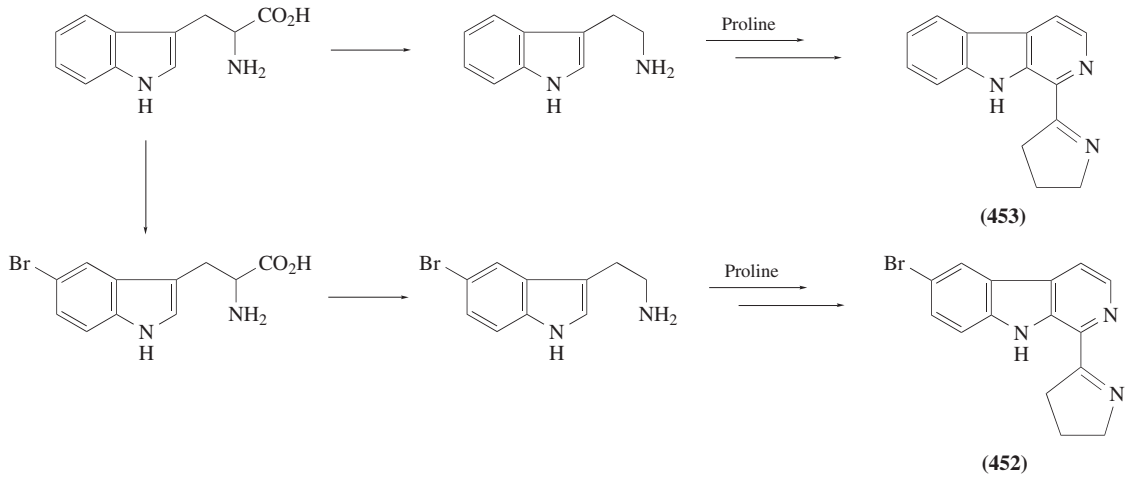
(482)



(483)

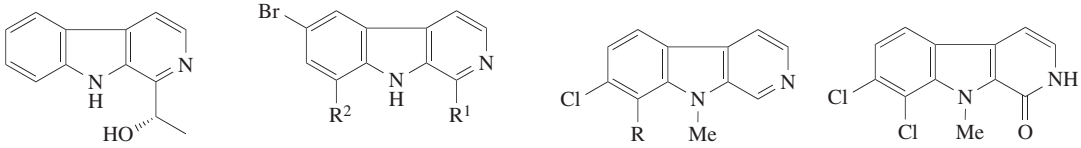
Biosynthetic studies of eudistomin H (**452**) in the Floridan tunicate *Eudistoma olivaceum* indicate that both 6-bromotryptamine and 6-bromotryptophan are incorporated into eudistomin H (**452**) and both are better than the nonbrominated precursors (Scheme 16).[488] The origin of eudistomin I

(**453**) in the same tunicate has been also investigated by *in vivo* techniques.[488] Tryptophan and proline are the primary precursors to eudistomin I (**453**), while tryptamine serves as an intermediate (Scheme 16).



Scheme 16

Marine bryozoans and hydroids also contain β-carboline alkaloids. (*S*)-1-(1′-Hydroxyethyl)-β-carboline (**484**) was obtained from the Tasmanian bryozoan *Costaticella hastata* together with some known β-carbolines previously reported from terrestrial plants.[489] Three new brominated β-carbolines (**485**)–(**487**) were isolated from the Mediterranean hydroid *Aglaophenia pluma*,[490] and the structures were firmly established by synthesis. The terrestrial blue-green alga *Dichothrix baueriana*, which was collected from the Na Pali coast of Kauai, Hawaii, produces bauerines A–C (**488**)–(**490**), which are active against *Herpes simplex* virus.[491]



(**484**)

(**485**) R^1 = Et, R^2 = H
(**486**) R^1 = Me, R^2 = H
(**487**) R^1 = Et, R^2 = Br

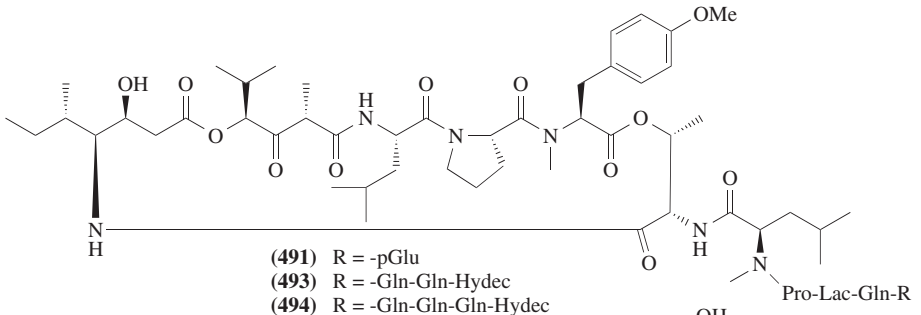
(**488**) R = H
(**489**) R = Cl

(**490**)

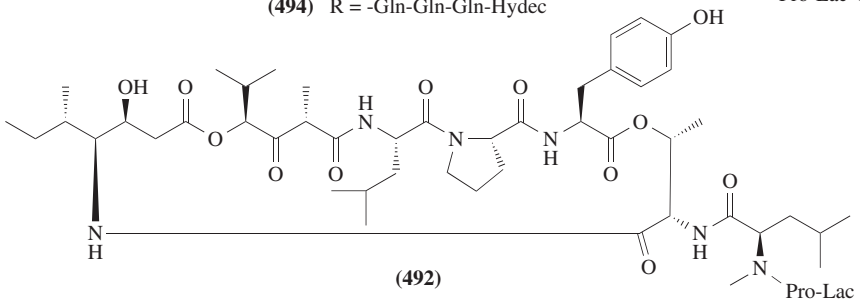
8.07.9.2.3 New tunicate metabolites

Tunicates have been recognized as a source of novel bioactive secondary metabolites with unique chemical structures, most of them being nitrogenous metabolites,[492] represented by didemnins (see Section 8.07.9.1)[493] and ecteinascidins.[494,495] This section deals with several natural products isolated from tunicates, particularly those published after 1994.

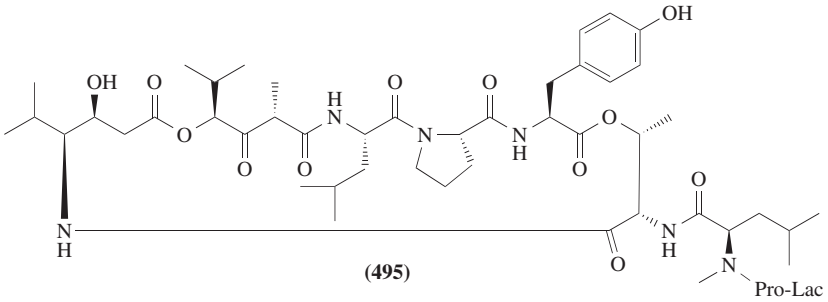
Seven additional didemnins—didemnins M (**491**), N (**492**), X (**493**), and Y (**494**), nordidemnin N (**495**), epididemnin A_1 (**496**), and acylclodidemnin A (**497**)—were isolated by Rinehart and co-workers from a large amount of extract of the Caribbean tunicate *Tridemnum solidum* (170 kg),[496] which was obtained during preparation of didemnin B for phase I and phase II studies. A polar fraction after a silica gel column chromatography of the extract was subjected to on-line LC/FAB mass analysis employing the moving belt technique and was separated by using high-speed centrifugal countercurrent chromatography (HSCCC), polystyrene-divinylbenzene copolymer gel, reversed- and normal-phase HPLC to give new didemnins. The structures of these compounds were assigned, based on spectral data and chemical degradation studies.



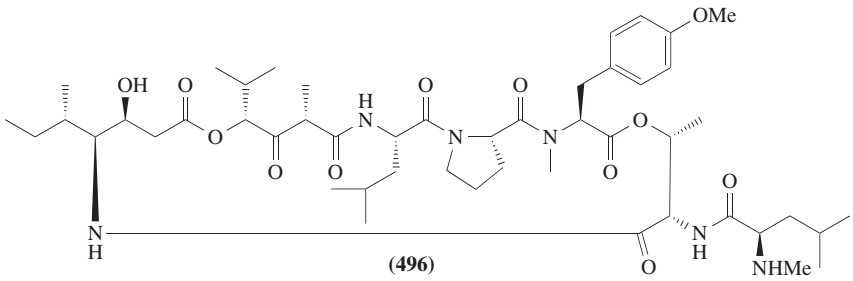
(491) R = -pGlu
(493) R = -Gln-Gln-Hydec
(494) R = -Gln-Gln-Gln-Hydec



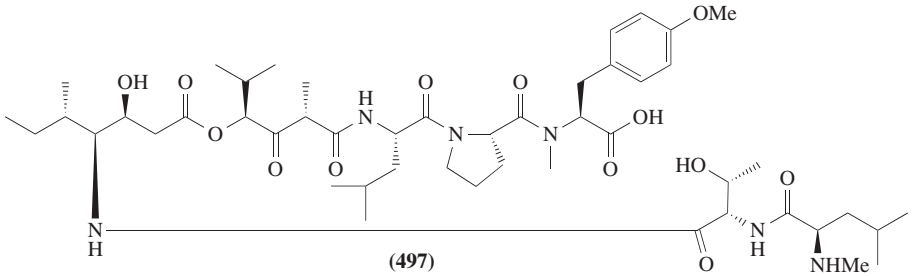
(492)



(495)



(496)

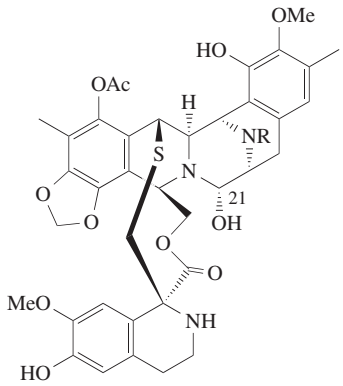


(497)

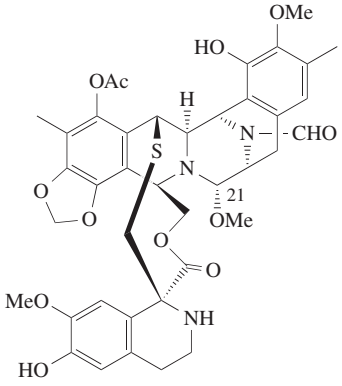
The bioactivities of 42 didemnin congeners, isolated from marine tunicates *Tridemnum solidum* and *Aplidium albicans* or prepared semisynthetically, were compared by using the growth inhibition of various murine and human tumor cells and plaque reduction of HSV-1 and VSV grown on cultured mammalian cells to assess cytotoxicity and antiviral activity. Biochemical assays for macromolecular synthesis (protein, DNA, and RNA) and enzyme inhibition (dihydrofolate reductase, thymidylate synthase, DNA polymerase, RNA polymerase, and topoisomerases I and II) were also performed to specify the mechanisms of action of each analogue. The immunosuppressive activity of the didemnins was determined using a mixed lymphocyte reaction (MLR) assay. These assays revealed that the native cyclic depsipeptide core was an essential structural requirement for

most of the bioactivities of the didemnins, especially for cytotoxicity and antiviral activities. The linear side-chain portion of the peptide could be altered with a gain, in some cases, of bioactivities. In particular, dehydrodidemnin B, tested against several types of tumor cells and in *in vivo* studies in mice, as well as didemnin M, tested for the mixed lymphocyte reaction and graft vs. host reaction in murine systems, showed remarkable gains in their *in vitro* and *in vivo* activities compared with didemnin B.[497]

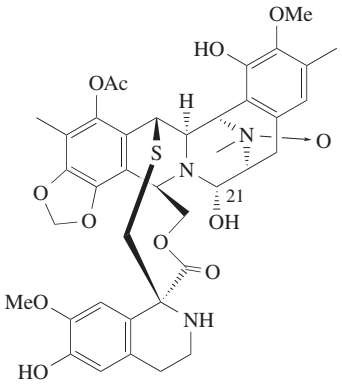
Ecteinascidins 729 (**498**) and 743 (**499**) were reported in 1990 to be isolated from the Caribbean tunicate *Ecteinascidia turbinata* independently by researchers at Harbor Branch Oceanographic Institution[494] and Rinehart's group.[495] Ecteinascidins protect mice *in vivo* against P388 lymphoma, B16 melanoma, M5076 ovarian sarcoma, Lewis lung carcinoma, and the LX-1 human lung and MX-1 human mammary carcinoma xenografts. The crystal structures of two derivatives, 21-*O*-methyl-N^{12}-formyl derivative (**500**) of (**498**) and naturally occurring ecteinascidin 743 N^{12}-oxide (**501**), were reported.[498]



(498) R = H
(499) R = Me

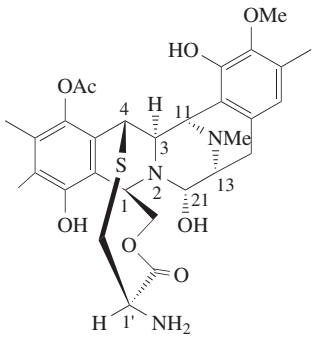


(500)



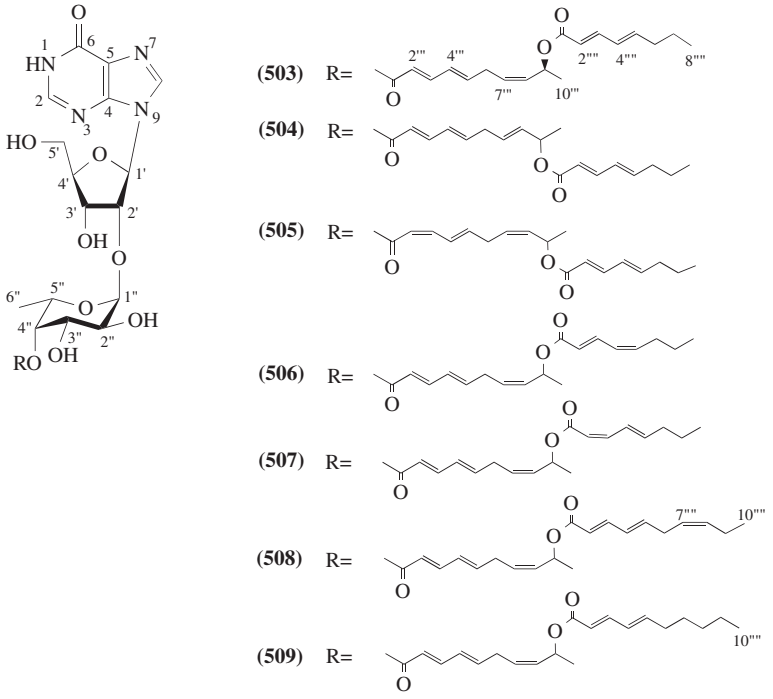
(501)

Ecteinascidins belong to a class of antibiotics that contain tetrahydroisoquinoline units including saframycins,[499] which was known to form a covalent adduct with DNA, most likely with the formation of a bond between the carbinolamine carbon (C-21) and N-2 of guanine in the minor groove of the DNA double helix. Ecteinascidins also bind strongly with DNA, but they do not form permanent covalent adducts. The three-dimensional structure of adducts of ecteinascidins and DNA was analyzed by use of a DNA heptamer model, d(TTGGGAA), and the program QUANTA; it was shown that the ring B platform stacked on the backbone of DNA on one side of the minor groove, following the right-handed curvature of the B-DNA helix, and ring C stacked on the backbone of the other side of the minor groove.[498] Further bioactive ecteinascidins such as (**502**), putative biosynthetic precursors of previously described ecteinascidins, were isolated from the Caribbean tunicate *Ecteinascidia turbinata*. The absolute configuration of the L-cysteine unit of (**502**) was assigned by chiral GC, while a 2D ROESY (rotating-frame Overhauser enhancement spectroscopy) spectrum of its acetate completed the assignment of the stereochemistry of (**502**) as 1*R*,2*R*,3*R*,4*R*,11*R*,13*S*,21*S*,1′*R*.[500]

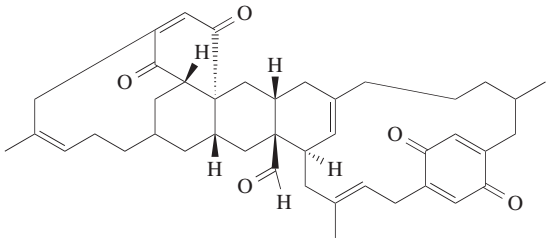


(502)

From the Okinawan marine tunicate *Aplidium multiplicatum*, shimofuridin A (**503**),[501] a nucleoside derivative embracing an acylfucopyranoside unit, was isolated and its structure including all absolute configurations was determined by spectral and chemical means. Compound (**503**) exhibited cytotoxicity against murine lymphoma L1210 cells with an IC_{50} value of 9.5 μg ml^{-1} *in vitro*, and antimicrobial activity against fungus *Trichophyton mentagrophytes* (MIC value, 133 μg ml^{-1}) and Gram-positive bacterium *Sarcina lutea* (MIC, 66 μg ml^{-1}). Compound (**503**) also showed endothelin converting enzyme (ECE) inhibition activity (31.2% at 100 μg ml^{-1}). Minor analogues, shimofuridins B–G (**504**)–(**509**)[502] were also isolated from the Okinawan marine tunicate *A. multiplicatum* by HPLC separation and they were stereoisomers of alkenes in the acyl side-chains ((**504**)–(**507**)), or homologues with two more carbons in the second acyl chain ((**508**) and (**509**)).



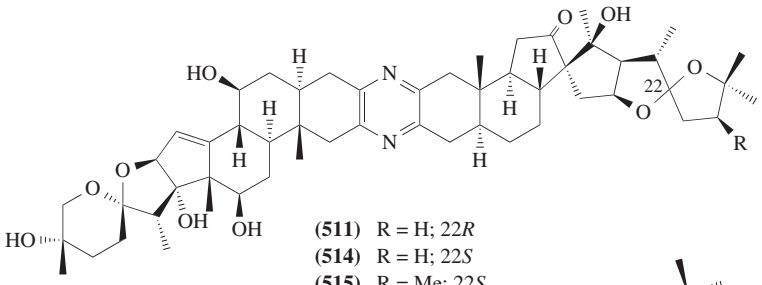
It seems likely that tunicates rarely contain terpenoids and steroids. However, a diterpenoid dimer and steroidal dimers have been obtained from extracts of tunicate. An unprecedented dimeric farnesylated benzoquinone, $C_{42}H_{46}O_5$, named longithorone A (**510**) was isolated from the tunicate *Aplidium longithorax* (Monniot) collected in Palau. The structure was established by single crystal X-ray diffraction. Longithorone A (**510**) was comprised of two subunits each derived from a farnesyl moiety bridging the 2,5-positions of a benzoquinone. Diels–Alder reactions may account for the union of the two subunits and additional ring formations. Longithorone A (**510**) contained five six-membered rings plus bridging 10- and 16-membered rings. Mild cytotoxicity to murine leukemia cells was observed for longithorone A (**510**).[503]



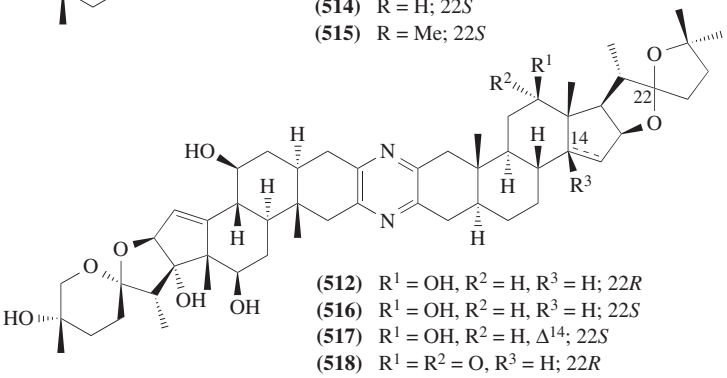
(510)

A cytotoxic dimeric steroidal alkaloid, ritterazine A (**511**),[504] was isolated from the tunicate *Ritterella tokioka*, and its structure elucidated by extensive spectroscopic analyses. It exhibited

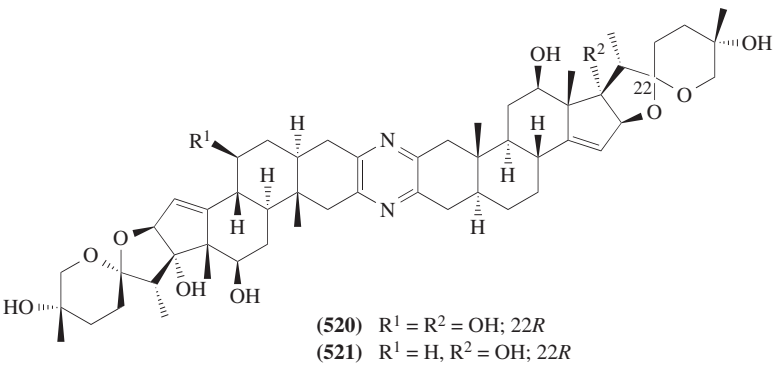
cytotoxicity against P388 murine leukemia cells with an IC_{50} value of 3.8×10^{-3} μg ml^{-1}. Further examination of this tunicate resulted in isolation of a total of 26 analogues, ritterazines A (**511**)–Z (**536**).[505–507] The structures including absolute stereochemistries were determined by extensive spectroscopic analyses and chemical means. Their structural features were reminiscent of the cephalostatins isolated from the Indian Ocean hemichordate *Cephalodiscus gilchristi*.[508] The most difficult aspect of the structural elucidation of this class of compounds lay in determining the orientation of the steroidal units with respect to the pyrazine ring. Fusetani and co-workers, who isolated the ritterazines, succeeded in preparing *N*-methyl derivatives of ritterazine B (**512**) in order to use the *N*-methyl groups for NOESY experiments.[505] This study revealed that the orientation of the steroidal units in (**512**) was identical with that in cephalostatin 1 (**537**),[508] which was established by X-ray analysis. Furthermore, Fusetani's group succeeded in applying ^{15}N HMBC spectroscopy to determine the two steroidal orientations in ritterazine A (**511**).[509]



(**511**) R = H; 22*R*
(**514**) R = H; 22*S*
(**515**) R = Me; 22*S*

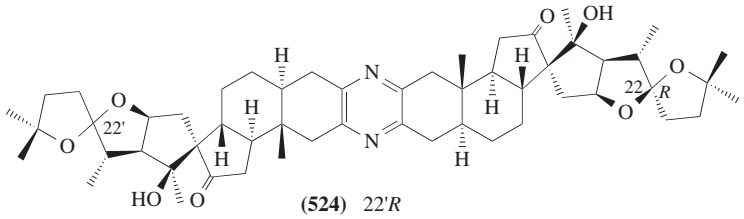


(**512**) R^1 = OH, R^2 = H, R^3 = H; 22*R*
(**516**) R^1 = OH, R^2 = H, R^3 = H; 22*S*
(**517**) R^1 = OH, R^2 = H, Δ^{14}; 22*S*
(**518**) $R^1 = R^2$ = O, R^3 = H; 22*R*
(**519**) $R^1 = R^2$ = O, R^3 = H; 22*S*

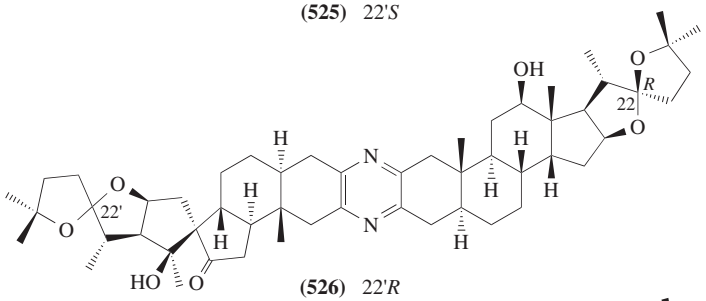


(**520**) $R^1 = R^2$ = OH; 22*R*
(**521**) R^1 = H, R^2 = OH; 22*R*
(**522**) $R^1 = R^2$ = H; 22*R*
(**523**) $R^1 = R^2$ = H; 22*S*

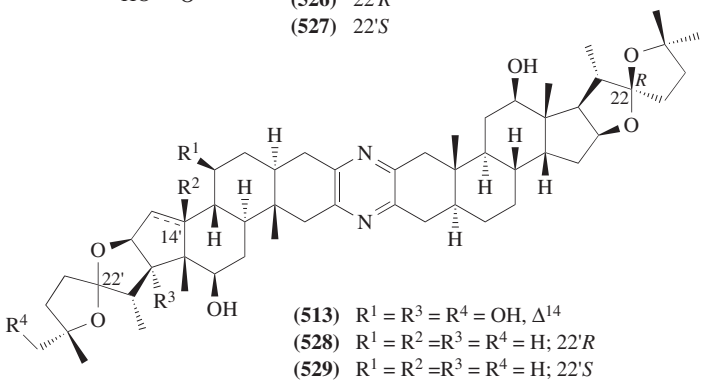
The structure–activity relationship of the 26 natural ritterazines together with several derivatives (**538**)–(**544**) prepared from the most active compound, ritterazine B (**512**), was investigated, and the



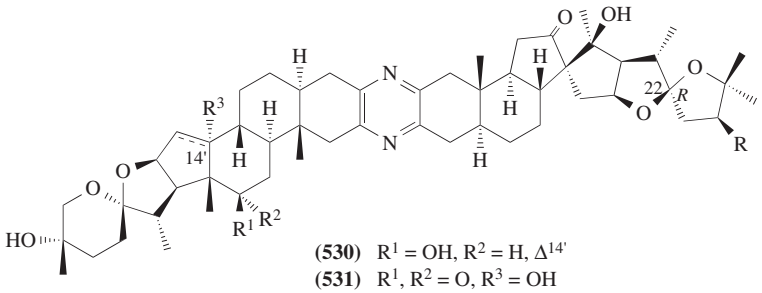
(524) 22′*R*
(525) 22′*S*



(526) 22′*R*
(527) 22′*S*

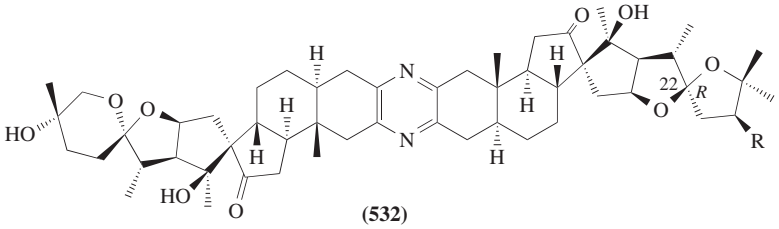


(513) $R^1 = R^3 = R^4 = OH, \Delta^{14}$
(528) $R^1 = R^2 = R^3 = R^4 = H$; 22′*R*
(529) $R^1 = R^2 = R^3 = R^4 = H$; 22′*S*

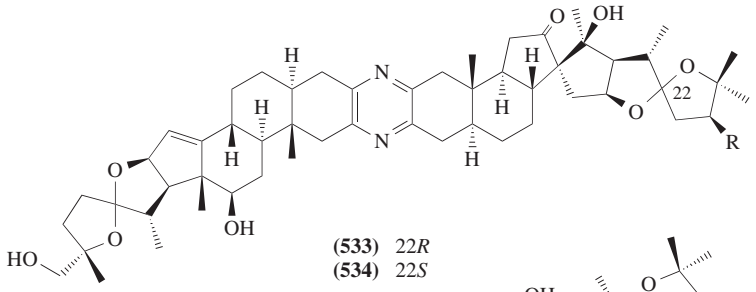


(530) $R^1 = OH, R^2 = H, \Delta^{14'}$
(531) $R^1, R^2 = O, R^3 = OH$

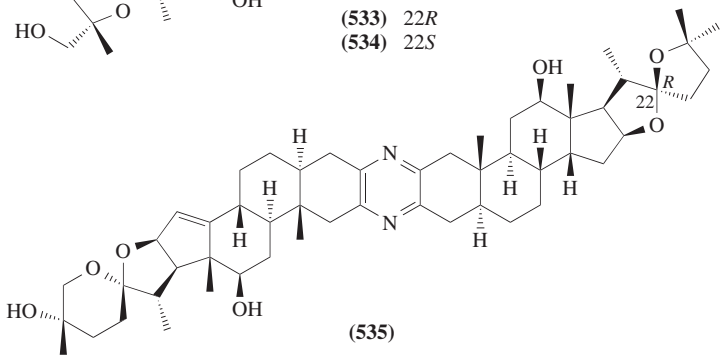
cytotoxicity data are shown in Table 21.[507] Ritterazine B (**512**) which was dissymmetric was the most potent cytotoxin; ritterazines A (**511**), D (**514**), E (**515**), F (**516**), G (**517**), H (**518**), I (**519**), J (**520**), K (**521**), L (**522**), M (**523**), and Y (**535**) were also highly cytotoxic. These compounds had the 5/6 spiro ring and 12′,25′-diol functionalities common in the western hemisphere. Ritterazines N (**524**), O (**525**), P (**526**), Q (**527**), R (**528**), S (**529**), W (**533**), X (**534**), and Z (**536**) having no 5/6 spiroketal were marginally active. Therefore, the 5/6 spiroketal was supposed to possess an important function for the expression of cytotoxicity, because the cleavage of the 5/6 spiroketal decreased their activity. Furthermore, ritterazine T (**530**) which had no conventional steroidal skeletal with OH-12 in the eastern hemisphere, but the 5/6 spiro ring in the western hemisphere, showed weak cytotoxicity. Therefore, the presence of a 5/6 spiro ring in one end, a 5/5 spiro ring in the other end, and the hydroxyl group on C-12 were shown to be important for cytotoxic activity. However, acetylation of secondary hydroxyl groups gave interesting information. Compared with oxidation, acetylation



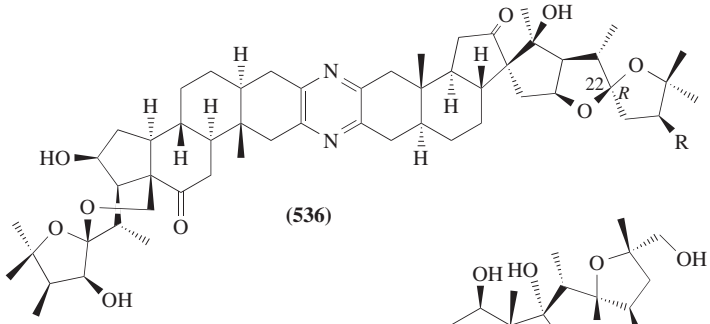
(532)



(533) 22*R*
(534) 22*S*



(535)



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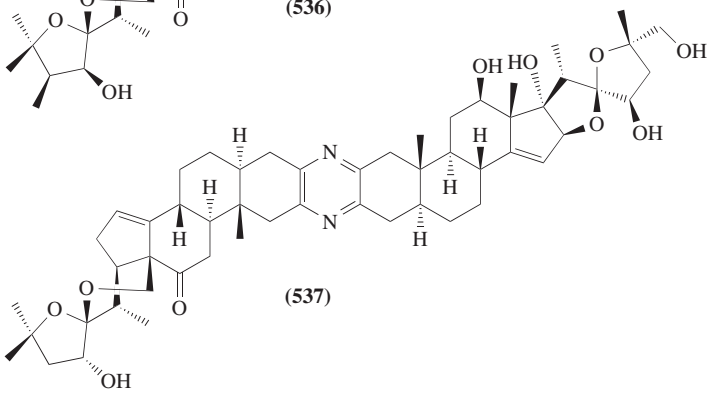
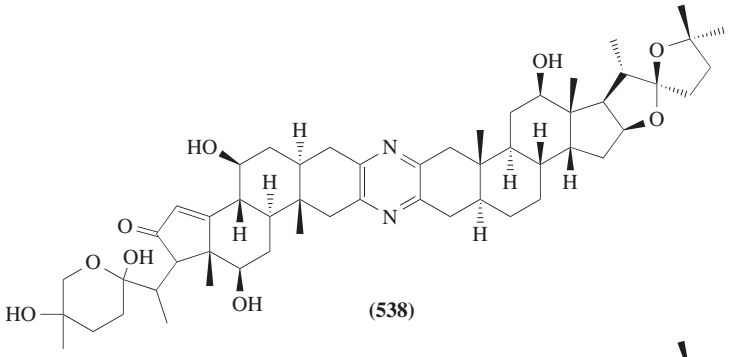


(537)

of OH-12 did not decrease cytotoxicity, but induction of acetyl groups in the western hemisphere showed weaker activity. It was possible to consider that the steric hindrance by acetyl groups in the western hemisphere influenced the expression of the cytotoxicity.

It may be generally recognized that peptides are frequently contained in tunicates, represented by didemnins described above. Many of these peptides also show high levels of pharmacological activity.[492,510] Tunicates of the genus *Lissoclinum* are well known as prolific producers of cytotoxic



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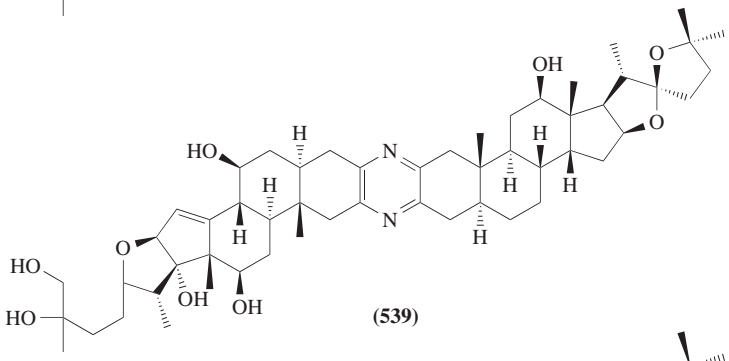

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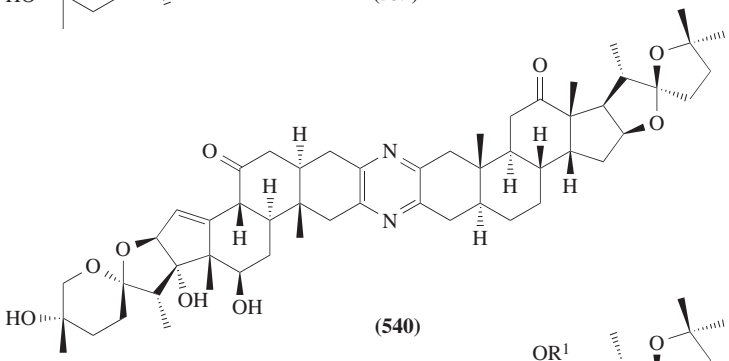
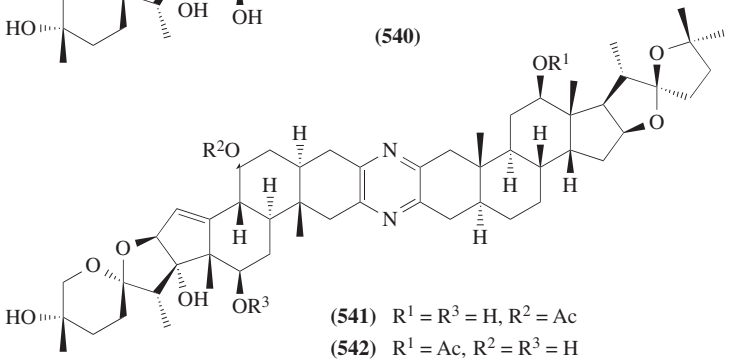


(540)

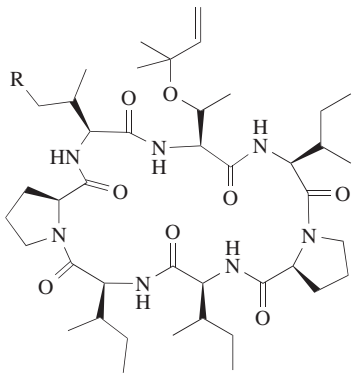


(541) $R^1 = R^3 = H$, $R^2 = Ac$
(542) $R^1 = Ac$, $R^2 = R^3 = H$
(543) $R^1 = R^2 = Ac$, $R^3 = H$
(544) $R^1 = R^2 = R^3 = Ac$

cyclic peptides with highly modified amino acids such as those containing oxazole, oxazoline, thiazole, and thiazoline groups.[511] Two cyclic heptapeptides, nairaiamide A (**545**) and B (**546**), were isolated from the ascidian *Lissoclinum bistratum* collected at Nairai Island, Fiji, and their structures were determined by a combination of spectroscopic techniques, including a natural abundance two-dimensional $^{1}H[^{13}C,^{1}H]$-HMQC–TOCSY experiment which was pivotal to assigning and identifying the amino acid residues.[512] $^{1}H[^{13}C]$-HMBC and $[^{1}H\text{-}^{1}H]$-ROESY experiments provided the correlations necessary to sequence the peptides. In both peptides, the Ile–Pro amide bonds adopted *cis* configurations.

Table 21 Cytotoxic activities of 34 ritterazine derivatives (**511**)–(**544**) (IC_{50} values against P388 leukemia cells, ng ml^{-1}).

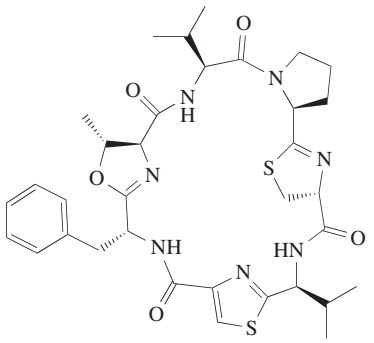
Compound	IC_{50}
(**511**)	3.5
(**512**)	0.15
(**513**)	92
(**514**)	16
(**515**)	3.5
(**516**)	0.73
(**517**)	0.73
(**518**)	16
(**519**)	14
(**520**)	13
(**521**)	9.5
(**522**)	10
(**523**)	15
(**524**)	460
(**525**)	2100
(**526**)	710
(**527**)	570
(**528**)	2100
(**529**)	460
(**530**)	460
(**531**)	2100
(**532**)	2100
(**533**)	3200
(**534**)	3000
(**535**)	3.5
(**536**)	2000
(**537**)	0.001–0.000001
(**538**)	2100
(**539**)	240
(**540**)	18
(**541**)	92
(**542**)	3.5
(**543**)	800
(**544**)	7600



(**545**) R = H
(**546**) R = Me

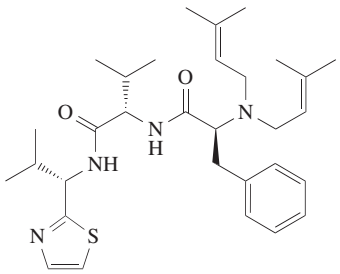
Cyclodidemnamide (**547**), a cyclic heptapeptide, was isolated from the marine ascidian *Didemnum molle* collected in the Cuyo Islands in the Philippines.[513] From 21.4 g of freeze-dried animal a total of 11.5 mg of (**547**) was isolated. The structure of the new, weakly cytotoxic, heptapeptide was unambiguously assigned using comprehensive NMR analysis in combination with hydrolysis and chiral GC–MS analysis of its component amino acids. The results clearly showed that the phenylalanine unit had the unnatural D configuration, while other amino acid residues obtained from the hydrolysis contained natural L configurations. Computer analysis led to the assignment of a solution

conformation that fully rationalized the NOE correlations and shielding effects observed by ^{1}H NMR. One methyl proton (C-32) on a valine residue was observed at unusually high field shift (δ_H 0.15), indicating that this methyl group was directed within the ring interacting with the π-cloud of the thiazole unit. Consistently, NOE correlations were observed from the C-32 methyl protons to the thiazole proton and the NH proton of phenylalanine residue.

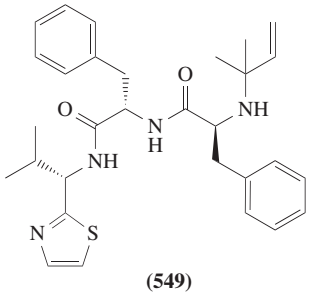


(547)

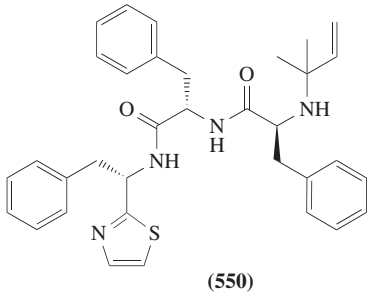
Three linear cytotoxic tripeptides, virenamides A–C (**548**)–(**550**), were isolated from extracts of the didemnid ascidian *Diplosoma virens* collected on the Great Barrier Reef, Australia.[514] Their structures were deduced from 1D and 2D NMR spectral data and confirmed by HPLC analysis of the constituent amino acids after hydrolysis of the peptides and derivatization with 1-fluoro-2,4-dinitrophen-5-yl-L-alanine amide using Marfey's procedure.[515] The virenamides showed modest cytotoxicity toward a panel of cultured cells: virenamide A (**548**) gave IC_{50} of 2.5 μg ml^{-1} against P388, and 10 μg ml^{-1} against A549, HT29, and CV1 cells. It exhibited topoisomerase II inhibitory activity (IC_{50} 2.5 μg ml^{-1}). Virenamides B (**549**) and C (**550**) both gave IC_{50} of 5 μg ml^{-1} against P388, A549, HT29, and CV1 cells.



(548)

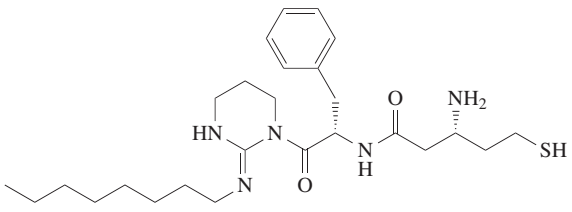


(549)



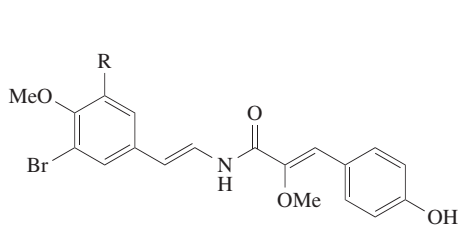
(550)

Caledonin (**551**), a modified peptide, was isolated from the marine tunicate *Didemnum rodriguesi*.[516] Caledonin (**551**) comprised a central L-phenylalanine residue connected via its amino group to (*S*)-3-amino-5-mercaptopentanoic acid (a new sulfur-containing *β*-amino acid), and by its carboxyl group to a six-membered cycloguanidine moiety, bearing an *n*-octyl chain. Tunicates are known to produce metal complexing metabolites, such as the tunichromes.[517] Some features of caledonin (**551**), and in particular the hydrophobic chain at one end and the penicillamine-like *β*-amino acid at the other, suggested that this metabolite was a natural bolaphile which strongly bound Zn^{II} and Cu^{I} ions and may be involved in ion transport across the membranes. A solution of (**551**) in CD_3OD was titrated with $ZnCl_2$ and monitored by ^{1}H NMR spectroscopy. The 2:1 caledonin/$ZnCl_2$ adduct was isolated and characterized by FABMS and NMR spectroscopy.

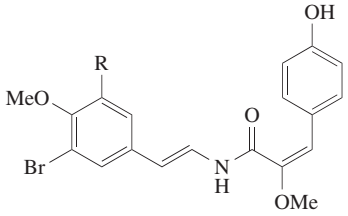


(551)

Four bromotyrosine derivatives, botryllamides A–D (**552**)–(**555**), having striking structural similarities to tunichromes,[517] were isolated from the brilliantly colored Styelid ascidian *Botryllus* sp. from Siquijor Island, the Philippines, and from *B. schlosseri* from the Great Barrier Reef, Australia.[518] Their structures were deduced from 1- and 2-dimensional NMR spectral data. Botryllamide D (**555**) showed marginal cytotoxicity, after 72 h exposure, against the human colon cancer cell line HCT116 ($IC_{50} = 17$ μg ml^{-1}) but was inactive *in vivo*.

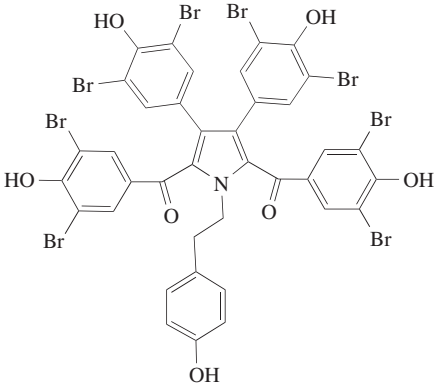


(552) R = Br
(553) R = H

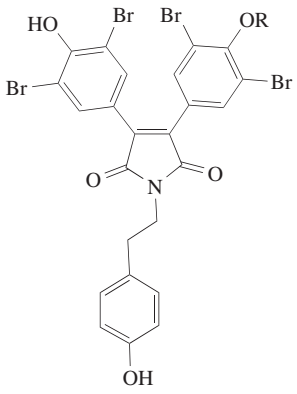


(554) R = Br
(555) R = H

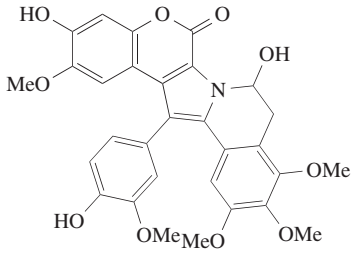
Three bromotyrosine-related compounds, polycitone A (**556**) and polycitrins A (**557**) and B (**558**), were isolated from the marine ascidian *Polycitor* sp.[519] The structures of these compounds were established mainly on the basis of NMR spectroscopic data and, in the case of (**556**), also by single-crystal X-ray diffraction analysis using the crystal of its penta-*O*-methyl derivative. Polycitone A (**556**) and polycitrins A (**557**) and B (**558**) represent the first examples of two new classes of marine products which might biogenetically be close to the lamellarins (lamerarin A (**559**)).[520] The penta-*O*-methyl derivative of (**556**) was found to inhibit the growth of SV40 transformed fibroblast cells in a concentration of 10 μg ml^{-1}.



(556)



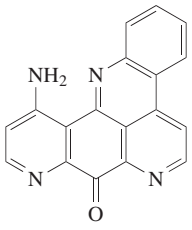
(557) R = H
(558) R = Me



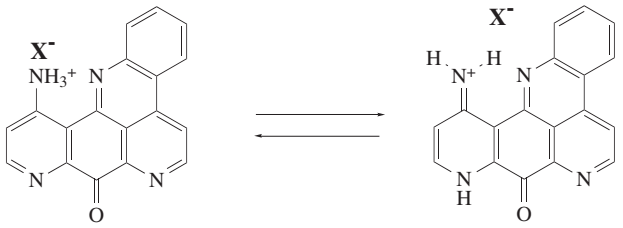
(559)

A fused pentacyclic aromatic alkaloid, cystodamine (**560**), was isolated from a Mediterranean ascidian *Cystodytes dellechiajei* (Polycitoridae).[521] Compound (**560**) was the first example of a marine product displaying a ^{1}H–^{14}N coupling during ^{1}H NMR analysis, which revealed in DMSO-d_6 solution the existence of an aminopyridine moiety (Equation (5)) as the pyridine ring was changed into a pyridone-imine one (**560b**) and one ammonium group was also found (2H, triplet system, $J = 52$ Hz). This triplet system was due to the ^{1}H–^{14}N coupling, the NH absorption band as an ammonium ion resolving itself into a triplet from spin–spin interaction with the ^{14}N

nucleus ($I = 1$). Cystodamine (**560**) showed cytotoxicity against CEM human leukemic lymphoblasts (IC_{50} 1.0 μg ml^{-1}).



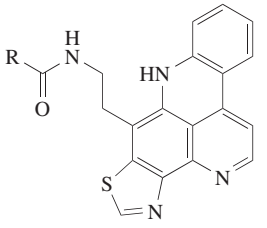
(560)



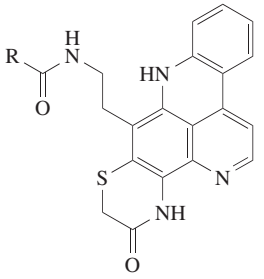
(5)

(560a) **(560b)**

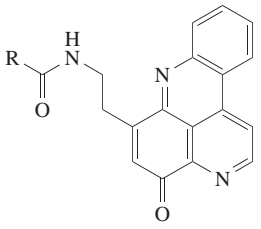
Several additional pyridoacridine alkaloids, dehydrokuanoniamine B (**561**), shermilamine C (**562**), and cystodytin J (**563**),[522] in addition to the known compounds cystodytin A (**564**),[523] kuanoniamine D (**565**),[524] shermilamine B (**566**),[525] and eilatin (**567**),[526] were isolated from a Fijian *Cystodytes* sp. ascidian. These compounds along with a previously reported pyridoacridine, diplamine (**568**),[527] showed dose-dependent inhibition of proliferation in human colon tumor (HCT) cells *in vitro*. All compounds inhibited the topoisomerase (TOPO) II-mediated decatenation of kinetoplast DNA (kDNA) in a dose-dependent manner. The pyridoacridines' ability to inhibit TOPO II-mediated decatenation of kDNA correlated with their cytotoxic potencies and their ability to intercalate into calf thymus DNA. These results suggested that disruption of the function of TOPO II, subsequent to intercalation, is a probable mechanism by which pyridoacridines inhibit the proliferation of HCT cells. Incorporation studies showed that pyridoacridines disrupt DNA and RNA synthesis with little effect on protein synthesis. It appeared that DNA is the primary cellular target of the pyridoacridine alkaloids.



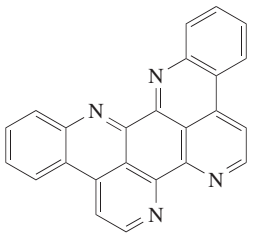
(561) R = $-CH_2=C(Me)_2$
(565) R = -Me



(562) R = $-CH_2=C(Me)_2$
(566) R = -Me



(563) R = -Me
(564) R = $-CH_2=C(Me)_2$



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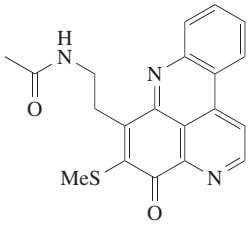
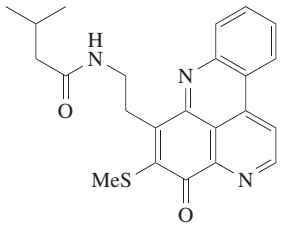
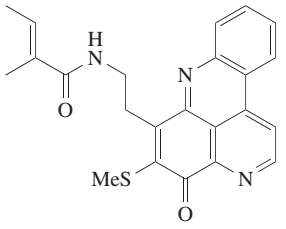


(568)

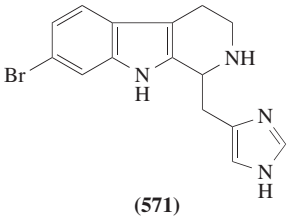
Further pyridoacridine alkaloids, lissoclin A (**569**) and B (**570**), were isolated from *Lissoclinum* sp. collected from the Great Barrier Reef, Australia, along with a tetrahydro-β-carboline, lissoclin C (**571**). Lissoclin A (**569**) underwent photorearrangement to a benzo-1,3-oxathiazoline (**572**).[528]



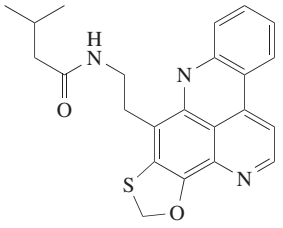
(**569**)



(**570**)

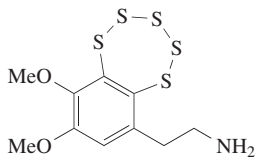


(**571**)

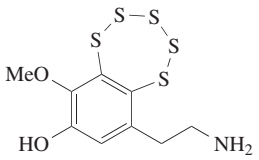


(**572**)

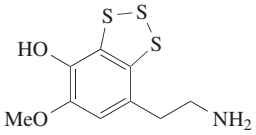
Several unique polysulfides biogenetically related to dopamine were isolated from the tunicate of the genus *Lissoclinum*. Varacin (**573**) was the first benzopentathiepin natural product and the first naturally occurring polysulfide amino acid isolated from *Lissoclinum vareau*, a lavender-colored encrusting species collected in the Fiji Islands. Varacin (**573**) exhibited potent antifungal activity against *Candida albicans* (14 mm zone of inhibition of 2 μg of varacin/disk) and cytotoxicity toward the human colon cancer HCT 116 with an IC_{90} of 0.05 μg ml^{-1}, 100 times the activity of 5-fluorouracil. Varacin (**573**) also exhibited a 1.5 differential toxicity toward the CHO cell line EM9 (chlorodeoxyuridine sensitive) versus BR1 (BCNU resistant), providing preliminary evidence that varacin (**573**) damages DNA.[529] The structure of varacin (**573**) was unambiguously confirmed by total synthesis.[530–532] From *Lissoclinum performatum* collected in Dinard, France, lissoclinotoxin A (**574**) was isolated as a potent antimicrobial and antifungal metabolite with modest cytotoxicity.[533] Its initial structure (**575**) containing a 1,2,3-trithiane ring was later revised to that containing a pentathiepin ring (**574**).[534] Additionally, another pentathepin derivative, lissoclinotoxin B (**576**), was isolated from the same tunicate *L. performatum*.



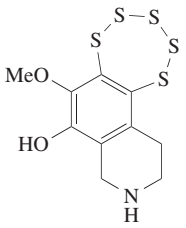
(**573**)



(**574**)



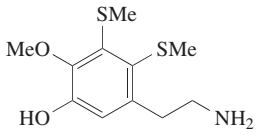
(**575**)



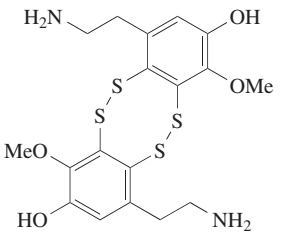
(**576**)

Varacin (**573**) and lissoclinotoxin A (**574**) were found to be chiral, and evidence was provided from unusual stereoisomerism due to restricted inversion about the benzopentathiepin ring, which induced asymmetry into the molecule and caused the protons to become diastereotopic.[528,535] Another polysulfide, lissoclinotoxin C (**577**), and a dibenzotetrathiepin compound, lissoclinotoxin D (**578**), were isolated from the tunicate *Lissoclinum* sp. collected from the Great Barrier Reef,

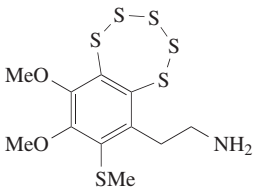
Australia.[528] The dimeric compound (**578**) exhibited antifungal activity against *Candida albicans*. The ascidian *Lissoclinum japonicum* from Palau contained the antimicrobial and antifungal metabolites, *N*,*N*-dimethyl-5-(methylthio)varacin (**579**) and 3,4-dimethoxy-6-(2′-*N*,*N*-dimethylaminoethyl)-5-(methylthio)benzotrithiane (**580**).[536] An inseparable 2:3 mixture of 5-(methylthio)varacin (**581**) and the corresponding trithiane (**582**) was isolated from a different *Lissoclinum* species from Pohnpei, Micronesia and 3,4-desmethylvaracin (**583**) was obtained from a species of *Eudistoma* from Pohnpei. These pentathiepins and trithianes selectively inhibited protein kinase C.[536]



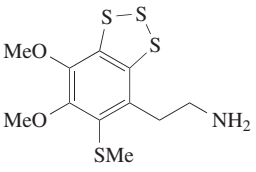
(**577**)



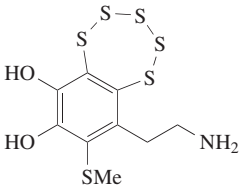
(**578**)



(**579**) R = Me
(**581**) R = H

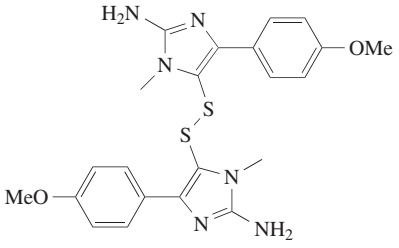


(**580**) R = Me
(**582**) R = H



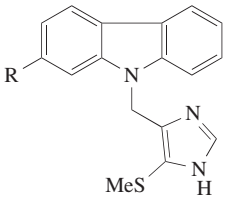
(**583**)

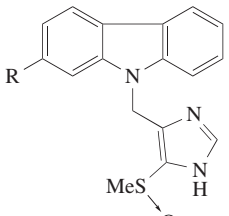
A dimeric disulfide alkaloid, polycarpine (**584**), was isolated almost at the same time independently by two research groups from tunicate *Polycarpa clavata* collected in Western Australia[537] and *Polycarpa aurata* collected in Chuuk, Micronesia.[538] The disulfide (**584**) inhibited the enzyme inosine monophosphate dehydrogenase, and the inhibition could be reversed by addition of excess dithiothreitol.[538] The dihydrochloride of (**584**) was cytotoxic against the human colon tumor cell line HCT-116 at 0.9 μg ml^{-1}.[537]



(**584**)

Four β-carboline-based metabolites, didemnolines A–D (**585**)–(**588**) were isolated from an ascidian of the genus *Didemnum*, collected near the island of Rota, Northern Mariana Islands.[539] These β-carboline-based metabolites differed from most previously isolated compounds in that they are substituted at the N-9 position of the β-carboline ring, rather than at the C-1 position. Didemnolines A–C (**585**)–(**587**) were moderately cytotoxic toward human epidermoid carcinoma KB cells, with sulfoxide-containing (**587**) exhibiting the greatest activity. Compounds (**585**) and (**587**) also exhibited antimicrobial activity.

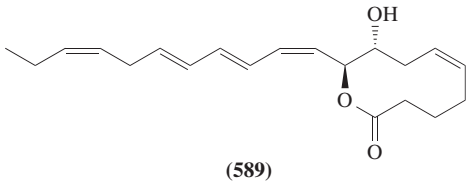




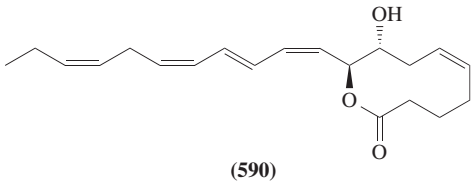
(585) R = Br
(586) R = H

(587) R = Br
(588) R = H

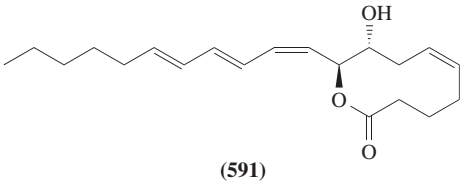
Three fatty acid metabolites didemnilactone A (**589**) and B (**590**) and neodidemnilactone (**591**) were isolated from the tunicate *Didemnum moseleyi* (Herdman). Their structures, including absolute stereochemistry, were established on the basis of spectral studies and chemical synthesis. Didemnilactones exhibited inhibitory activity against lipoxygenase and weak binding activity to leukotriene B4 receptors.[540]



(589)

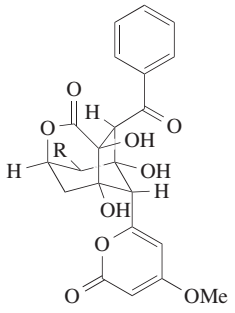


(590)

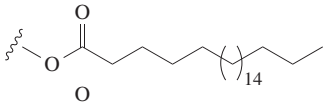


(591)

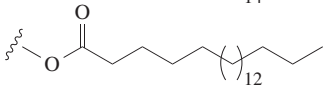
A known microbial antibiotic, enterocin (**592**), and its derivatives (**593**)–(**595**) were isolated from the brown, encrusting ascidian, *Didemnum* sp. collected in Western Australia.[541] Enterosin (**592**) and 5-deoxyenterocin (**593**) were originally isolated from three strains of soil-derived *Streptomyces* species.[542] This was the first observation of enterosins being isolated from nonmicrobial sources, adding further support for the concept that bacteria may produce some of the interesting molecules isolated from marine invertebrates. The limited production of enterocin antibiotics by various *Streptomyces*, and the uniqueness of this structural class, created significant questions about their true origins in biologically complex marine invertebrates. Microscopic examination of a thin section of the alcohol-fixed ascidian tunic of this *Didemnum* sp. showed the presence of large amounts of V-shaped bacteria similar in size and shape to *Arthrobacter* species. Although considerable effort was expended to collect fresh samples and cultivate these endobiotic microorganisms, none of the 20 strains obtained could be confirmed, in culture, to produce the enterocin-based metabolites isolated from the whole animal.[541]



(592) R = OH
(593) R = H

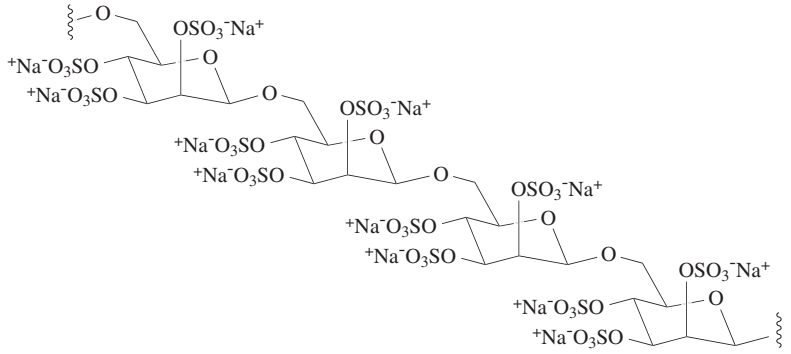


(594) R =



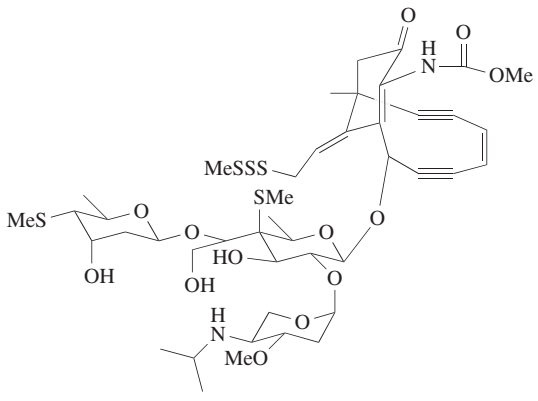
(595) R =

An unusual sulfated mannose homopolysaccharide, kakelokelose (**596**), was isolated from the mucous secretion of the Pacific tunicate *Didemnum molle* collected in Pohnpei, Micronesia, and in Manado, Indonesia, by sequential ultrafiltration, guided by anti-HIV tests. From 1.5 kg of *D. molle* (wet weight), 193 mg of a white solid was obtained, which showed *in vitro* anti-HIV activity. It slowly dissolved in water to give a very viscous solution. Analysis of the NMR data revealed that it consisted of a sequence of 2,3,4-trisulfated mannose units joined through β (1,6) glycosidic linkages.[543]



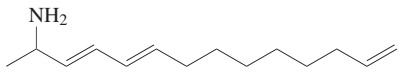
(596)

An enediyne antitumor antibiotic, namenamicin (**597**), was isolated from a methanolic extract of the thin encrusting orange ascidian *Polysyncraton lithostrotum* (order: Aplousobranchia, family: Didemnidae) collected at Namenalala Island, Fiji in 10^{-4}% yield (1 mg from 1 kg of frozen tissue), guided by bioautography-directed fractionation of the extract using the biochemical induction assay (BIA).[544] Namenamicin (**597**) contained the same "enediyne warhead" as the calicheamicins; however, the attached carbohydrate moiety differed in replacement of the N—O sugar linkage between the A and B sugars with a C—O, an *S*-methyl substituent at the 4 position of the A sugar, and the absence of a benzoate ring appended to the B sugar. Namenamicin (**597**) exhibited potent *in vitro* cytotoxicity with a mean IC_{50} of 3.5 ng ml^{-1} and *in vivo* antitumor activity in a P388 leukemia model in mice (ILS 40% at 3 μg kg^{-1}). Namenamicin (**597**) also showed potent antimicrobial activity and DNA cleavage experiments indicated that namenamicin (**597**) cleaved DNA with a slightly different recognition pattern than calicheamicin γ_1^I. Bacteria form highly specific symbiotic relationships with marine plants and animals which leads one to speculate on the true biosynthetic origin of namenamicin (**597**). The fact that all of the enediyne antitumor antibiotics previously isolated were products of antinomycetes and namenamicin's extremely low and variable yield from the ascidian lend support to the hypothesis of a microbial origin for this natural product. In order to address the question of compound origin, isolation experiments were carried out in search of a possible producing microorganisms. As a result, 16 micromonospora were isolated from the tissue of *Polysyncraton lithostrotum* and three of these micromonospora were found to produce potent DNA-damaging compounds.

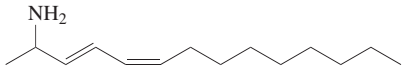


(597)

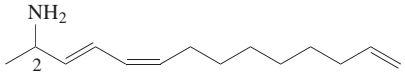
Long-chain amines (**598**)–(**601**) were isolated from New Zealand tunicate *Pseudodistoma novaezelandae*, exhibiting cytotoxic activity against P388 murine leukemia cells and moderate antifungal activity vs. *Candida albicans*.[545] The amines were isolated as a racemate, and (**598**) and (**600**) were obtained as a mixture of (3*E*,5*Z*)-amine and (3*E*,5*E*)-amine in a ratio of 3:2. The synthesis of 2*S*-(**600**) was achieved with high enantiomeric purity in excellent overall yield.[546]



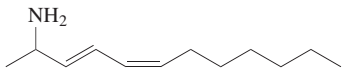
(**598**)



(**599**)

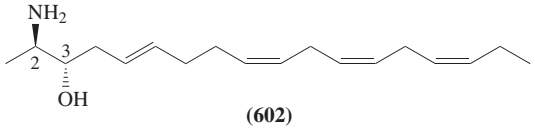


(**600**)

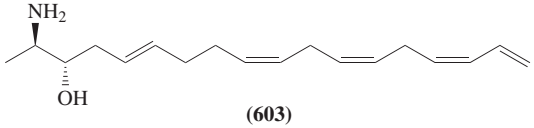


(**601**)

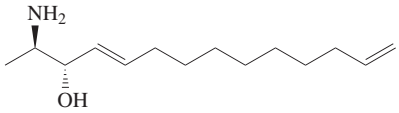
Crucigasterins 277 (**602**), 275 (**603**), and 225 (**604**), three polyunsaturated amino alcohols, were isolated from the Mediterranean tunicate *Pseudodistoma crucigaster*.[547] The structures of these compounds were assigned based on NMR and FABMS data. The absolute stereochemistry of the amino alcohol portion in (**602**) was assigned to be 2*R*,3*S* based on chiral GC comparison of 3-hydroxy-4-aminopentanoic acid, a chemical degradation product of (**602**), with a synthetic sample prepared from L-alanine. Compounds (**602**)–(**604**) exhibited moderate cytotoxicity and antimicrobial activity. These amino alcohols were long-chain 2-amino-3-ols from an ascidian. The 2*R* stereochemistry suggested that these compounds were biosynthesized from D-alanine, unlike the usual plant and mammalian sphingosines, which are derived from L-serine.



(**602**)

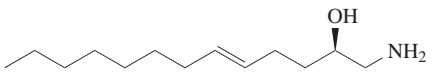


(**603**)



(**604**)

From the ethanol extract of an ascidian *Didemnum* sp., collected from the Great Barrier Reef, Australia, an antifungal amino alcohol, (*R*)-(*E*)-1-aminotridec-5-en-2-ol (**605**), together with (**606**) and (**607**), which were characterized as the *N*-Boc derivatives, were isolated and their absolute stereochemistry was determined by CD spectrum of the dibenzoyl derivative of (**605**) based on exciton coupling theory.[548] In the agar plate disk diffusion assay, amino alcohol (**605**) trifluoroacetate showed moderate activity against *Candida albicans*. The amino alcohols (**606**) and (**607**) (trifluoroacetate salts) both showed activity comparable to that of (**605**), whereas the free base of (**605**), formed upon treatment of the trifluoroacetate salt with K_2CO_3, showed slightly enhanced activity. The structures of compounds (**605**)–(**607**) join an expanding family of modified marine sphinganoids. Sphingosine itself derives from palmitoyl CoA and (*S*)-serine, but the implied biosynthesis of (**605**) appears to require a C_{12} fatty acid and glycine rather than (*S*)-serine.



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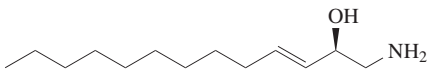
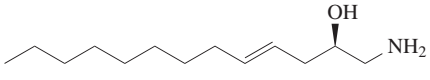
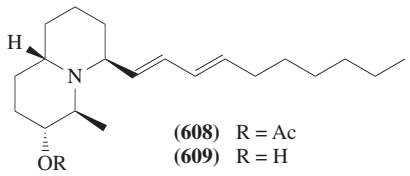


(**606**)

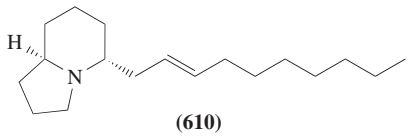


(**607**)

The ascidians of the genus *Clavelina* were found to contain a number of cyclic nitrogenous compounds with unsaturated alkyl side-chains. The ascidian *Clavelina cylindrica* obtained in Bermuda gave the quinolizidine alkaloids clavepictins A (**608**) and B (**609**)[549] together with the indolizidine alkaloids piclavines A_1–A_4 (**610**)–(**613**), B (**614**), and C (**615**).[550] The same ascidian obtained from Venezuela gave pictamine (**616**).[551] Clavepictine A (**608**) and pictamine (**616**) differed only by the length of the side-chain. The ascidian *Clavelina lepadiformis* yielded a decahydroquinoline alkaloid lepadin A (**617**).[552]



(**608**) R = Ac
(**609**) R = H



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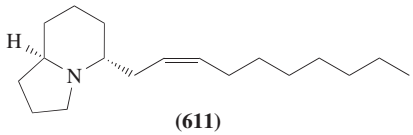

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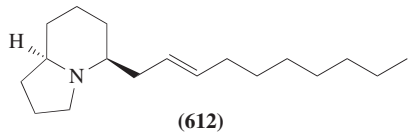
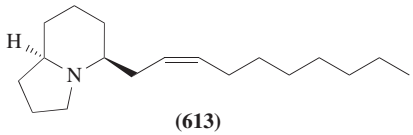
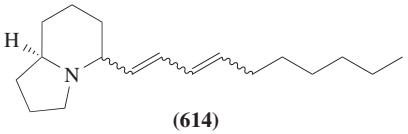


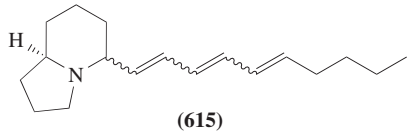
(**612**)



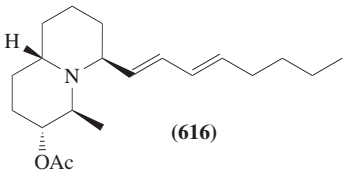
(**613**)



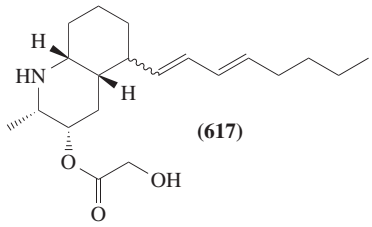
(**614**)



(**615**)

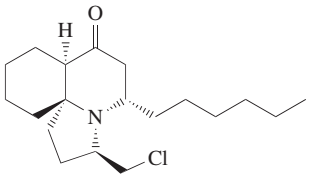


(**616**)

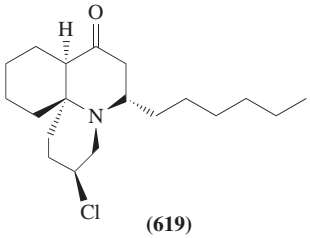


(**617**)

The ascidian *Clavelina cylindrica*, which was collected in Tasmania, yielded two alkaloids, cylindricine A (**618**) and B (**619**).[553] Cylindricine B (**619**) was the first example of the new pyrido-[2,1-*j*]quinoline ring system while cylindricine A (**618**) was the first pyrrolo[2,1-*j*]quinoline known from nature. Single-crystal X-ray studies of both compounds supported the assignment of the structures of the two alkaloids. The absolute configurations were not assigned.



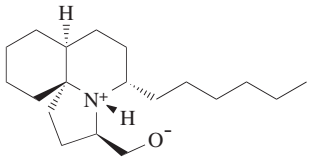
(**618**)



(**619**)

Solutions of either cylindricine A (**618**) or cylindricine B (**619**) both gave, after 6 days, the same equilibrium mixture of 3:2 of (**618**) and (**619**). This process could be followed easily by NMR spectroscopy. The interconversion between (**618**) and (**619**) only occurred when the compounds were present as free bases. Their salts, for example, the picrates, were quite stable in solution at room temperature for several weeks. The interconversion involves the chlorine and the nitrogen participating in a stereospecific ring-opening ring-closing reaction. The reaction, which involves inversion of the nitrogen, may be concerted or involve an aziridinium ion intermediate. The equilibrium mixture of alkaloids (**618**) and (**619**) exhibited bioactivity; it was active in the brine shrimp bioassay causing significant mortality at a level of 3×10^{-2} mmol.

A cytotoxic alkaloid, lepadiformine (**620**), with the same heterocyclic skeleton as (**618**) was isolated from the ascidian *Clavelina lepadiformis*.[554] The structure of (**620**) was established on the basis of chemical properties and by spectroscopic means, including a unique zwitterionic-like moiety. Lepadiformine (**620**) had moderate cytotoxic activity against cell lines of KB, HT29, P388, doxorubicin-resistant P388, and NSCLC-N6 (non-small-cell lung carcinoma). The alkaloid (**620**) also had cycle-dependent and phase-dependent properties. A partial, dose-dependent, G1 phase blockade of the cells was noted after 72 h of growth in a continuous drug exposure experiment. Due to the moderate cytotoxicity and effects on the cell cycle, lepadiformine (**620**) seemed to be of little interest with regard to antitumoral activity. However, the special zwitterionic structure may be of strong interest in terms of biological ''proton-transfer mechanisms'' since it seemed to be a natural example of models described as ''*cis*-decalin amino acid'' derivatives having such important properties.



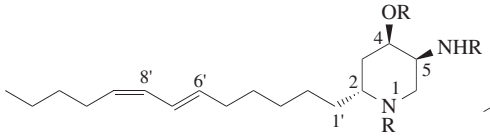
(620)

8.07.9.2.4 Pseudodistomins

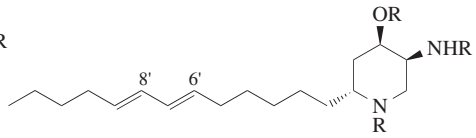
This section describes the work of the authors on the unique piperidine alkaloids, pseudodistomins, from an Okinawan marine tunicate.[555]

In 1986 the authors investigated the bioactive substances from an Okinawan tunicate *Pseudodistoma kanoko*, which is an orange-colored compound tunicate and looks like a strawberry (Japanese name, ''ichigo-boya''). The material was collected off Ie Island, Okinawa, by SCUBA (-5 m to -10 m). The methanol–toluene (3:1) extract of *P. kanoko* was partitioned between toluene and water. The aqueous layer was successfully extracted with chloroform, ethyl acetate, and 1-butanol. By preliminary screening using mammalian muscle preparations, the chloroform-soluble fraction was found to exhibit marked antispasmodic activity on the isolated guinea-pig ileum; the contractile responses to carbachol and histamine were abolished by this fraction. The chloroform-soluble fraction was therefore subjected to bioassay-guided fractionations using silica gel flash column chromatography eluted with $CHCl_3$/*n*-BuOH/H_2O/AcOH (1.5:6:1:1) followed by reversed-phase HPLC separation (Develosil ODS-5, 50% MeCN with 0.1% TFA) to afford an active fraction, which was positive on the ninhydrin-test on TLC. This active fraction was shown to be a mixture of two components (pseudodistomins A (**621**) and B (**622**)), the separation of which was first carried out after converting them into acetates (**623**) and (**624**), respectively, by ODS-HPLC (YMC-Pack, AM) with 88% MeOH. Acetates (**623**) and (**624**) were used for characterization and structural studies. A small amount of (**621**) and (**622**) (before acetylation) was obtained by careful HPLC (Develosil ODS-5) eluting with 37% MeCN with 0.2% TFA for bioassay purposes. In addition to antispasmodic activity, pseudodistomins A (**621**) and B (**622**) exhibited cytotoxic activity against murine leukemia cells, L1210 and L5178Y, *in vitro* (IC_{50} values: 2.5 μg ml^{-1} and 0.4 μg ml^{-1} against L1210, respectively; 2.4 μg ml^{-1} and 0.7 μg ml^{-1} against L5178Y, respectively). Both compounds (**621**) and (**622**) also exhibited calmodulin antagonistic activity; they both inhibited

calmodulin-activated brain phosphodiesterase with IC_{50} values of 3×10^{-5} M, being approximately 3 times more potent than W-7, a well-known synthetic calmodulin antagonist.[556]

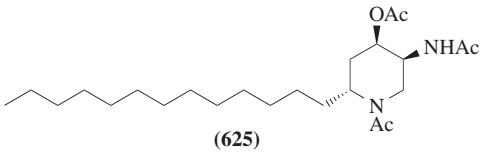


(621) R = H **(623)** R = Ac

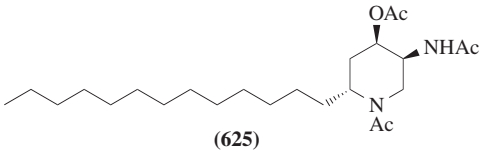


(622) R = H **(624)** R = Ac

Structural studies were mostly carried out using pseudodistomin B acetate (**624**). In the ^{1}H NMR spectrum of the acetate (**624**), several signals appeared broad and split in an approximately 4:1 ratio; this phenomenon might be ascribed to the presence of two slowly interconverting conformations due to the rotation of the secondary amide group. Although the elucidation of the 1D NMR data was not helped by this phenomenon, 2D NMR data of (**624**) with good quality was recorded on a 400 MHz spectrometer, and analysis of the ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY spectral data led to a structure for (**624**) containing a piperidine nucleus and an alkyl side-chain with two *E* double bonds. Spectral data for pseudodistomin A acetate (**623**) suggested that pseudodistomin A possesses the same type of structure with different alkene geometry (one *E* and one *Z*). Catalytic hydrogenation of each of the acetates (**623**) and (**624**) afforded the identical tetrahydro derivative (**625**). The relative stereochemistry of the chiral centers at C-2, C-4, and C-5 was determined by the coupling constants and NOE data using the tetrahydroacetate (**625**) (Figure 17). Although it seemed unfavorable that the alkyl side-chain at C-2 is axially oriented, it was proposed that the conformation shown in Figure 17 may be stabilized by an intramolecular hydrogen bond between the N(5)-H and N(1), which was inferred from the FT IR spectrum of a dilute solution of (**625**). Knapp and Hale, who achieved the synthesis of the tetrahydroacetate (**625**) in 1993,[557] indicated that this hydrogen bond would be highly strained, but they also agreed that the conformation in Figure 17 was preferred to the alternative one because of a steric interaction between the N(1)-acetyl group and the alkyl side-chain, which was also supported by the Macromodel calculation.[557]



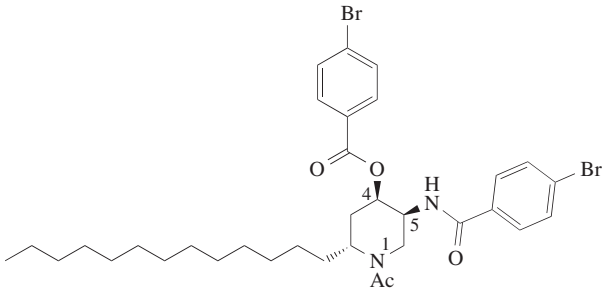
(625)



(625)

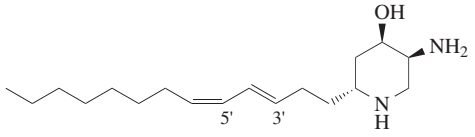
Figure 17 Perspective drawings of (**625**) and (**626**).

The absolute stereochemistry of the C-2, C-4, and C-5 positions was deduced on the basis of the exciton chirality method. The 1-acetyl-4,5-bis(*p*-bromobenzoyl) derivative (**626**) was prepared by partial hydrolysis of (**625**), followed by *p*-bromobenzoylation. The CD spectrum of (**626**) showed a positive Cotton effect, implying the 2*R*, 4*R*, and 5*S*-configurations (Figure 17).

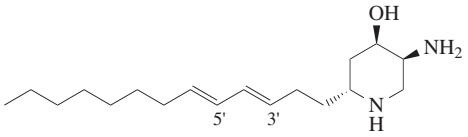


(626)

The authors initially proposed the conjugated diene position in the side-chain of pseudodistomins A and B to be at the 3′,5′-position ((**627**) and (**628**), respectively);[556] this position was, however, later revised to be at the 6′,8′-position ((**621**) and (**622**), respectively) by subsequent studies.

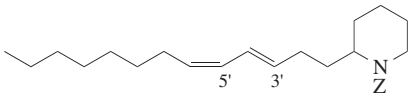


(627)

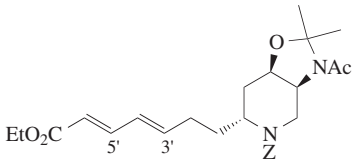


(628)

Following publication of the authors' work on the isolation and structure elucidation of pseudodistomins, these compounds were chosen as a target of organic synthesis by several groups, probably because of their unique bioactivities as well as their interesting structure apparently related to sphinganoids. In 1990, Nakagawa *et al*. reported the synthesis of compound (**629**) as a model for (**627**) (initial structure), and they also prepared an optically active compound (**630**) from L-aspartic acid as a key intermediate.[558]



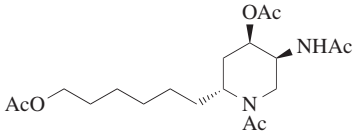
(629)



(630)

Total synthesis of the tetrahydroacetate (**625**) was achieved by three groups. Natsume and co-workers prepared (±)-(**625**) from a 1,2-dihydropyridine derivative through the singlet oxygen addition reaction.[559] Naito *et al*. described the synthesis of (±)-(**625**) via a route involving the reductive photocyclization of enamide and α-acylamino radical allylation,[560] and they afterwards synthesized optically active (+)-(**625**) through the cycloaddition of a nitrone to (+)-2-aminobut-3-en-1-ol.[561] Knapp and Hale, *vide supra*, prepared (+)-(**625**) as the optically active form from D-serine,[557] and the optical rotation of the synthetic sample of (+)-(**625**) corresponded to that of the natural one. From these studies the piperidine ring absolute stereochemistry of pseudodistomins A (**621**) and B (**622**) was unambiguously confirmed as that described initially by the authors.[556]

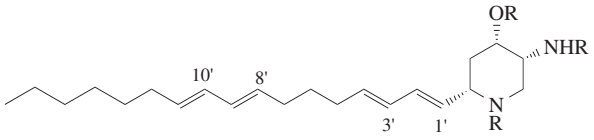
Naito and co-workers, however, questioned the side-chain diene position during their synthesis of (±)-(**625**); they prepared the triacetates of (**628**) (3′,5′-diene; the authors' initial structure for pseudodistomin B), and found that the spectral data of the synthetic triacetate were not superimposable onto those of the natural specimen.[562] The side-chain diene position of pseudodistomin B was therefore reinvestigated by chemical degradation experiments. The triacetate (**624**) prepared from a natural specimen of pseudodistomin B (**622**) was treated with ozone followed by reduction with $NaBH_4$ and acetylation with Ac_2O and pyridine afforded the tetraacetate (**631**), the FABMS of which clearly afforded the $(M+H)^+$ ion at m/z 385, implying the side-chain diene position of pseudodistomin B (**622**) to be at the 6′,8′-position.[562] After being made aware of this result, Naito *et al*. prepared the triacetate with the 6′,8′-diene (**624**), whose spectral data proved to be identical with those of the authentic sample of (**624**).[563]



(631)

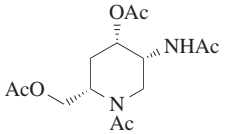
As the result of the above work, the structure of pseudodistomin A also had to be reexamined. Since a natural specimen of pseudodistomin A was unavailable at that time, the extracts of the tunicate *P. kanoko* were reinvestigated. Pseudodistomins A and B were first isolated from the chloroform-soluble fraction obtained by partition experiments. The toluene-soluble fraction, which had been shown to be less active on the antispasmodic activity assay, was reexamined to detect a ninhydrin-positive spot on TLC. The toluene-soluble fraction was therefore separated by the same procedures as above to succeed in reisolating pseudodistomin A as its acetate (**623**). The pseudodistomin A acetate (**623**) thus obtained was subjected to ozonolysis by the same procedures used for pseudodistomin B acetate (**624**) to give the identical product (**631**) on the basis of TLC, ^{1}H NMR, and EIMS (m/z 325, (M—CH_3CONH_2)+) data. Thus, pseudodistomin A was also revealed to have the 6′,8′-diene (**621**), and the 6′*E*,8′*Z*-configuration was deduced from the combination of the HOHAHA spectrum and coupling constant data of the acetate (**623**).[564]

In the course of the reisolation of pseudodistomin A (**621**), the authors also aimed at isolating other new alkaloids since marine aliphatic amino alcohols related to sphinganoids are of interest to many scientists working in a broad range of biological sciences. The ninhydrin-positive fraction was, after acetylation, carefully examined by HPLC (Develosil ODS-5) eluting with 85% MeOH to afford a new piperidine alkaloid, named pseudodistomin C (**632**), as its acetate (**633**). Before acetylation, (**632**) was also obtained by preparative silica gel TLC ($CHCl_3$/MeOH/H_2O, 6:4:0.7), and was revealed to exhibit cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* (IC_{50} values, 2.3 μg ml^{-1} and 2.6 μg ml^{-1}, respectively).[565]

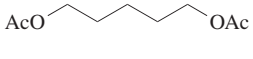


(632) R = H
(633) R = Ac

The ^{1}H NMR spectrum of the acetate (**633**) in $CDCl_3$ showed signals so broad that no signals were able to be assigned, and was quite different from the ^{1}H NMR spectrum of pseudodistomin B acetate (**624**). The 2D NMR experiments (^{1}H-^{1}H COSY, HSQC, and HMBC) of (**633**) were carried out in a CD_3OD solution, which showed relatively resolved signals. Since the ^{1}H NMR spectrum of the natural compound (**632**) in a C_5D_5N solution appeared better in resolution, the ^{1}H-^{1}H COSY and HSQC spectra of (**632**) were recorded in this solution. From these spectral data, pseudodistomin C (**632**) was suggested to consist of a piperidine moiety and an unsaturated side-chain; the piperidine ring has the same substituents (4-hydroxyl and 5-amino groups) as those of pseudodistomins A and B, (**621**) and (**622**), and the side-chain attached to C-2 contains two dienes. The positions of the two dienes were clarified unambiguously by the following degradations. Pseudodistomin C (**632**) was treated with ozone followed by $NaBH_4$ reduction and acetylation to give a crude product, from which the tetraacetate ((**634**), EIMS m/z 315 $(M+H)^+$ and 255 (M—CH_3CONH_2)$^+$) was obtained by HPLC purification, thus revealing one of the two dienes to be located at the 1′,3′-position. The second diene was deduced to be on the 8′,10′-position since 1,5-pentanediol diacetate (**635**) was detected by reversed-phase TLC and HPLC analyses from the crude mixture of the ozonolysis products. These alkenes were inferred to be all *E* from the coupling constants and the ^{13}C chemical shifts of the allylic methylenes.



D-(**634**)

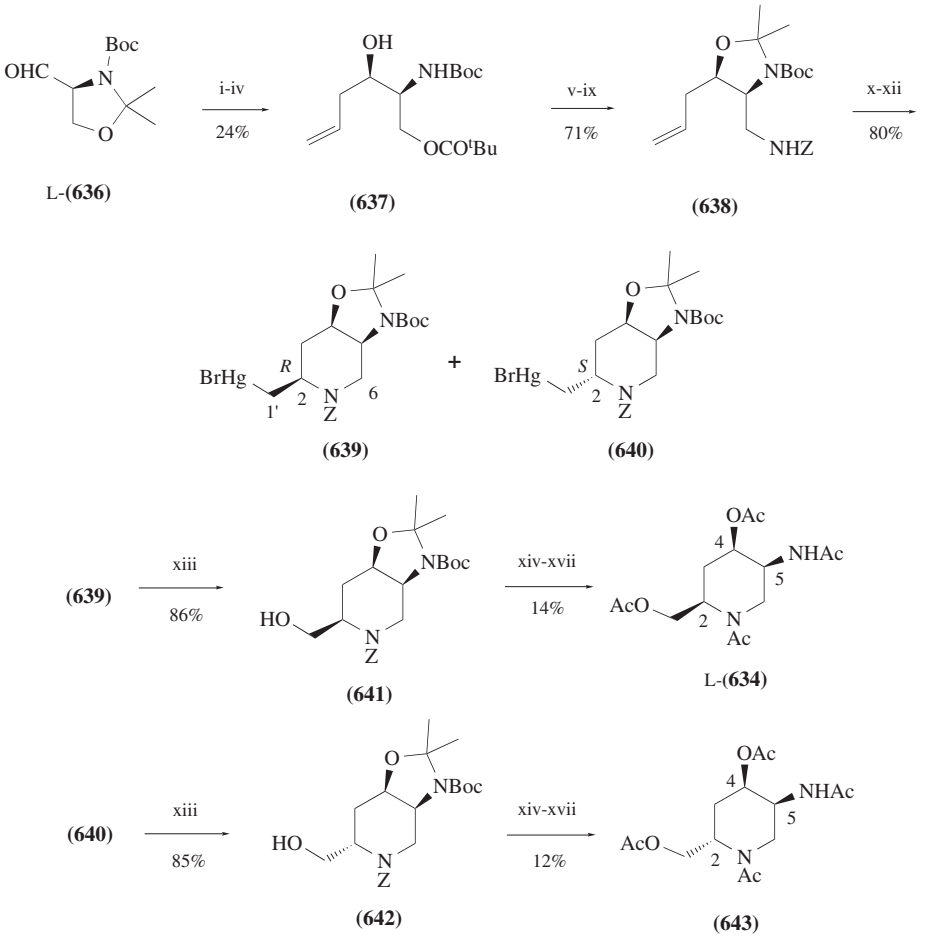


(635)

Since the ^{1}H NMR spectrum of pseudodistomin C acetate (**633**) appeared quite different from that of pseudodistomin B acetate (**624**), stereochemical evidence of the piperidine ring portion of pseudodistomin C (**632**) was required. Thus, the tetraacetate (**634**), which was obtained by ozonolysis of (**632**), was prepared as an optically active form, as shown in Scheme 17. Oxazolidine aldehyde (**636**), prepared from L-serine,[566] was treated with allylmagnesium bromide to give a 1:1 diastereomeric mixture of allyl alcohols. After deprotection of the acetonide group and conversion into pivaloyl ester, the unnecessary *threo*-isomer was removed by silica gel column chromatography. The *erythro*-monopivaloate (**637**) was transformed via five steps into a benzyl carbamate (**638**), which was subjected to amide mercuration to give 2*R*- and 2*S*-piperidine derivatives (**639**) and (**640**) in the ratio of 54:46. Oxidative demercuration of (**639**) and (**640**) gave primary alcohols (**641**) and (**642**), respectively, which were deprotected and acetylated to afford tetraacetates L-(**634**) and (**643**), respectively. The ^{1}H NMR spectrum of the tetraacetate (**634**) obtained from a natural specimen of pseudodistomin C (**632**) was identical with that of the 2*R*,4*R*,5*S*-derivative L-(**634**). Since the tetraacetate (**643**) possesses the same relative configurations at the C-2, C-4, and C-5 positions on the piperidine ring as those of pseudodistomins A (**621**) and B (**622**), the relative configurations of pseudodistomin C (**632**) proved to be different from those of (**621**) and (**622**). The sign of optical rotation of synthetic L-(**634**) ($[\alpha]_D$ -19°) was opposite to that of the tetraacetate (**634**) ($[\alpha]_D$ $+16^\circ$) derived from a natural specimen of (**632**). The absolute configuration of pseudodistomin C (**632**) was therefore revealed as 2*S*, 4*S*, and 5*R*. This result was, however, unexpected since the piperidine alkaloids isolated from the same tunicate possess different stereochemistries at the C-4 and C-5 positions. To obtain further unambiguous confirmation of this conclusion, the enantiomer D-(**634**) was prepared from D-serine by the same procedures as above, and subjected to chiral HPLC analysis (CHIRALPAK AD, Daicel Chemical Ind. Ltd.; 4.6×250 mm; flow rate: 0.5 ml min^{-1}; UV detection at 215 nm; eluent: hexane/2-propanol, 8:2), which established that the tetraacetate (**634**) (t_R 16.5 min) derived from natural specimen (**632**) showed the same retention time as the enantiomer D-(**634**) prepared from D-serine (D-(**634**): t_R 16.5 min; L-(**634**): t_R 15.1 min), thus firmly establishing the 2*S*, 4*S*, and 5*R*-configurations for pseudodistomin C (**632**).[565]

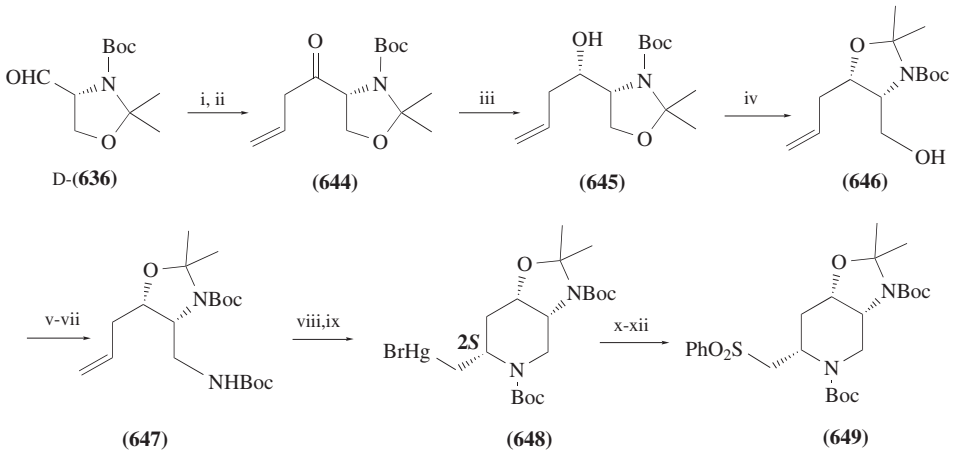
To provide further unambiguous evidence for the whole structure of pseudodistomin C (**632**), the total synthesis of (**632**) was investigated by the authors as follows.[567] The synthesis began with Garner's aldehyde, D-(**636**),[566] derived from D-serine as summarized in Scheme 18. The Grignard reaction of allylmagnesium bromide with D-(**636**) afforded a 1:1 mixture of *erythro*- and *threo*-homoallyl alcohols. To obtain the *erythro*-alcohol (**645**) practically, the diastereomeric mixture was oxidized with Dess–Martin periodinane in DMF to give the ketone (**644**), which was reduced with $Zn(BH_4)_2$ to give *erythro*-alcohol (**645**) in 96% *de* (60% *ee*, *vide infra*). The *erythro*-alcohol (**645**) was transformed into the *t*-butyl carbamate (**647**) via isomeric alcohol (**646**) in seven steps by the previous method;[565] the terminal amine was protected by the Boc group to simplify the deprotection. Amide mercuration of (**647**) with $Hg(OAc)_2$ in $ChCl_3$ afforded (2*S*)-piperidine (**648**) and its (2*S*)-isomer in a ratio of 1.5:1. The (2*S*)-piperidine derivative (**648**) was oxidatively demercurated to give alcohol, which was treated with diphenyl disulfide and tri-*n*-butylphosphine in pyridine followed by oxidation of the sulfide group with diphenyl diselenide and hydrogen peroxide to furnish phenylsulfone (**649**).

The side-chain moiety of (**632**) was prepared as shown in Scheme 19, starting from the known 1-bromo-3*E*,5*E*-decadienoyl bromide (**650**).[568] Condensation of (**650**) and sodium dimethyl malonate afforded dimethyl ester (**651**), which was heated with sodium chloride in wet DMSO at 190 °C to afford the corresponding ester (**652**). Reduction of the ester group of (**652**) with DIBAL in toluene and the Wittig reaction provided ethyl tetradecatrienoate (**653**) with all *E*-configurations revealed from coupling constants. The ester (**653**) was reduced with DIBAL and the resulting alcohol was oxidized with pyridinium chlorochromate (PCC) to afford the corresponding aldehyde, which was subjected to Julia alkenation with the phenylsulfone (**649**). The sulfone (**649**) was treated with *n*-butyllithium in THF in the presence of HMPA at -78 °C to produce the orange sulfone anion, which was allowed to react with the aldehyde obtained from (**653**), and then quenched with benzoyl chloride to afford a diastereomeric mixture of β-benzoyloxy sulfones. Treatment of the crude mixture with sodium amalgam resulted in formation of the tetraene (**654**) possessing the backbone skeleton of pseudodistomin C (**632**). The ^{1}H NMR of (**654**) revealed that the last generated $\Delta^{1',2'}$-double bond was *E* ($J_{1',2'} = 14.0$ Hz), and the HPLC analysis of the tetraene (**654**) using a reversed-phase column showed a single peak predominantly, suggesting that the tetraene (**654**) possesses all *E*-configurations. Removal of the protective groups of (**654**) with 3N HCl afforded pseudodistomin C (**632**), whose ^{1}H NMR and EIMS spectra as well as R_f values on TLC were completely identical with those of the natural specimen.[565] The synthetic pseudodistomin C (**632**) was acetylated with acetic anhydride in pyridine to furnish pseudodistomin C triacetate (**633**), which was also identified



i, CH_2=$CHCH_2MgBr$; ii, *p*-TsOH, MeOH; iii, PivCl, pyridine; iv, SiO_2 column, hexane/EtOAc (3:1); v, DMP, BF_3·OEt; vi, 2.5 N KOH, MeOH; vii, Phthalimide, DIAD, PPh_3; viii, H_2NNH_2·$2H_2O$, EtOH; ix, ZCl, 2N NaOH; x, $Hg(OCOCF_3)_2$, $CHCl_3$; xi, $NaHCO_3$; xii, NaBr; xiii, $NaBH_4$,O_2, DMF; xiv, TFA, CH_2Cl_2; xv, Ac_2O, pyridine; xvi, H_2, Pd/C, EtOH; xvii, Ac_2O, pyridine.

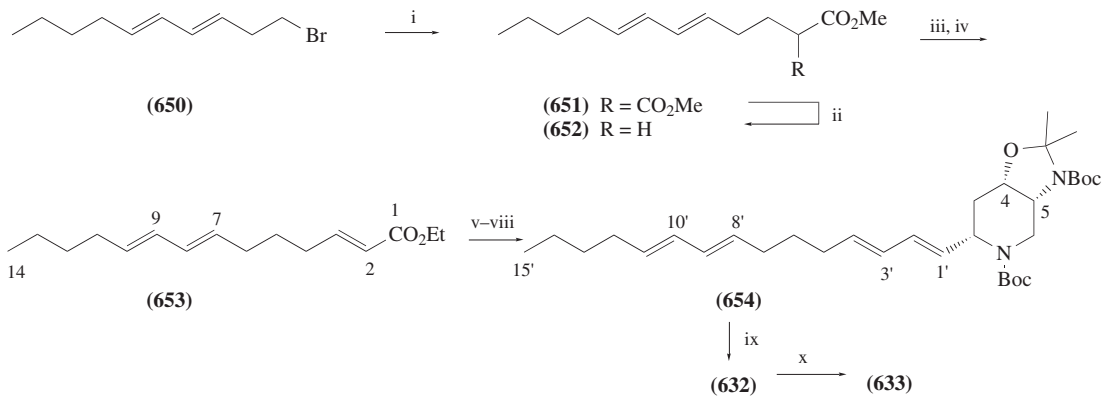
Scheme 17



i, CH_2=$CHCH_2MgBr$ (quant.); ii, Dess–Martin periodinane, DMF (68%); iii, $Zn(BH_4)_2$, benzene-Et_2O (quant.); iv, ref. 2, (4 steps, 56%); v; Phthalimide, DIAD, PPh_3; vi, H_2NNH_2 •H_2O, EtOH; vii, $(Boc)_2O$, 1N NaOH, dioxane (3 steps, 80%); viii, $Hg(OAc)_2$, $CHCl_3$; ix, NaBr, $NaHCO_3$ (2 steps, 56%; 2*R*-isomer, 28%); x, O_2, $NaBH_4$, DMF (90%); xi $(PhS)_2$, $Bu^n{}_3P$, pyridine (79%); xii, Ph_2Se_2, 30% H_2O_2, CH_2Cl_2-Et_2O (73%)

Scheme 18

with the triacetate (**633**)[565] derived from a natural specimen of (**632**) on the basis of ^{1}H NMR and EIMS spectra as well as TLC and HPLC examinations. The sign of the optical rotation of synthetic triacetate (**633**) ($[\alpha]_D$ +43° (*c* 1, $CHCl_3$)) was also the same as that of the natural one ($[\alpha]_D$ +85° (*c* 0.98, $CHCl_3$)).[565]



i, Na, MeOH, $CH_2(CO_2Me)_2$ (74%); ii, NaCl, DMSO, H_2O, 190 °C (74%); iii, DIBAL, toluene; iv, $Ph_3P{=}CHCO_2Et$, CH_2Cl_2 (2 steps, 68%); v, DIBAL, CH_2Cl_2 (80%); vi, PCC, CH_2Cl_2 (74%); vii, Bu^nLi, **649**, THF-HMPA; viii, Na-Hg, MeOH (13% from **649**); ix, 3N HCl, EtOAc (34%); x, Ac_2O, pyridine (56%)

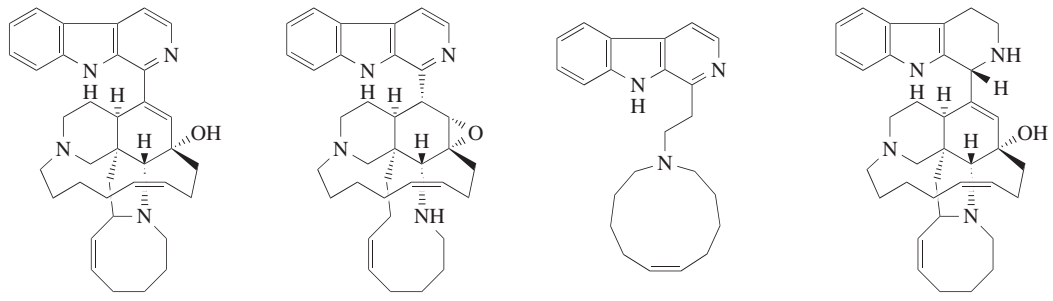
Scheme 19

Thus the further structural confirmation of (**632**) by total synthesis was completed, although the absolute value of the optical rotation of the synthetic triacetate (**633**) was smaller than that of the natural specimen of (**633**). The optical purity of the synthetic compound was examined by means of chiral HPLC analysis after conversion of the corresponding alcohol derived from (**648**) into tetraacetate (**634**), which had been obtained by ozonolysis of (**632**),[565] to reveal that the synthetic tetraacetate (**634**) obtained in this study was 60% *ee*. The optical purity of synthetic (**633**) was estimated to be parallel to this result. This result may be attributable to partial racemization during the oxidation–reduction process to obtain the *erythro*-alcohol (**645**). In the authors' previous study,[565] chiral HPLC had shown that no crucial racemization occurred since the *erythro*-alcohol (**645**) and its *threo*-isomer were separated after conversion into monopivaloyl esters ((**637**) and its isomer) by four repeated silica gel chromatographies.

8.07.9.3 Sponge Metabolites

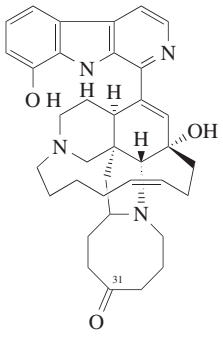
8.07.9.3.1 Manzamines and related alkaloids

In 1968 Higa and co-workers isolated a novel cytotoxic β-carboline alkaloid, named manzamine A (**655**), from a marine sponge *Haliclona* sp. collected off Manzamo, Okinawa, and the structure including absolute configuration was established by X-ray analysis.[569] In the following year the isolation of manzamines B (**656**), C (**657**), and D (**658**) from the *Haliclona* sponge was reported.[570,571]



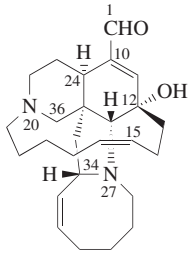
(**655**) (**656**) (**657**) (**658**)

At almost the same time Kobayashi and co-workers independently isolated keramamines A and B (=manzamines A (**655**) and F (**659**), respectively) from an Okinawan marine sponge *Pellina* sp.[572] These unusual ring systems have attracted great interest as challenging targets for total synthesis and for their unprecedented biosynthetic path, to be resolved.

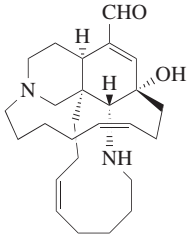


(659)

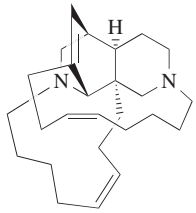
In 1992 Baldwin and Whitehead proposed a biogenetic path for manzamines A–C (**655**)–(**657**), as shown in Scheme 20.[573] The proposal suggested that *bis*-3-alkyldihydropyridine macrocycle (a), which can be derived from ammonia, a C_3 unit, and a C_{10} unit, might be converted through a Diels–Alder-type [4+2] intramolecular cycloaddition into a pentacyclic intermediate (b), which in turn led to manzamines A (**655**) and B (**656**) via a tetracyclic intermediate (c). Although Baldwin and Whitehead's biogenetic path was elegant and fascinated many chemists, this proposal was only a hypothesis without any experimental basis. In the same year Kobayashi and co-workers isolated two novel alkaloids, ircinals A (**660**) and B (**661**) from an *Ircinia* sponge,[574] which are very close to intermediate (c). Subsequently, from an *Amphimedon* sponge, they obtained some novel manzamine-related alkaloids, keramaphidin B[575] (**662**), which is quite similar to intermediate (b); keramaphidin C[576] (**663**) and keramamine C[576] (**664**), which seem to be biogenetic precursors of manzamine C (**657**); and ircinols A (**665**) and B[577] (**666**), which correspond to antipodes of the alcohol forms of ircinals A (**660**) and B (**661**).



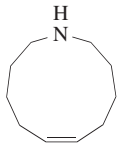
(660)



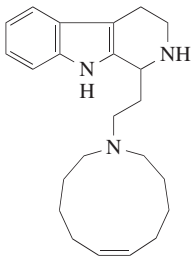
(661)



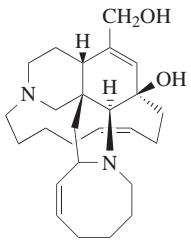
(662)



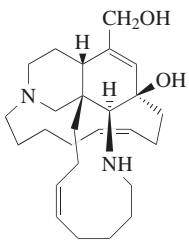
(663)



(664)

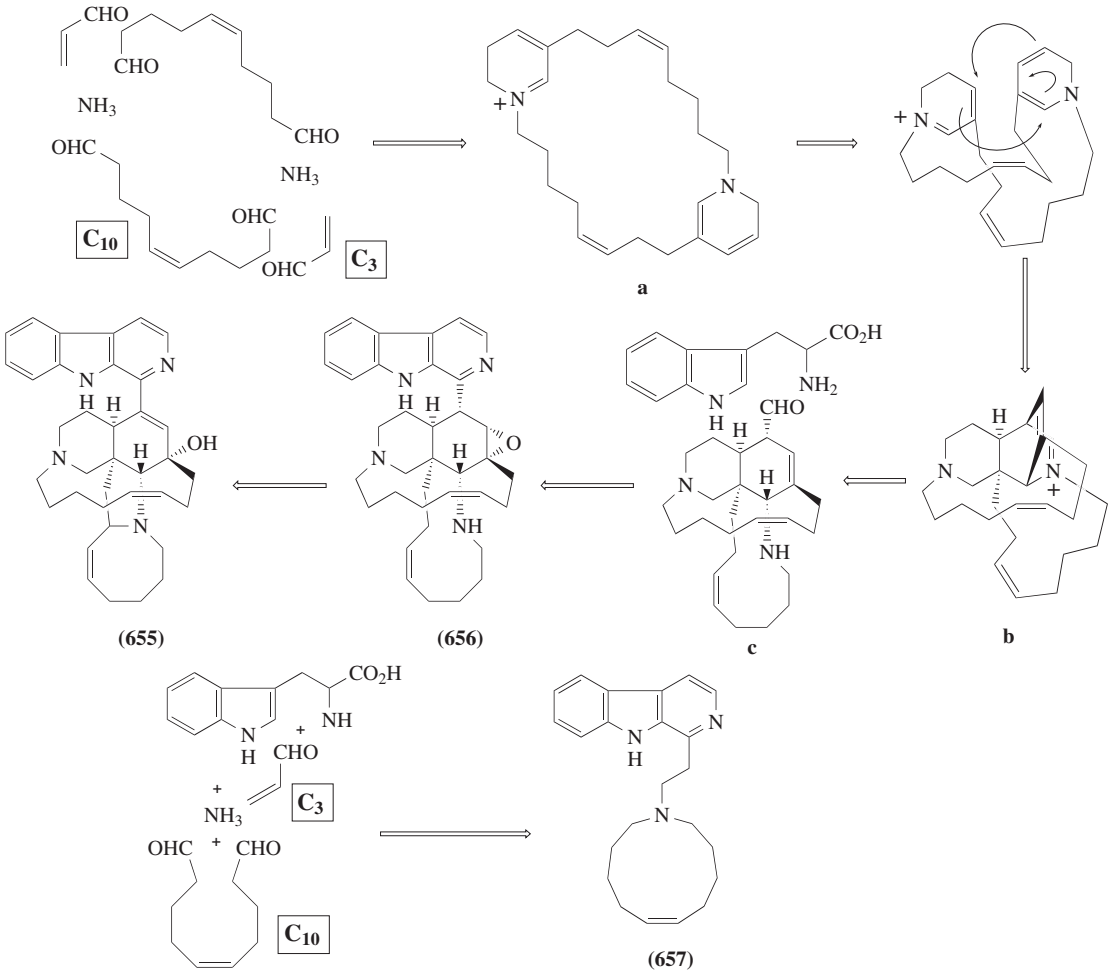


(665)



(666)

The absolute configuration of ircinal A (**660**) was confirmed by the chemical correlation with manzamine A (**655**) (Scheme 21).[574] The Pictet–Spengler cyclization of (**660**) with tryptamine afforded manzamine D (**658**), which was then transformed into manzamine A (**655**) through ddq oxidation. The structure of ircinal B (**661**) was established by the chemical correlation with



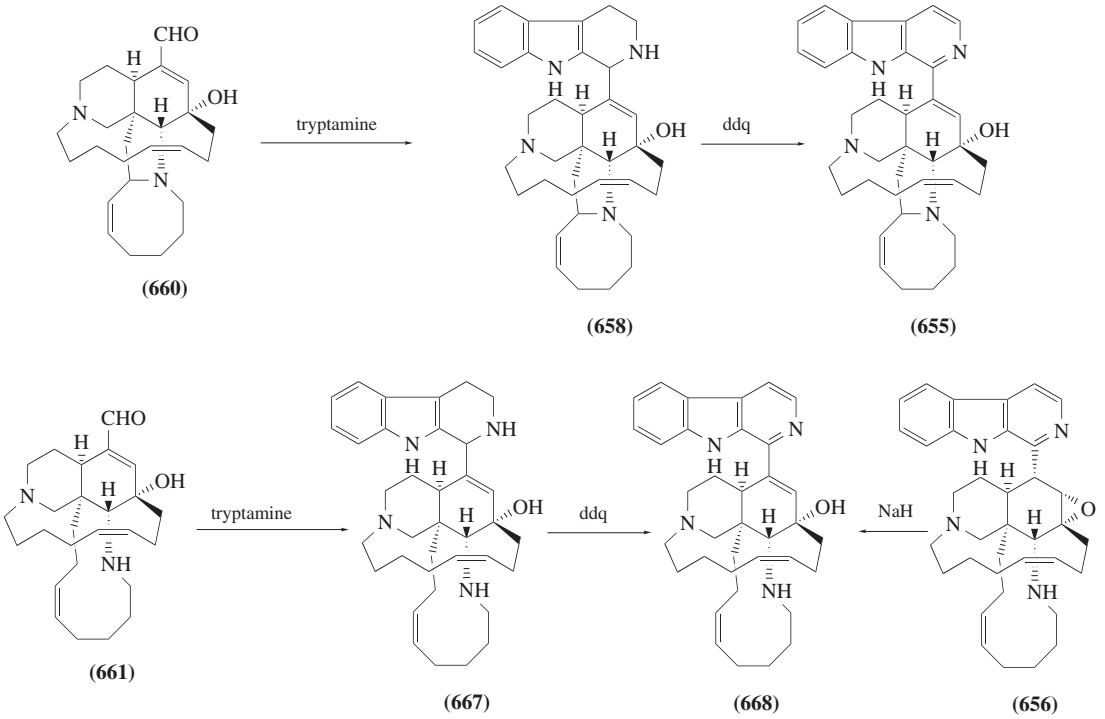
Scheme 20

manzamine B (**656**), as shown in Scheme 21. The Pictet–Spengler cyclization of (**656**) with tryptamine afforded manzamine H (**667**), which was then transformed into manzamine J (**668**) through ddq oxidation. On the other hand, treatment of manzamine B (**656**) with NaH also gave manzamine J (**668**). Manzamines A (**655**) and B (**656**) may be biogenetically generated from ircinals A (**660**) and B (**661**) through the Pictet–Spengler reaction with tryptamine or tryptophan followed by dehydrogenation, as shown in Scheme 21.

The planar structure of keramaphidin B (**662**), $C_{26}H_{40}N_2$, was elucidated by detailed analyses of ^1H and ^{13}C NMR data aided by 2D NMR experiments. The relative stereostructure was established by X-ray analysis using a suitable crystal of (**662**) grown in acetonitrile. Interestingly, the X-ray study revealed that the crystal of (**662**) was racemic though it possesses four asymmetric centers. The structure of keramaphidin B (**662**) corresponds to that of the pentacyclic intermediate (b) proposed by Baldwin and Whitehead.

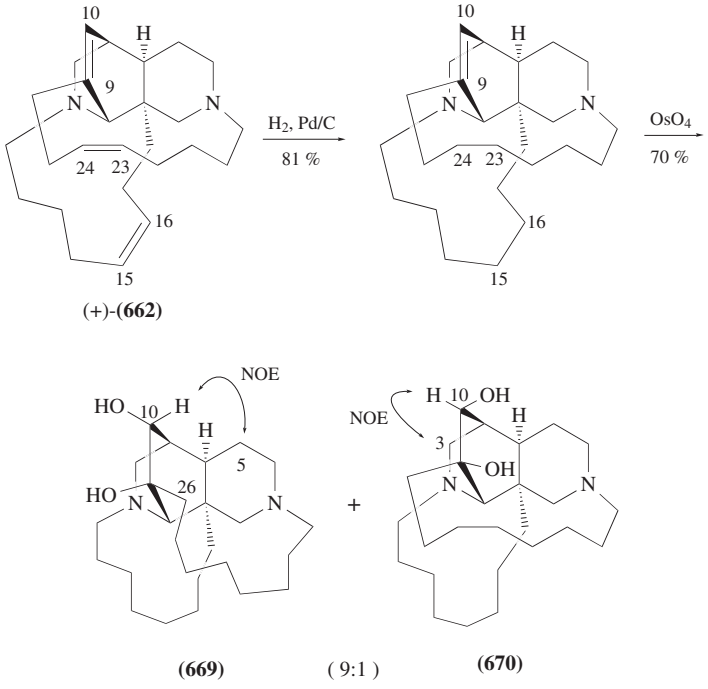
Chiral HPLC analyses of the crystals and the filtrate of keramaphidin B (**662**) revealed that the ratio of (+)- and (−)-forms was ca. 1:1 for the crystals and 20:1 for the filtrate, respectively. On the other hand, the ratio of the crystal and the filtrate of keramaphidin B (**662**) was 5:95. These results suggested that (+)-keramaphidin B {(+)-(**662**)} was estimated to be ca. 97% of this sponge.[578]

(+)-Keramaphidin B ((+)-(**662**)) was subjected to reduction with Pd/C, and then oxidation with OsO_4 to give two dihydroxy products (**669**) and (**670**) in the ratio of ca. 9:1 (Scheme 22). The relative stereochemistry at C-9 and C-10 of (**669**) and (**670**) was elucidated from NOE data. Treatment of (**669**) with (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPACl) gave the (*S*)- and (*R*)-MTPA esters, respectively. The ^1H chemical shift differences ($\Delta\delta = \delta_S - \delta_R$) indicated that the absolute configuration at C-10 of (**669**) was *R*. Thus the absolute



Scheme 21

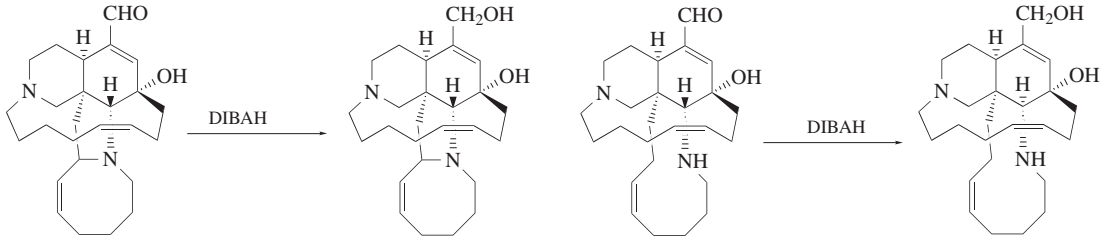
configurations at C-1, C-4, C-4a, and C-8a of (+)-keramaphidin B ((+)-(**662**)) were concluded to be *R*, *S*, *R*, and *S*, respectively.[579]



Scheme 22

Ircinol A (**665**) corresponded to the alcoholic form of (**660**). Treatment of ircinal A (**660**) with DIBAH afforded a reduction product (**671**) (Scheme 23), the spectral data of which were identical with those of ircinol A (**665**). However, the sign of the optical rotation was opposite ((**671**), $[\alpha]_D^{18}$

+20° (*c* 0.2, MeOH); (**664**), $[\alpha]_D^{18}$ −19° (*c* 0.5, MeOH)). This result revealed that ircinol A (**665**) was an enantiomer of the alcoholic form at C-1 of ircinal A (**660**), which has been shown to have the same absolute configuration as that of manzamine A (**655**).

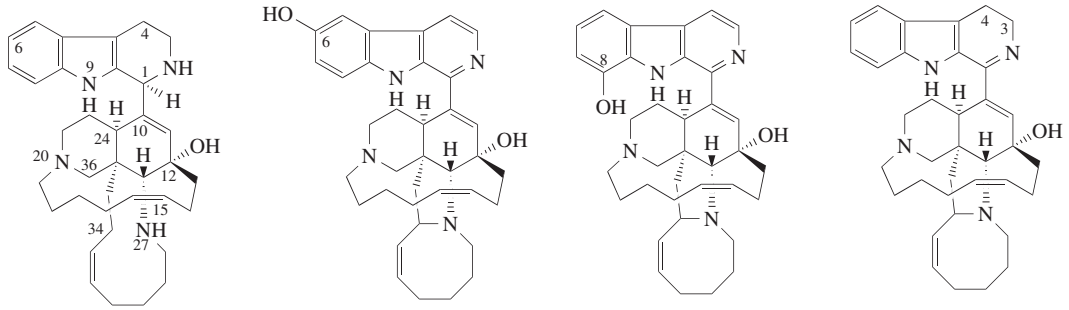


(660) **(671)** **(661)** **(672)**

Scheme 23

Reduction of ircinal B (**661**) with DIBAH yielded a reduction product (**672**) (Scheme 23), which showed identical spectral data to those of ircinol B (**666**) but the opposite sign of optical rotation ((**672**), $[\alpha]_D^{18}$ +4.2° (*c* 0.2, MeOH); (**666**), $[\alpha]_D^{18}$ −2.8° (*c* 0.12, MeOH)). Thus the absolute stereochemistry of ircinol B was concluded to be as shown in Structure (**666**).

Manzamine L[578] (**673**) was isolated from the fractions containing manzamines D (**658**) and H (**667**). The spectral data of manzamine L (**673**) were similar to those of manzamine H (**667**), and the structure of (**673**) was elucidated to be a stereoisomer at C-1 of (**667**) on the basis of conversion of (**673**) with ddq into manzamine J (**668**). The absolute configurations at C-1 of manzamines D (**658**), H (**667**), and L (**673**) were deduced from the CD data and the molecular mechanics calculations using Macro Model version 5.0 to be *R*, *R*, and *S*, respectively. Interestingly, the 1*S*-isomer of manzamine D (**658**) has not been isolated from the *Amphimedon* or *Ircinia* sponge, but from the latter ircinals A (**660**) and B (**661**) and manzamines H (**667**) and J (**668**) were obtained. 6-Hydroxymanzamine A[580] (=manzamine Y[581] (**674**)) is the first manzamine congener with a hydroxyl group at C-6, although 8-hydroxyl analogues of manzamines such as manzamine F[582] (**659**) and 8-hydroxymanzamine A[583] (=manzamine G[584] (**675**)) have been reported previously. On the other hand, 3,4-dihydromanzamine A[580] (**676**) is the first 3,4-dihydro analogue of manzamines and is easily converted into manzamine A (**655**) by daylight.



(673) **(674)** **(675)** **(676)**

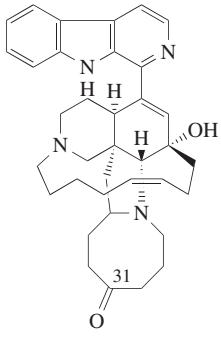
The *Amphimedon* sponge also contains several manzamine-related alkaloids with both dextrorotation and laevorotation. The minor enantiomer of keramaphidin B ((−)-(**662**)) is considered to possess the same configuration as most of the manzamine-related alkaloids with dextrorotation represented by manzamines A (**655**) and B (**656**), while the major enantiomer ((+)-(**662**)) may be correlated to those with laevorotation like ircinols A (**665**) and B (**666**). Altogether the ratio of dextrorotatory vs. laevorotatory alkaloids in this sponge was found to be about 15:1. On the other hand, the ratio of (−)- and (+)-form of keramaphidin B (**662**) was 3:97.

A plausible biogenetic path of manzamines A (**655**) and B (**656**) is shown in Scheme 24, which was elucidated on the basis of the structures and ratios of all the manzamine-related alkaloids isolated from this sponge. Baldwin and Whitehead's *bis*-3-alkyldihydropyridine (a) might be a biogenetic precursor of both enantiomers of keramaphidin B (**662**). The 2,3-iminium form of the

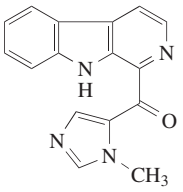
(−)-enantiomer ((−)-(**662**)) may be hydrolyzed to generate (+)-ircinals A (**660**) or B (**661**), which are probably converted through Pictet–Spengler cyclization with tryptamine into manzamines D (**658**), H (**667**), and L (**673**), respectively, and then dehydrogenated to manzamines A (**655**) and B (**656**), respectively. On the other hand, the (+)-enantiomer ((+)-(**662**)) may be associated with some antipodes of ircinals and manzamines such as ircinols A (**665**) and B (**666**).

Isolation of keramaphidin C (**663**) and keramamine C (**664**) together with manzamine C (**657**) and tryptamine seems to substantiate partly the biogenetic path of manzamine C (**657**), which may be derived from coupling of keramaphidin C (**663**) with tryptamine and a C_3 unit via keramamine C (**664**) (Scheme 25). On the other hand, keramaphidin C (**663**) is probably generated from a C_{10} unit and ammonia.

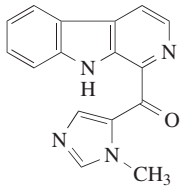
Many manzamines and related alkaloids have been reported from several marine sponges; manzamines E (**677**) and F (**659**) from an Okinawan sponge *Xespongia* sp.;[582] 8-hydroxymanzamine A (=manzamine G (**676**)) from an Indonesian sponge *Pachipellina* sp.;[583] xestomanzamines A (**678**) and B (**679**) and manzamines X (**680**) and Y (=6-hydroxymanzamine A) from Okinawan sponges *Xestospongia* and *Haliclona* sp.,[581] respectively; 1,2,3,4-tetrahydro-8-hydroxymanzamine A (**681**) and its 2-*N*-methyl derivative (**682**) from Papua New Guinean sponges *Petrosia* and *Cribochalina* sp.;[585] and 6-deoxymanzamine X (**683**) and the three 1-*n*-oxide derivatives (**684**)–(**686**) from a Philippine sponge *Xestospongia ashimorica*.[586] Unique β-carboline alkaloids, xestomanzamines A



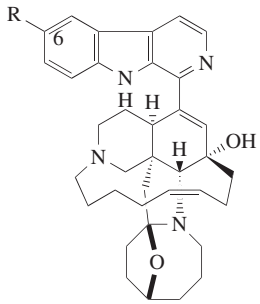
(**677**)



(**678**)

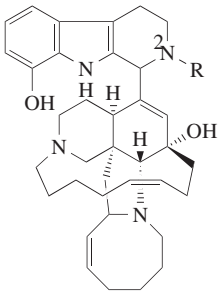


(**679**)



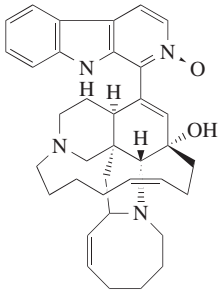
(**680**) R = OH

(**683**) R = H

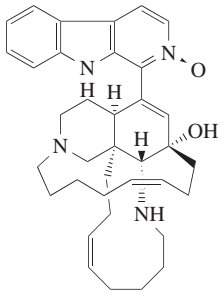


(**681**) R = H

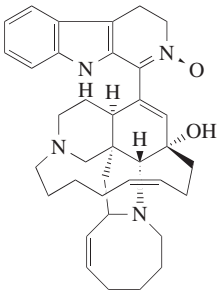
(**682**) R = Me



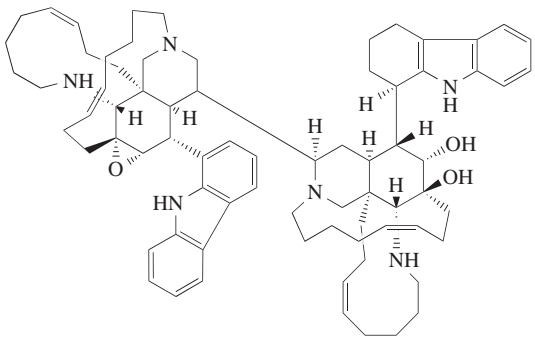
(**684**)



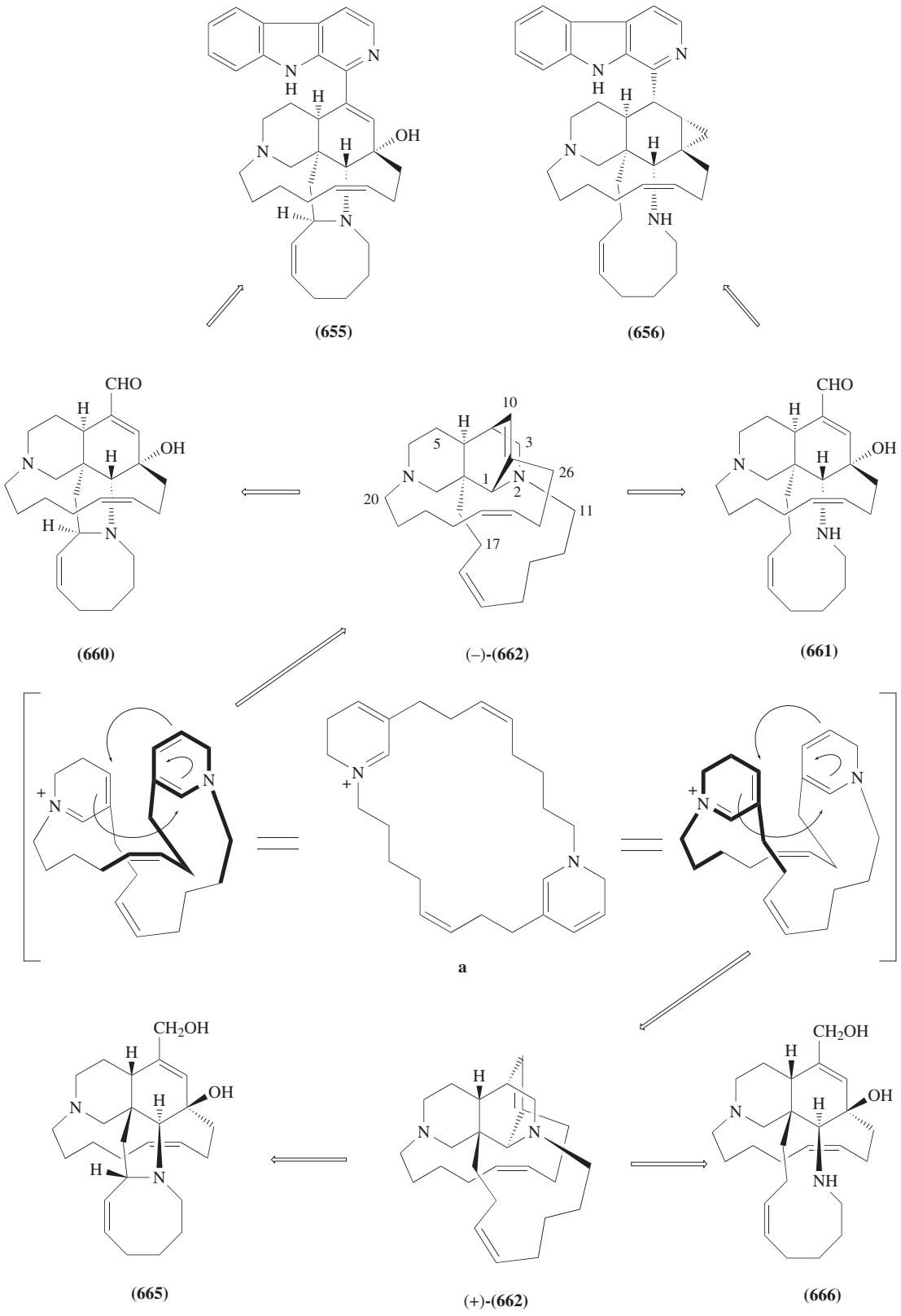
(**685**)



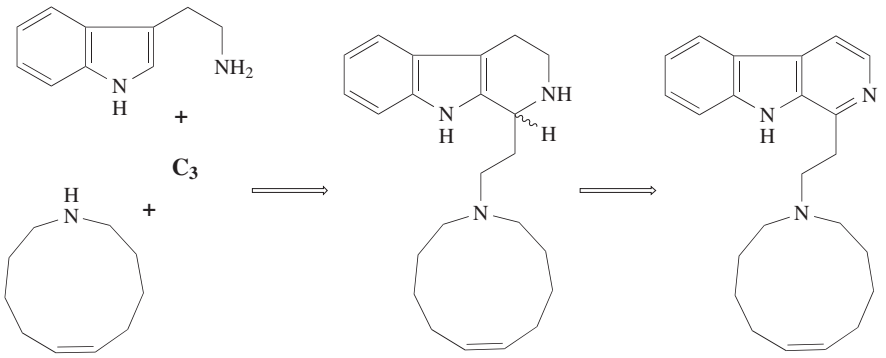
(**686**)



(**687**)



Scheme 24



(663) (664) (657)

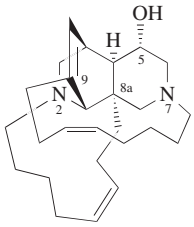
Scheme 25

(**678**) and B (**679**) may be biogenetically derived from *N*-methylhistidine and tryptamine. More recently, Scheuer and co-workers isolated the first manzamine dimer, named kauluamine (**687**), from an Indonesian sponge *Prianos* sp.[587]

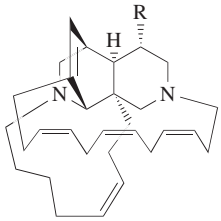
Several alkaloids similar to keramaphidin B (**662**) were reported from two groups independently just before or after isolation of (**662**). Andersen and co-workers isolated eight pentacyclic alkaloids, named ingenamine (**688**),[588] ingamines A (**689**) and B (**690**),[589] and ingenamines B–F (**691**)–(**695**),[590] from the sponge *Xestospongia ingens* collected in Papua New Guinea. Crews and co-workers reported two new alkaloids, xestocyclamines A (**696**)[591] and B (**697**),[592] from a Papua New Guinean sponge *Xestospongia* sp. Ingenamine (**688**) corresponds to the 5-hydroxyl form of keramaphidin B (**662**). The skeletons of ingamines A (**689**) and B (**690**) differ from those of ingenamine (**688**) and keramaphidin B (**662**) in having a C_{12} alkyl chain between N-7 and C-9 in place of the C_8 alkyl chain in (**688**) and (**662**). Ingenamines B (**691**), C (**692**), and D (**693**) have a C_9 or C_{10} alkyl chain between N-7 and C-9, while ingenamines E (**694**) and F (**695**) possess a twelve-carbon alkyl chain between N-2 and C-8a. If keramaphidin B (**662**) and ingenamine (**688**) are generated from a pair of symmetrical dialdehydes (C_{10} unit), C_3 units, and ammonia as Baldwin suggested,[573] it could be that the macrocyclic rings of ingamines A (**689**) and B (**690**) and ingenamines B–F (**691**)–(**695**) are derived from a C_{11}, C_{12}, or C_{14} dialdehyde unit in addition to a C_{10} dialdehyde unit. The absolute configurations of ingenamine (**688**), ingamine A (**689**), and ingenamine E (**694**), which were determined by application of a modified Mosher method at the hydroxyl group on C-6,[590] were the same as those of keramaphidin B (**662**). Xestocyclamine A (**696**) was revised to be the Δ_{14} isomer of (**688**) by reassignment of its 2D NMR data, although the initial structure of xestocyclamine A was elucidated to be (**698**). The structure of xestocyclamine B (**697**) corresponds to the 15,16-dihydro form of ingenamine D (**693**).

Some alkaloids from marine sponges, which are reminiscent of Baldwin's bisdihydropyridine macrocycle (a), have been reported. In 1989 Fusetani *et al*. isolated new bistetrahydropyridine alkaloids, haliclamines A (**699**) and B (**700**) as cytotoxic constituents from a sponge *Haliclona* sp. collected from the Uwa Sea, Japan.[593] The structures consisted of two tetrahydropyridines linked through C_9 and C_{12} alkyl chains. Cyclostellettamines A–F[594] (**701**)–(**706**), isolated from the hydrophilic extracts of the sponge *Stelletta maxima* collected off the Sata Peninsula, Shikoku, Japan, were macrocyclic bispyridines linked through C_{12}–C_{14} alkyl chains.

Unique bis-quinolizidine alkaloids, petrosin (**707**)[595] and petrosins A (**708**)[596,597] and B (**709**)[596] were isolated from the Papua New Guinean sponge *Petrosia serita*, and the C_2-symmetrical structure of petrosin (**707**) was established by X-ray analysis, while petrosin A (**708**) was elucidated as mesomeric.[597] In 1984 Nakagawa *et al*. reported isolation of novel macrocyclic 1-oxa-quinolizidine alkaloids, xestospongins A–D (**710**)–(**713**), from the Australian sponge *Xestospongia exigua*, and the structures were established on the basis of X-ray analysis of xestospongin C (**712**).[598] In 1989 Kitagawa and co-workers isolated 10 new alkaloids, araguspongines B–H and J[599] (**714**)–(**721**) and aragupetrosine A (**722**),[600] together with petrosin (**709**) and petrosin A (**710**) from an Okinawan sponge *Xestospongia* sp. Araguspongines are bis-1-oxa-quinolizidine alkaloids, while aragupetrosine A (**721**), having a quinolizidine and a 1-oxa-quinolizidine ring is a hybrid of petrosin (**707**) and araguspongin F (**717**). Araguspongines F (**718**), G (**719**), H (**720**), and J (**721**) were obtained as optically pure compounds, while araguspongines B (**714**), D (**716**), and E (**717**) were isolated as enantiomeric mixtures or mesomeric compounds. In 1986 an interesting biogenetic path for petrosin

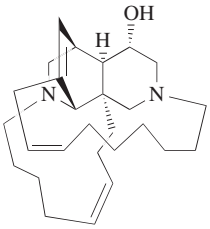


(688)

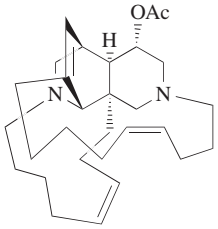


(689) R = OH

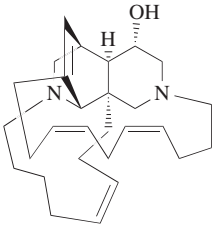
(690) R = H



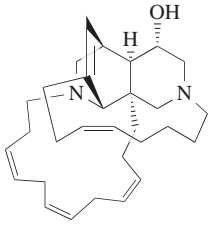
(691)



(692)

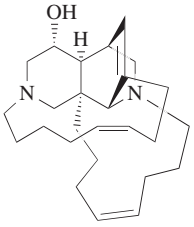


(693)

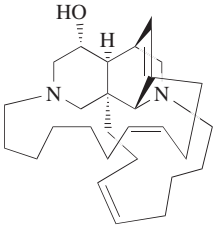


(694) R = OH

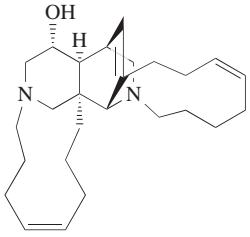
(695) R = H



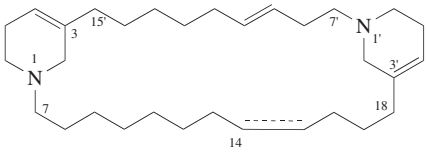
(696)



(697)

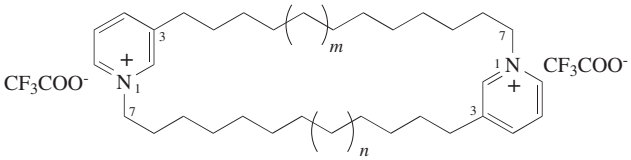


(698)



(699)

(700) :Δ^{14}



(701) $m = 1, n = 1$

(702) $m = 1, n = 2$

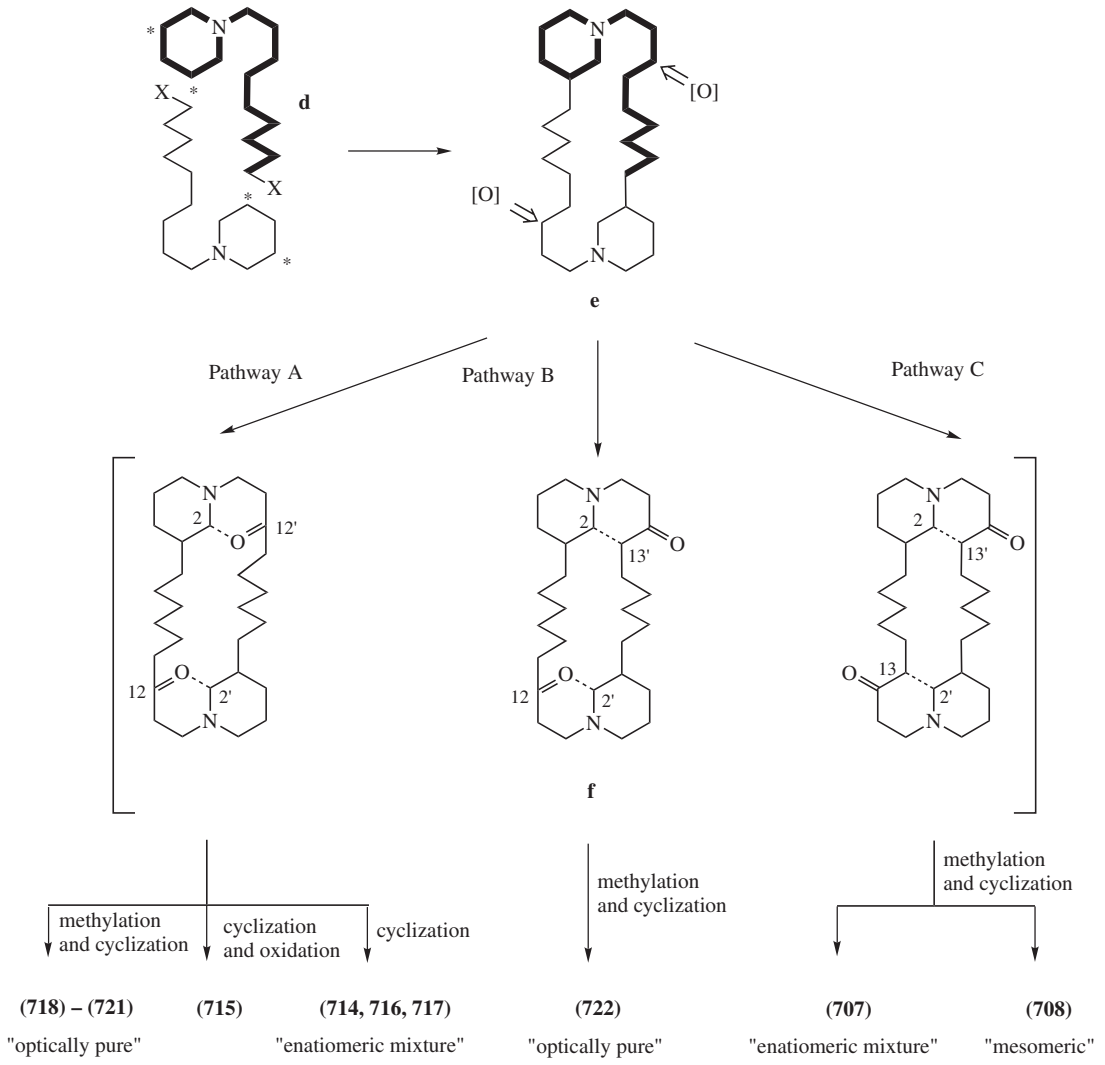
(703) $m = 2, n = 2$

(704) $m = 1, n = 3$

(705) $m = 2, n = 3$

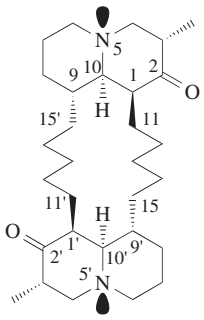
(706) $m = 3, n = 3$

(**707**), petrosin A (**708**), and xestospongin A–D (**710**)–(**713**) was proposed by Cimino *et al.*,[601] although those alkaloids were isolated from different genera of marine sponges. On the other hand, Kitagawa *et al.* indicated the biogenetic relationship between bisquinolizidine alkaloids and bis-1-oxa-quinolizidine alkaloids.[599,600] The biogenetic path is shown in Scheme 26; dimerization of two C_9-C_5N units (d) gives the macrocycle (e) followed by oxidation of C-12 and C-12′ to afford the diketone compound (f). Araguspongines B–H and J (**714**)–(**720**) may be generated from (e) via pathway A. On the other hand, aragupetrosine A (**722**) and petrosins (**707**) and (**708**) were probably generated via pathway B and C, respectively. Both precursors, (a) in Schemes 20 and 24 and (e) in Scheme 26 are similar to haliclamines A (**699**) and B (**700**) and cyclostellettamines A–F (**701**)–(**706**). Demethylxestospongin B (**723**) has been isolated together with xestospongins B (**708**) and D (**710**) and araguspongin F (**718**) from a New Caledonian sponge *Xestospongia* sp.[602]

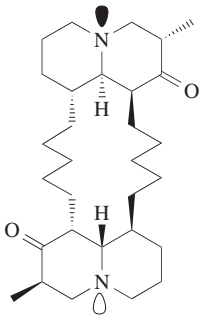


Scheme 26

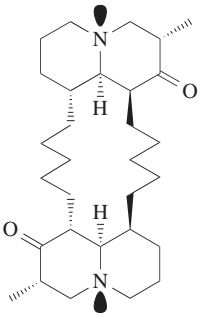
The metabolic pattern of the Mediterranean sponge *Reniera sarai* is characterized by the presence of a series of unique complex polycyclic alkaloids. Cimino *et al.* reported the isolation of unprecedented alkaloids, sarains 1–3 (**724**)–(**726**), isosarains 1–3 (**727**)–(**729**), and saraines A–C (**730**)–(**732**).[601,603–608] Sarains 1–3 (**724**)–(**726**)[601,608] possessed a *trans*-quinolizidine moiety linked to an unsaturated pyperidine ring directly and by two linear alkyl chains, while isosarains 1–3 (**727**)–(**729**),[604,606,608] obtained as minor constituents of the sponge, were isomers at C-1, C-2, and C-9 of sarains 1–3 (**724**)–(**726**), respectively. Saraine A (**730**), the structure of which was assigned on the



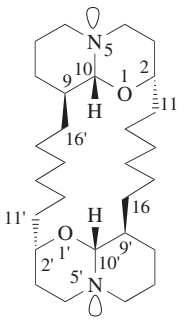
(707)



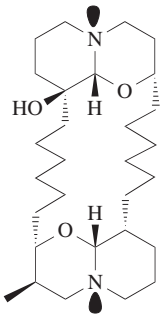
(708)



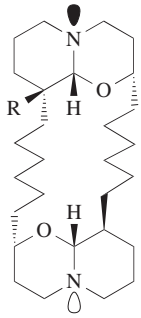
(709)



(710)

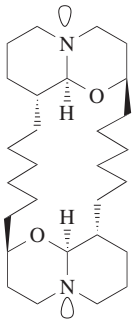


(711)



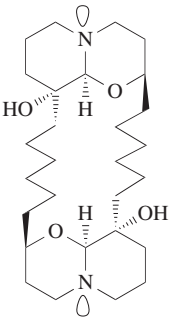
(712) R = H

(713) R = OH

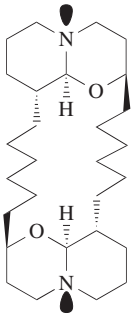


(+)-**(714)**

(+)-**(714)**:(–)-**(714)** 1:1

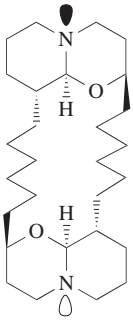


(715)



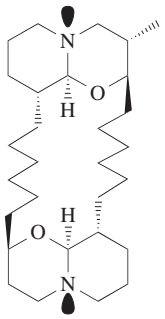
(–)-**(716)**

(+)-**(716)**:(–)-**(716)** 3:7

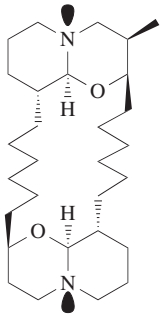


(+)-**(717)**

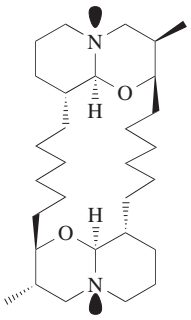
(+)-**(717)**:(–)-**(717)** 3:2



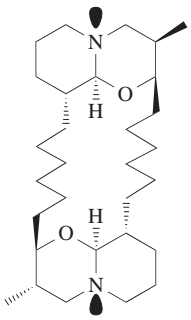
(718)



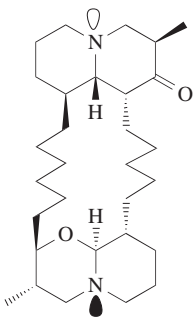
(719)



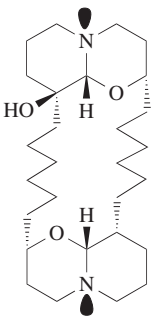
(720)



(721)

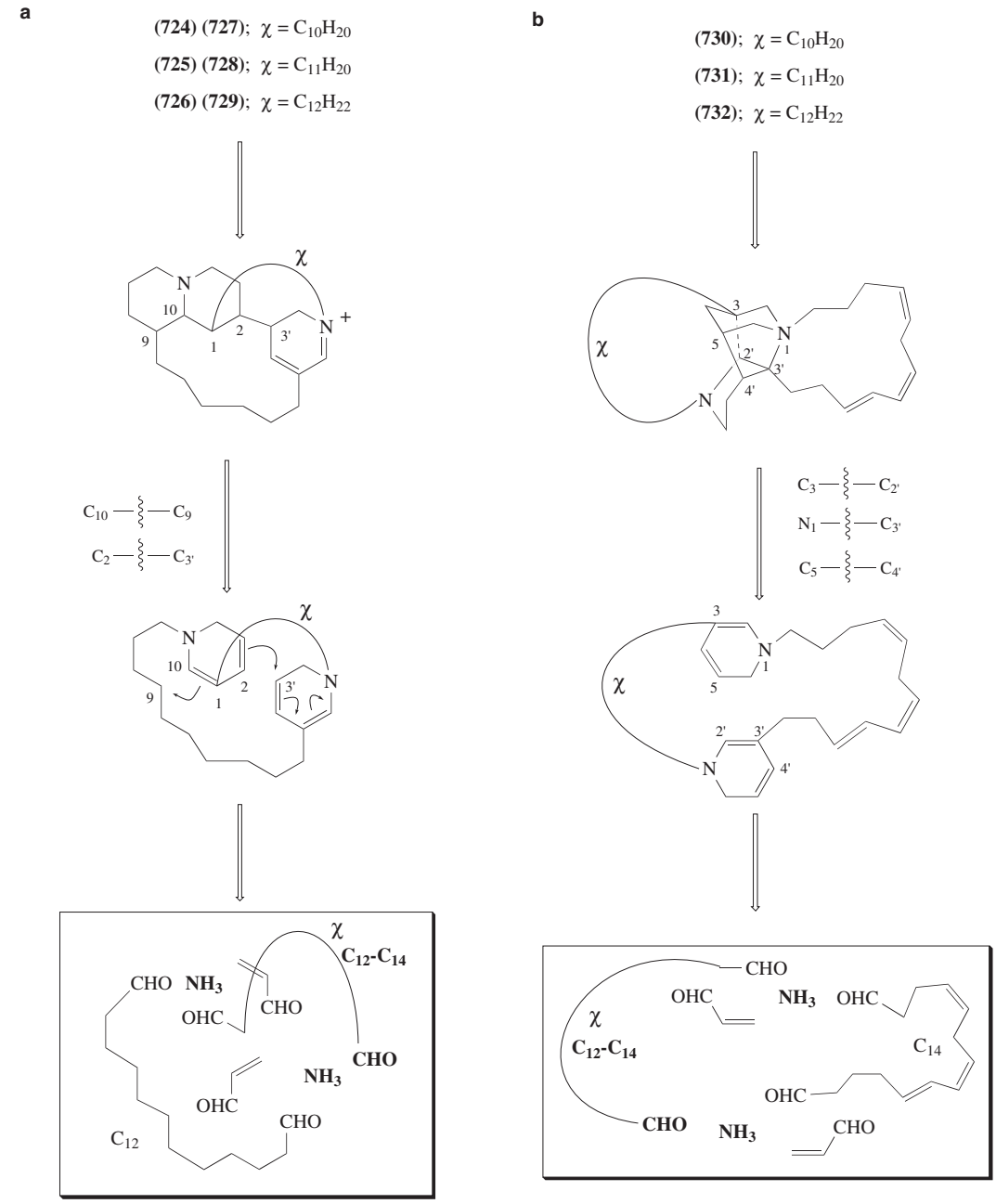


(722)



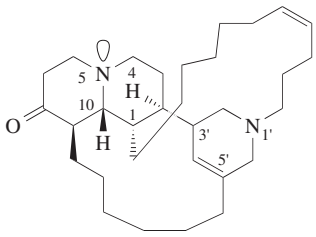
(723)

basis of combination of X-ray study of the acetyl derivative of (**730**)[603] and detailed spectral studies of (**730**) itself,[605] had a unique pentacyclic core with two macrocyclic rings. More recently structures of saraines B (**731**) and C (**732**) and the absolute stereochemistry of (**730**)–(**732**) were determined.[607] The biogenetic path of sarain-related alkaloids (**724**)–(**732**) proposed by Cimino *et al*. is analogous to Baldwin's proposal for manzamines (Scheme 21). The retro-biosynthesis of sarains 1–3 (**724**)–(**726**) and isosarains 1–3 (**727**)–(**729**) (Scheme 27(a)) proceeds through a partially reduced *bis*-3-alkylpyridine macrocycle which contains 10 carbons in an alkyl chain and 10, 11, or 12 carbons in another alkyl chain (χ). These macrocycles form sarains and isosarains through some intramolecular reaction.[608] On the other hand, macrocycles for saraines A–C (**730**)–(**732**) contain 12 carbons in an alkyl chain and 10, 11, or 12 carbons in another alkyl chain (χ) (Scheme 27(b)).[607]

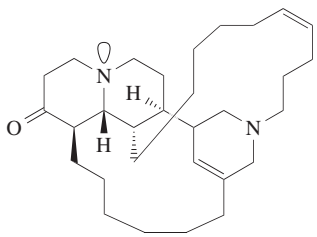


Scheme 27

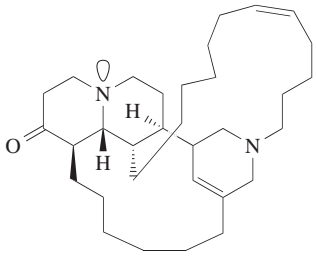
Papuamine[609] (**733**) and haliclonadiamine[610] (**734**) were isolated from *Haliclona* sponges collected off Papua New Guinea and Palau, respectively. The structure of (**733**) was elucidated mainly by



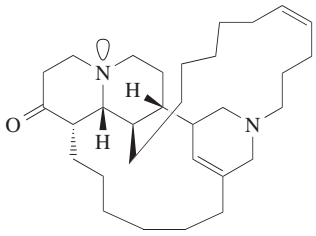
(724)



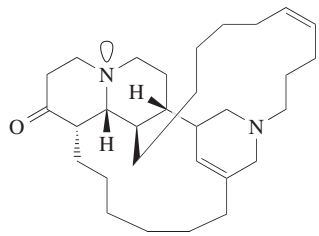
(725)



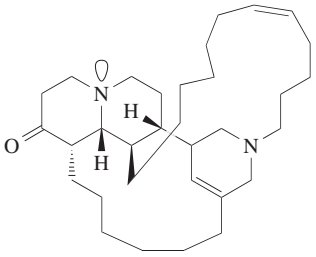
(726)



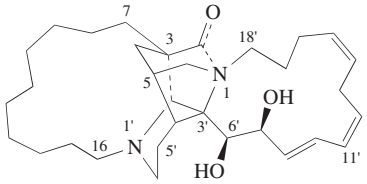
(727)



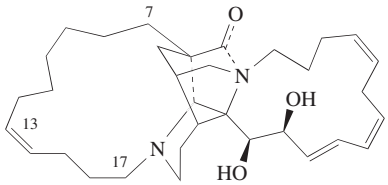
(728)



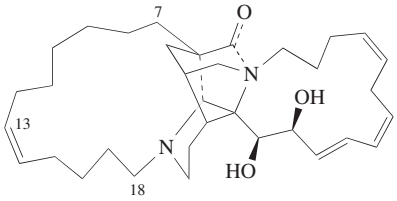
(729)



(730)

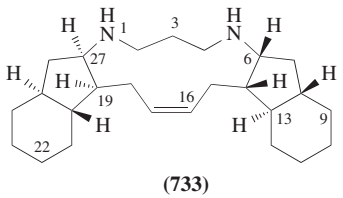


(731)

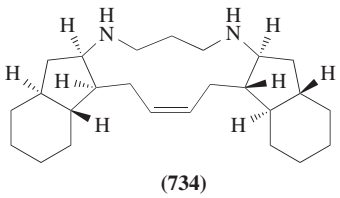


(732)

NMR data including INADEQUATE studies, while the structure of (**734**) was established by X-ray analysis of its diacetyl derivative. Haliclonadiamine (**734**) is an unsymmetrical diastereomer of papuamine (**733**). Crews *et al.* proposed the biogenetical and taxonomic consideration of marine diamine-containing alkaloids, in which they suggested that papuamine (**733**) and haliclonadiamine (**734**) may be formed by condensation of ammonia, acrolein, and acyclic aldehyde, which are Baldwin's biosynthetic building blocks of manzamines.[585]

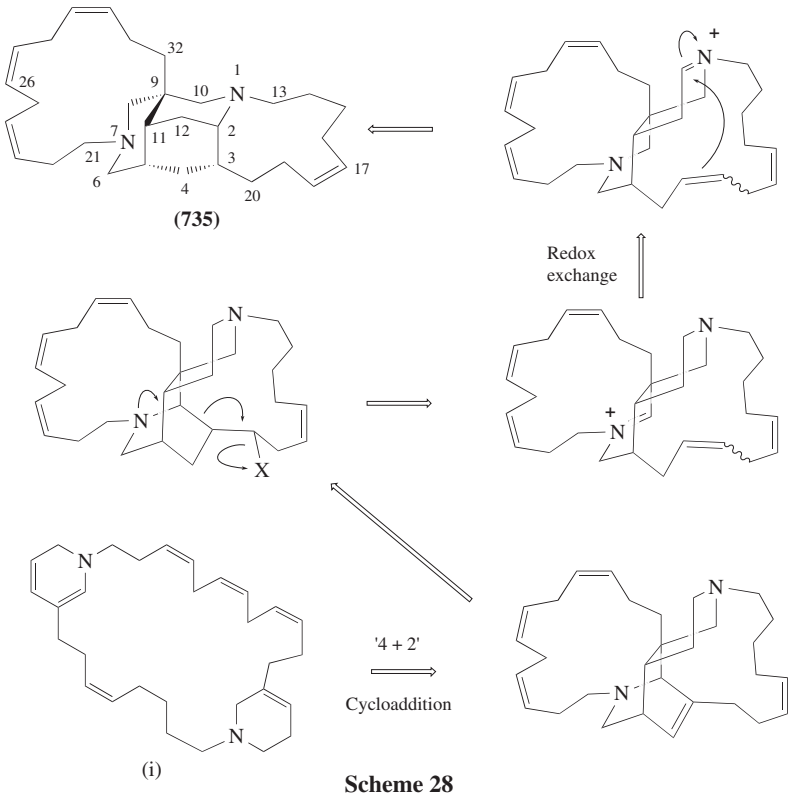


(733)



(734)

An example of a novel class of pentacyclic alkaloid, madangamine A[611] (**735**), was isolated by Andersen and co-workers from the Papua New Guinean sponge *Xestospongia ingens*, from which ingenamine (**688**) and ingamines A (**689**) and B (**690**) were also isolated. The proposed biogenesis for madangamine A (**735**) is outlined in Scheme 28, which partly resembles Baldwin's proposal for the biogenesis of manzamines. The ingenamine-type intermediate [= ingenamine F (**694**)], which may be generated from a bis-3-alkylpyridine macrocycle (i) similar to (a) (Scheme 20) through a "4+2" cycloaddition reaction, can undergo rearrangement to generate the madangamine A skeleton.

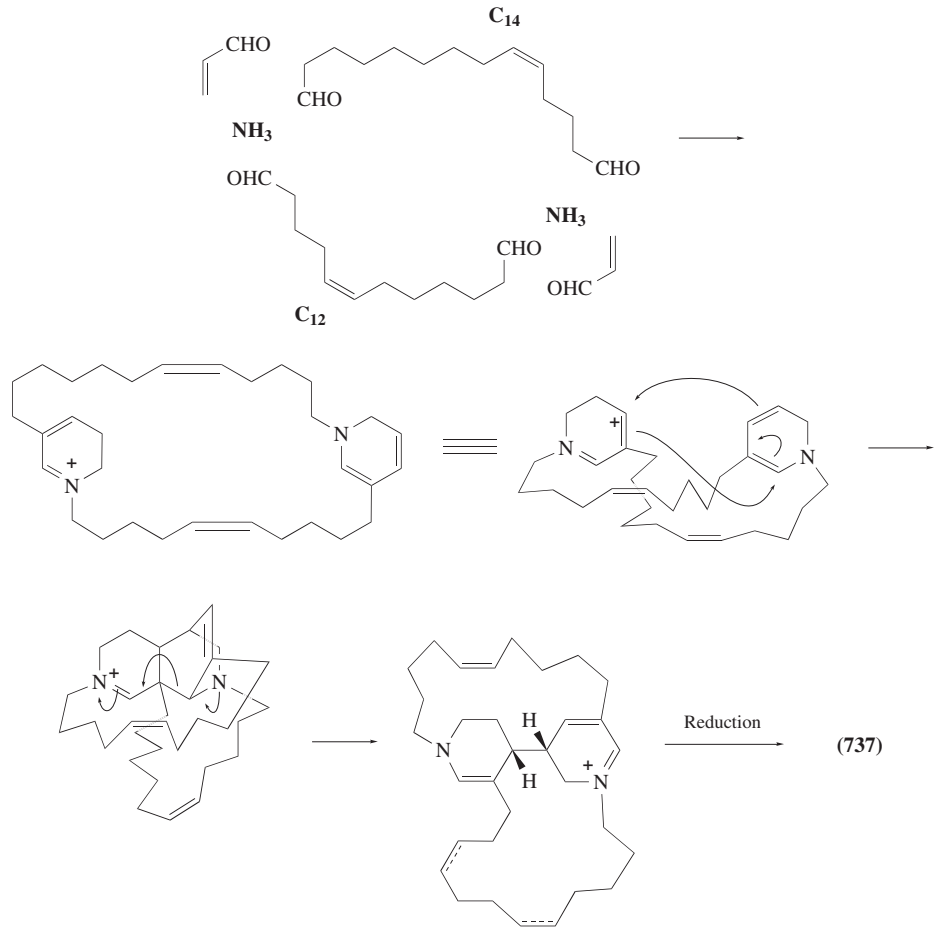


Scheme 28

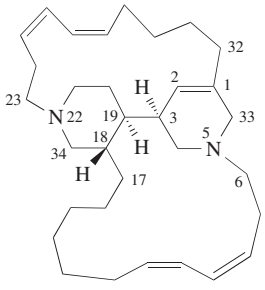
Crews and co-workers isolated a novel tetracyclic diamine alkaloid, named halicyclamine A (**736**), from an Indonesian sponge *Haliclona* sp., and elucidated the structure on the basis of spectroscopic data.[612] More recently isolation of haliclonacyclamines A (**737**) and B (**738**) from a *Haliclona* sponge collected off the Great Barrier Reef was reported by Garson and co-workers, and the structure of (**739**) was assigned by X-ray analysis.[613] The relative stereochemistry at C-2 in halicyclamine A (**736**) was different from those of haliclonacyclamines A (**737**) and B (**738**). Crews and co-workers also reported the isolation of halicyclamine B (**739**) from an Indonesian *Xestospongia* sponge and its structural determination based on X-ray analysis.[614] The biogenetic path of these tetracyclic alkaloids is proposed by Garson and co-workers (Scheme 29); cleavage of the C-1—C-8a bond of Baldwin's pentacyclic intermediate (b) analogue followed by reduction may give halicyclamine A (**738**), while Crews and co-workers proposed that halicyclamine B (**739**) was probably derived from a homologue of (**736**) via 1,3-sigmatropic shift.

The taxonomy of sponge materials containing manzamine-related alkaloids is shown in Table 22. This result indicates that all the alkaloids described in this review are obtained mainly from sponges belonging to the two orders Haplosclerida and Nepheliospongida except for sponges of genera *Ircinia*, *Prianos*, and *Stelletta*. In particular, the *Xestospongia* and *Haliclona* sponges are rich sources of manzamine-related alkaloids.

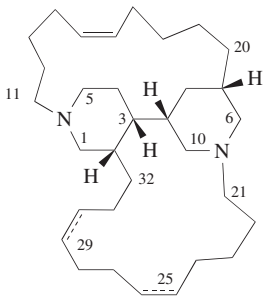
Manzamines and related alkaloids are very interesting marine sponge-derived metabolites possessing unusual ring systems which are challenging targets for total synthesis and for unprecedented biogenesis studies. Further progress in extensive studies on isolation, structure elucidation, biogenesis, total synthesis, and bioactivity is expected for these miracle compounds.



Scheme 29

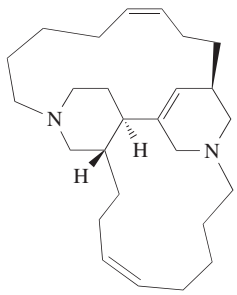


(736)



(737) : Δ^{25}

(738) : Δ^{29}



(739)

8.07.9.3.2 Metabolites of the genus Theonella

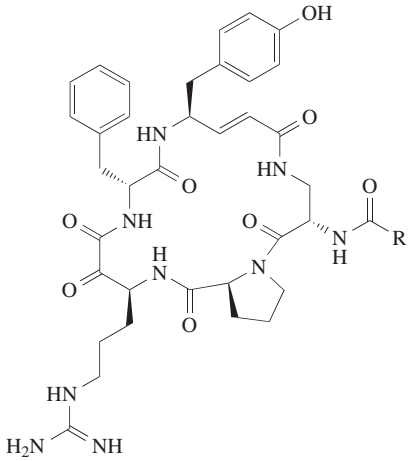
Marine sponges of the genus *Theonella* frequently afford a variety of interesting secondary metabolites including unusual cyclic peptides[615] as well as polyoxygenated aliphatic compounds,[616] most of which exhibit significant biological activity. This section deals with several natural products isolated from marine sponges of the genus *Theonella*.

Table 22 Taxonomy of sponges containing manzamine-related alkaloids.

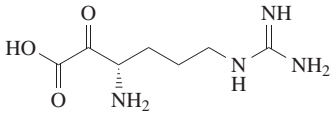
Order	*Family*	*Genus*	*Compounds*	*Ref.*
Subclass, Tetractinomorpha				
Chroristida	Stelletidae	*Stelletta*	(**701**)–(**706**)	593
Subclass, Ceractinomorpha				
Dictyoceratida	Thorectidae	*Ircinia*	(**655**), (**656**), (**658**)–(**660**), (**666**), (**667**)	574
Nepheliospongida	Oceanapiidae	*Pellina*	(**655**), (**656**)	572
		Pachypellina	(**655**), (**675**)	583
Nepheliospongiidae	*Petrosia*		(**707**), (**708**), (**709**)	595, 596
			(**681**), (**682**)	585
		Xestospongia	(**659**), (**675**), (**677**)	582, 584
			(**655**), (**675**), (**677**)–(**680**)	581
			(**710**)–(**713**)	598
			(**707**), (**708**), (**714**)–(**722**)	599, 600
			(**706**), (**708**), (**716**), (**723**)	601
			(**696**), (**697**)	591, 592
			(**662**), (**688**)–(**695**), (**735**)	588–590, 611
			(**739**)	612
Haplosclerida	Niphatidae	*Cribochalina*	(**682**)	586
		Amphimedon	(**655**)–(**658**), (**660**)–(**667**), (**673**)–(**676**)	575–578, 580
	Chalinidae	*Haliclona*	(**655**)–(**658**)	569–571
			(**699**), (**700**)	593
			(**655**)–(**657**), (**674**)	581
			(**733**)	609
			(**733**), (**734**)	610
			(**736**)	612
			(**737**), (**738**)	613
		Reneira	(**724**)–(**732**)	602–604, 607, 608
Poecilosclerida	Desmacidonidae	*Prianos*	(**687**)	587

Fusetani and his research group, during their screening program of Japanese marine invertebrates for potential biomedicals, found that a marine sponge of the genus *Theonella* inhibited various proteinases, particularly thrombin. The sponge was collected off Hachijo-jima Island, 300 km south of Tokyo, and was characterized by a brilliant yellow inner body which was different from that of another *Theonella* sponge containing theonellamides.[617] Two active substances, named cyclotheonamide A (**740**) and B (**741**) were isolated.[618] Cyclotheonamide A (**740**) was isolated from the 1987 collection by means of solvent partitioning, gel filtration, and three successive reverse-phase HPLCs, whereas cyclotheonamide B (**741**) was isolated from the 1989 collection. ^{1}H and ^{13}C NMR spectra indicated that cyclotheonamide A (**740**) contained proline, phenylalanine, 2,3-diaminoproprionic acid (Dpr), and two unusual amino acid residues: α-ketoarginine (K-Arg (**742**)) was an analogue of arginine with a carbonyl group between the carboxyl and methine carbons; the other one, V-Tyr (**743**), was a tyrosine analogue with an ethylene unit between the carboxyl and methine carbons. The α-amino group of Dpr was formylated. The sequence of the five residues was deduced by a combination of HMBC and NOESY experiments. Cyclotheonamide B (**741**) has an acetamide instead of the formylamide in (**740**). The stereochemistry of the amino acids except (**742**) was deduced by chiral GC analysis after degradation. Cyclotheonamide A (**740**) inhibited thrombin with IC_{50} 0.076 μg ml^{-1}. The stereochemistry of V-Tyr residue (**743**) was reassigned as having *S*-configuration during the study of total synthesis of (**741**) by Hagihara and Schreiber.[619] The K-Arg residue (**742**) was also revealed to possess *S*-configuration; this site was not defined in the original studies.

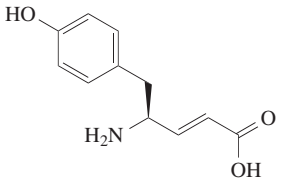
Further investigation of the extract of the marine sponges *Theonella swinhoei* led to isolation of three other analogues, cyclotheonamides C (**744**), D (**745**), and E (**746**).[620] Compounds (**744**) and (**745**) were isolated from *T. swinhoei* with a bright yellow interior from which (**740**) and (**741**) were obtained previously, while (**746**) was isolated from *T. swinhoei* with a white interior. Cyclotheonamides C (**744**), D (**745**), and E (**746**) inhibited thrombin with IC_{50} of 8.4, 8.2, and 28 nM, respectively, while they were inhibitory against trypsin with IC_{50} of 7.4, 63, and 370 mM, respectively. Cyclotheonamide C (**744**), in which the vinylogous tyrosine unit was replaced by a dehydrovinylogous tyrosine unit, showed thrombin-inhibiting activity comparable to that of cyclotheonamide A (**740**). Cyclotheonamide E (**746**), which included D-Ile and phenylacetyl-Ala amide



(740) R = H
(741) R = Me



(742)

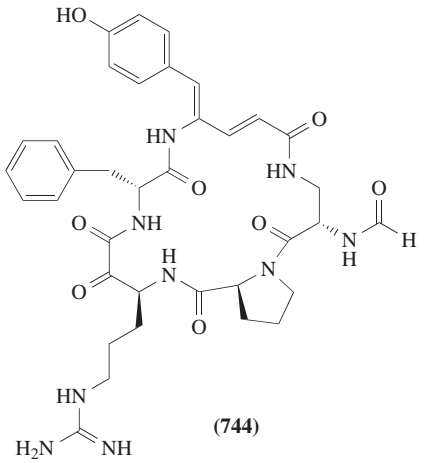


(743)

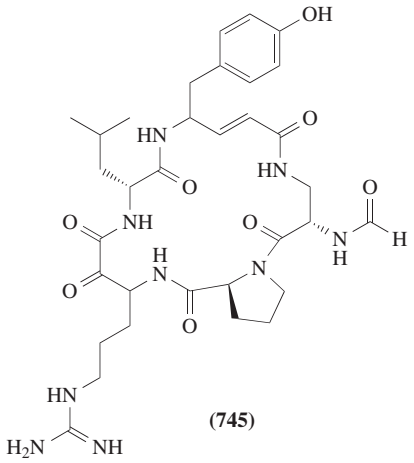
groups in place of D-Phe and formyl groups in (**740**), was 20-fold less active. Interestingly, cyclotheonamide A (**740**) was not detected in the white variety of *T. swinhoei*. Cyclotheonamide B (**741**) was present only in the 1990 collection of the yellow variety and was never encountered thereafter. These observations may indicate the involvement of microbial symbionts in the synthesis of cyclotheonamides.

The unique structural features of cyclotheonamides in combination with their serine-protease inhibiting properties have motivated many researchers to study their total synthesis and mechanisms of actions. Total syntheses of cyclotheonamides have been accomplished by the research groups of Schreiber,[619] Maryanoff,[621] Wipf,[622] Shioiri,[623,624] and Ottenheijm.[625] Maryanoff *et al*. studied X-ray crystallography of a complex of cyclotheonamide A (**740**) with human α-thrombin, a protease central to the bioregulation of thrombosis and hemostasis.[621] This work (2.3 Å resolution) confirmed the structure of cyclotheonamide A (**740**) and revealed intimate details about its molecular recognition within the enzyme active site. Interactions due to the ''Pro-Arg motif'' (Arg occupancy of the S_1 specificity pocket; formation of a hydrogen-bonded two-strand antiparallel β-sheet with Ser^{214}-Gly^{216}) and the α-keto amide group of (**740**) were primarily responsible for binding to thrombin, with the α-keto amide serving as a transition-state analogue. A special interaction with the ''insertion loop'' of thrombin (Tyr^{60A}-Thr^{60I}) was manifested through engagement of the hydroxyphenyl group of (**740**) with Trp^{60D} as part of an ''aromatic stacking chain.'' Biochemical inhibition data (K_i values at 37 °C) were obtained for (**740**) with thrombin and a diverse collection of serine proteases. Thus, (**740**) was just a moderate inhibitor of human α-thrombin ($K_i = 0.18\ \mu\ M^{-1}$) but a potent inhibitor of trypsin ($K_i = 0.023\ \mu M$) and streptokinase ($K_i = 0.035\ \mu M$). Maryanoff *et al*. also achieved total synthesis of cyclotheonamide A (**740**) and prepared several analogues (**747**)–(**751**) of (**740**) by a convergent synthetic protocol[626] in which a late-stage primary amine group was available for substitution. The ^{13}C NMR spectrum of (**740**) in D_2O showed virtually exclusive population by the hydrated form of the α-keto amide (*gem*-diol structure). They also prepared cyclotheonamide B (**741**) through an analogous transformation. The analogues (**747**)–(**751**), as well as (**740**) and (**741**), were examined for their ability to inhibit the serine protease α-thrombin, in comparison with suitable reference standards. They characterized Michaelis–Menten and slow-binding kinetics for the cyclotheonamide derivatives. An attempt was made to utilize the unoccupied hydrophobic S_3 subsite of thrombin via analogues (**747**) and (**748**). Also, removal of the hydroxyphenyl group, which was thought to be involved in an aromatic stacking interaction with Trp^{60D} of thrombin, was explored via analogue (**751**). The importance of the α-keto and alkene groups was

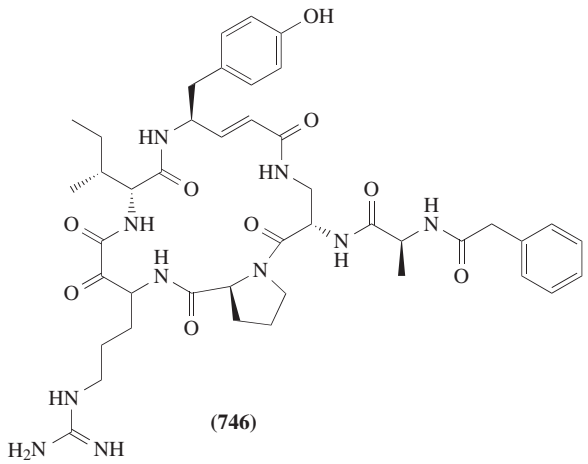
examined via analogues (**749**) and (**750**), respectively. The relation of structure and function with the analogues proved to be less predictable than anticipated.[627]



(**744**)



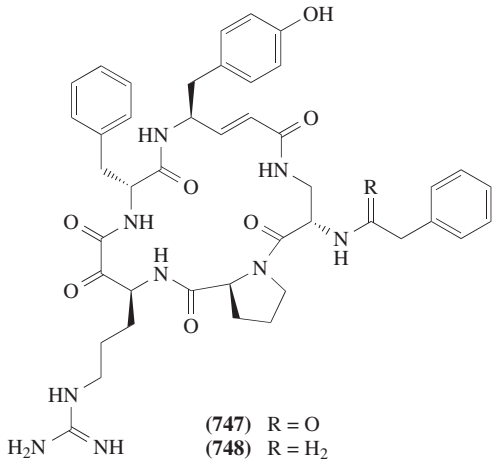
(**745**)



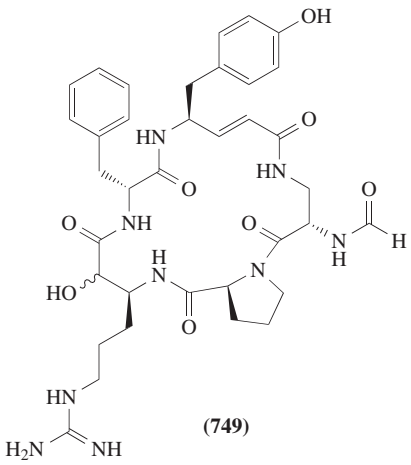
(**746**)

The treatment of cyclotheonamide A (**740**) with aqueous sodium carbonate or triethylamine at 23 °C generated two isomeric products. X-ray analysis of a complex with human α-thrombin indicated a ring-opened pentapeptide from cleavage at the α-keto amide bond. However, mass spectral data and a model study suggested a different product.[628] Clardy and co-workers independently studied a 2.0 Å resolution X-ray diffraction analysis of the cyclotheonamide A (**740**) complexed to bovine *β*-trypsin, which revealed the key interactions responsible for the slow and tight binding:[629] (**740**) formed a covalently linked hemiketal with Ser 195 O*γ* as well as an extensive series of hydrogen bonds and hydrophobic interactions; in particular, the arginine side-chain used both direct and water-mediated hydrogen bonds to fill the S_1 pocket, the hemiketal oxygen utilized several main-chain hydrogen bonds to fill the oxyanion hole, and the side-chain of D-Phe used aromatic stacking to form a binding pocket.

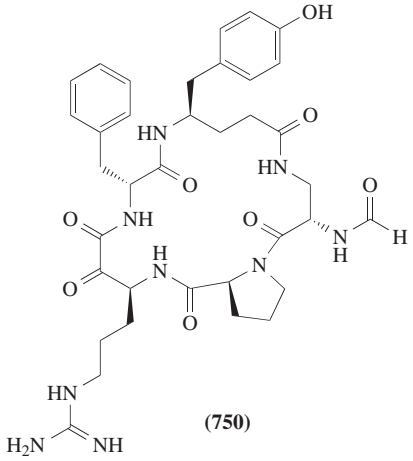
From the same *Theonella* sponge collected at Hachijo-jima Island that contained cyclotheonamides, orbiculamide A (**752**), a cytotoxic cyclic peptide, was isolated.[630] It is a cyclic peptide with three unusual amino acid residues: 2-bromo-5-hydroxytryptophan (**753**); theoleucine (**754**), which is an α-keto homologue of leucine; and theoalanine (**755**), which may be formed by cyclization of a dipeptide, alanyl-vinylogous-serine. Standard amino acid residues were assigned by amino acid analysis and HPLC analysis of the acid hydrolyzate after derivatization with Marfey's reagent. The total structure was elucidated by interpretation of 2D NMR spectral data in CD_3OH and DMSO-d_6 and by chemical degradation. The absolute configurations of the unusual amino acid residues (**753**)–(**755**) were determined by chiral GC analysis after degradations (e.g., $NaIO_4/KMnO_4$. H_2O_2/aqueous NaOH). The 3-methylvaleric acid residue was revealed to have *S* stereochemistry by GC (OV-1) analysis after conversion into the (*S*)-1-naphthylethylamide. α-Keto-*β*-amino acids such as (**754**) appear to be a characteristic feature of peptides from sponges of the genus *Theonella*.



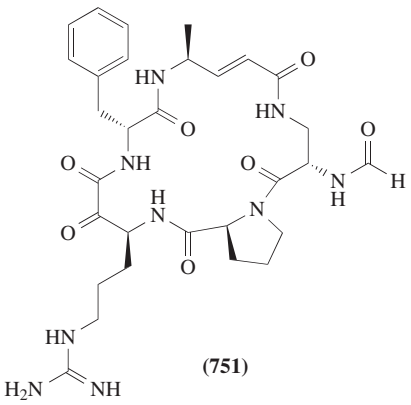
(747) R = O
(748) R = H_2



(749)



(750)



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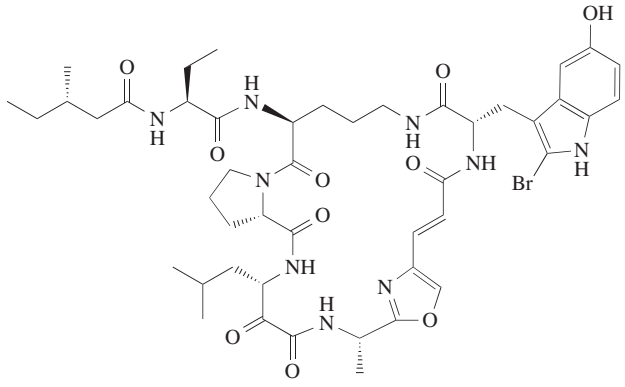

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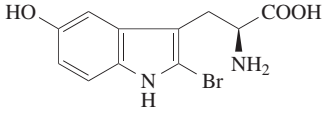

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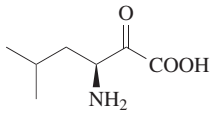

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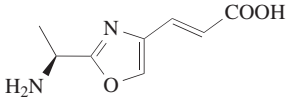
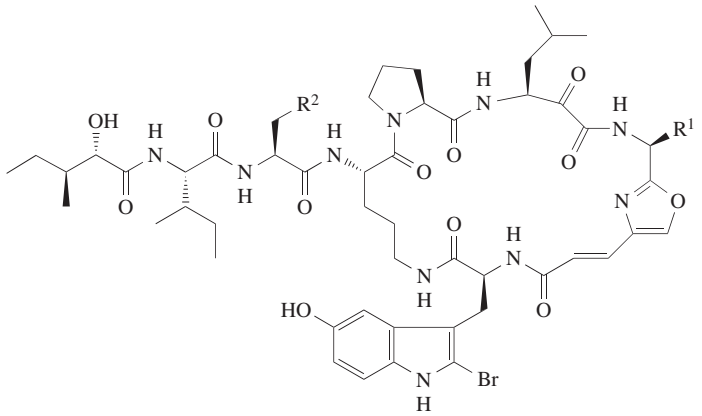
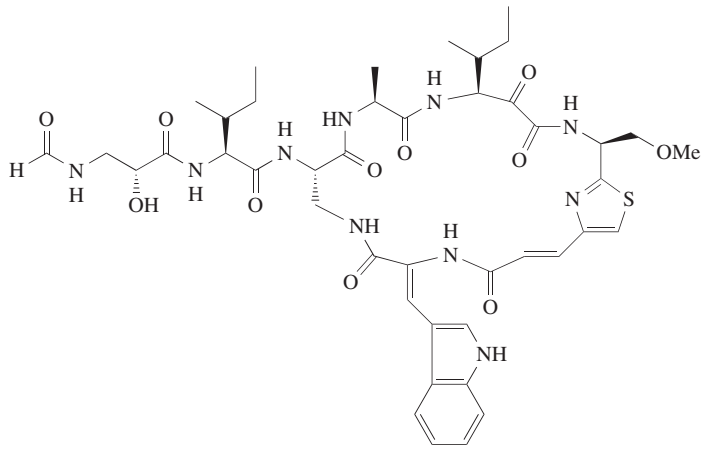


(755)

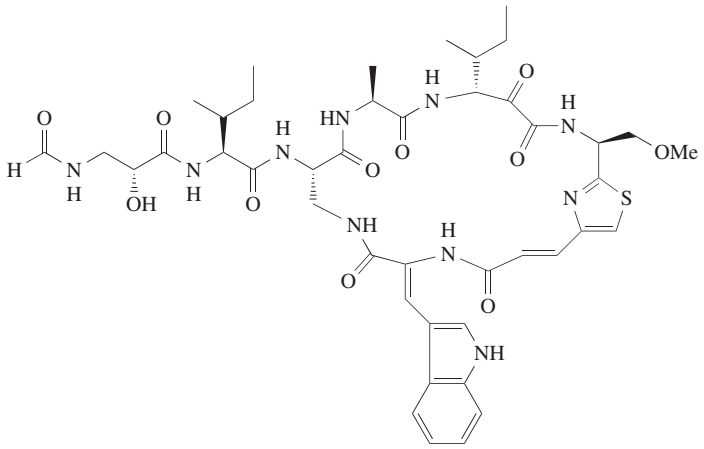
From a sponge of the genus *Theonella* collected off the Kerama Islands, Okinawa, Kobayashi *et al.* isolated keramamides B–D (**756**)–(**758**),[631] which were structurally related to orbiculamide A (**752**). Keramamides B–D (**756**)–(**758**) (5×10^{-8} M) inhibited the superoxide generation response of the human neutrophils elicited with a chemotactic peptide, *N*-formyl-Met-Leu-Phe (fMLP), but did not inhibit that induced by phorbol myristate, acetate, or immune complex. Another related peptide, keramamide F (**759**) was also obtained from the same sponge.[632] Keramamides B–D (**756**)–(**758**) and F (**759**) also contained α-keto β-amino acid residues. Keramamide F (**759**) was moderately cytotoxic. In addition, four related cyclic peptides, keramamides E (**760**), **G** (**761**), H (**762**), and J (**763**) were isolated from the sponge *Theonella* sp., and their structures were elucidated on the basis of spectral data, particularly 2D NMR and FAB MS/MS data, as well as by chemical means.[633] The 2-hydroxy-3-methylpentanoic acid which was generated from (**760**) by alkaline hydrolysis was revealed to have 2*S*,3*S*-configuration by comparison of retention times in chiral HPLC with those of 2*S*,3*S*-, 2*R*,3*R*-, 2*S*,3*R*-, and 2*R*,3*S*-isomers derived from L-Ile, D-Ile, L-allo-Ile, and D-allo-Ile through deamination with $NaNO_2$, respectively. Similarly the absolute configurations of the 2-hydroxy-3-methylpentanoic acid moiety in keramamides B–D (**756**)–(**758**) were determined to be 2*S*,3*S* by the same methods. Keramamide E (**760**) exhibited cytotoxicity against L1210 murine leukemia cells and KB human epidermoid carcinoma cells with IC_{50} values of 1.60 μg ml^{-1} and 1.55 μg ml^{-1}, respectively, whereas keramamides G (**761**), H (**762**), and J (**763**) showed no cytotoxicity ($IC_{50} > 10$ μg ml^{-1}).



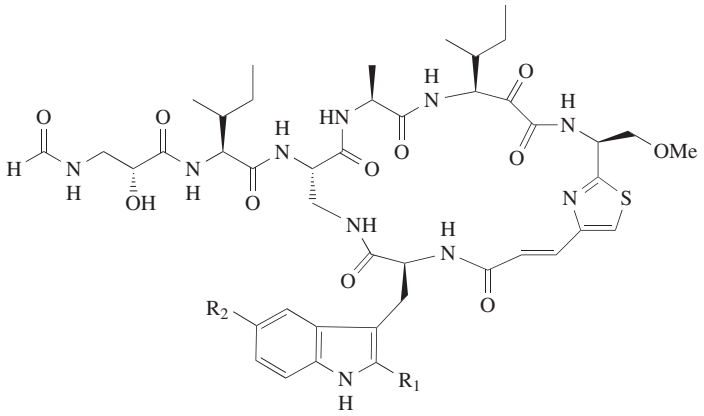
(**756**) $R^1 = CH_2Me$, $R^2 = CH_2Me$
(**757**) $R^1 = CH_2Me$, $R^2 = Me$
(**758**) $R^1 = Me$, $R^2 = Me$
(**760**) $R^1 = Me$, $R^2 = CH_2Me$



(**759**)



(761)

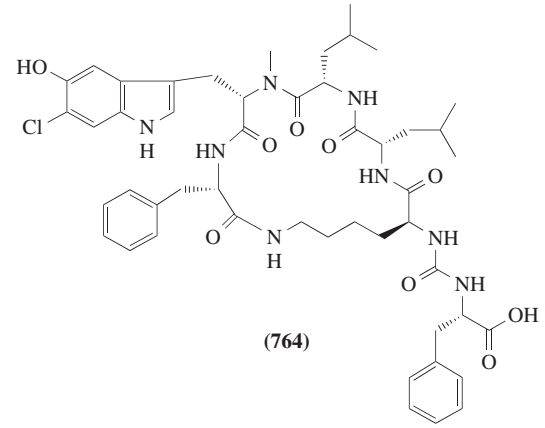


(762) R^1 = Br, R^2 = OH

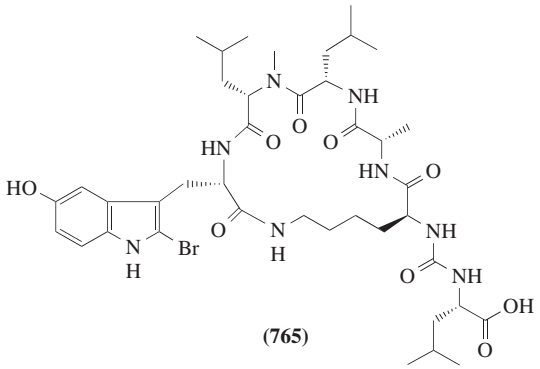
(763) $R^1 = R^2$ = H

Kobayashi *et al*. also isolated a different class of cyclic peptides from *Theonella* sponges. From the same sponge that contained keramamides B–D (**756**)–(**758**), which was brown-colored with a yellow inner body, keramamide A (**764**) was isolated,[634] and the structure was established as a unique hexapeptide containing a hitherto unknown amino acid, 6-chloro-5-hydroxy-*N*-methyltryptophan, and possessing an unusual ureido bond. The structural assignment was made on the basis of spectroscopic results. In particular, the proposed structure based on the NMR data was wholly supported by FABMS/MS evidence. Comparison of the daughter ions obtained by the CAD spectra of two precursor ions containing ^{35}Cl and ^{37}Cl, respectively, established the presence or not of a chlorine atom in a particular daughter ion. Keramamide A (**764**) exhibited inhibitory activity against sarcoplasmic reticulum Ca^{2+}-ATPase (IC_{50} 3×10^{-4} M). From another *Theonella* sponge with a white inner body, konbamide (**765**), with calmodulin antagonistic activity, was isolated.[635] Konbamide (**765**) was elucidated to be a unique hexapeptide with an ureido bond and unusual amino acids such as 2-bromo-5-hydroxytryptophan and *N*-methylleucine. The absolute configurations of the unusual tryptophan-derived amino acid residues, 2-bromo-5-hydroxytryptophan and 6-chloro-5-hydroxy-*N*-methyltryptophan, contained in keramamide A (**764**) and konbamide (**765**), respectively, were determined to be both L on the basis of chiral GC and HPLC analyses.[636] Schmidt and Weinbrenner synthesized both the D- and L-2-bromo-5-hydroxytryptophan containing forms of konbamide (**765**).[637] The physical properties of the natural and the synthetic konbamide were compared; HPLC data, optical rotation data, and NMR spectra showed slight variations, whereas the mass spectra were the same. They suggested that the proposed structure of konbamide (**765**) cannot be corrected. Thus, the structure of konbamide (**765**) as well as keramamide A (**764**) is under reinvestigation by Kobayashi's group.

Five tridecapeptide lactones, named theonellapeptolides Ia (**766**), Ib (**767**), Ic (**768**), Id (**769**),[638], and Ie (**770**), were isolated from the Okinawan marine sponge *Theonella swinhoei*.[639] A new HPLC

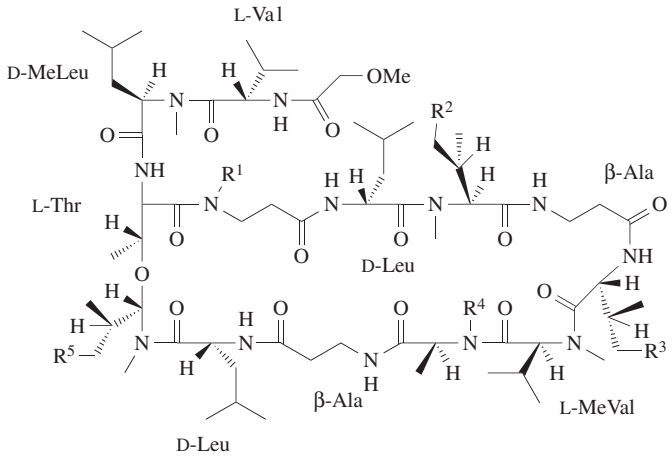


(764)



(765)

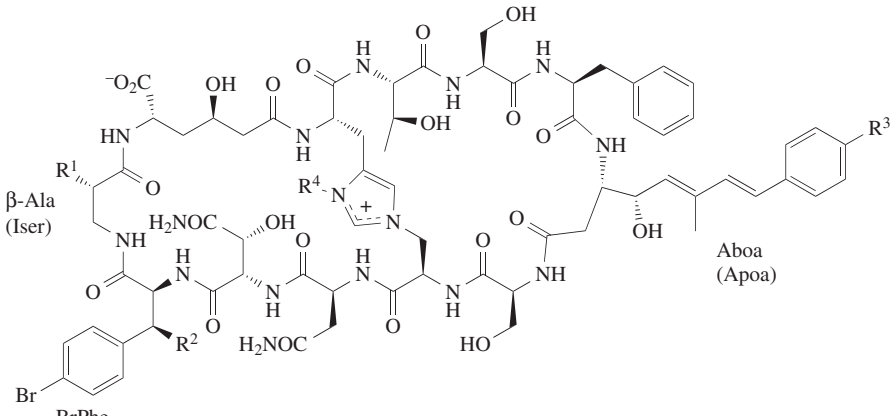
method for analyzing the amino acid composition of (**769**) containing *N*-Me amino acids was devised. The structures of these peptide lactones were determined on the basis of chemical and physicochemical examinations including HPLC–CD combined analysis of the amino acid compositions. Theonellapeptolides Ib (**767**), Ic (**768**), Id (**769**), and Ie (**770**) exhibited moderate cytotoxic activity towards for L1210 *in vitro* (IC_{50} 1.6, 1.3, 2.4, and 1.4 μg ml^{-1}, respectively), and theonellapeptolide Ie (**770**) exhibited ion-transport activities for Na^+ and K^+. Following the characterization of theonellapeptolides Ia–Ie (**766**)–(**770**), another new tridecapeptide lactone named



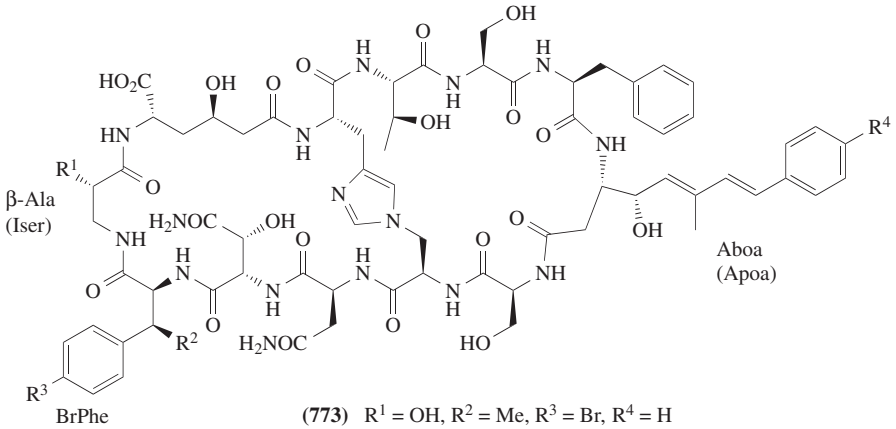
(766) R^1 = H, R^2 = Me, R^3 = H, R^4 = Me, R^5 = Me
(767) R^1 = H, R^2 = H, R^3 = Me, R^4 = Me, R^5 = Me
(768) R^1 = H, R^2 = Me, R^3 = Me, R^4 = Me, R^5 = H
(769) R^1 = H, R^2 = Me, R^3 = Me, R^4 = Me, R^5 = Me
(770) R^1 = Me, R^2 = Me, R^3 = Me, R^4 = Me, R^5 = Me
(771) R^1 = H, R^2 = Me, R^3 = Me, R^4 = H, R^5 = Me

theonellapeptolide IId (**771**) was isolated from the Okinawan marine sponge *Theonella swinhoei*.[640] Theonellapeptolide IId (**771**) prevented fertilization of the sea urchin *Hemicentrotus pulcherrimus* at concentrations of 25 μg ml^{-1} or greater but did not affect early embryonic development of fertilized eggs up to the gastrula stage.

Matsunaga and Fusetani isolated five cytotoxic bicyclic peptides, theonellamides A–E (**772**)–(**776**),[641] from the same *Theonella* sponge which contained theonellamide F (**777**),[617] an antifungal bicyclic peptide bridged by a histidinoalanine residue. Theonellamides A (**772**) and B (**773**) differed from theonellamide F (**777**) in three amino acid residues ((**772**), (**773**)/(**777**): 2*S*-isoserine (Iser)/*β*-alanine (*β*Ala), *β*-methyl-*p*-bromophenylalanine (MeBrPhe)/*p*-bromophenylalanine (BrPhe), and (5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-phenyl-5,7-octadienoic acid (Apoa)/(5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-(*p*-bromophenyl)-5,7-octadienoic acid (Aboa)). Additionally, theonellamide A (**772**) bore a *β*-D-galactose linked to the free imidazole nitrogen. Theonellamide C (**774**) was debromotheonellamide F. Theonellamide D (**775**) and E (**776**) were the α-L-arabinoside and *β*-D-galactoside of theonellamide F (**777**). Their structures were assigned on the basis of spectral data and chromatographic analyses of degradation products. The histidinoalanine residue (Hisala) in theonellamide F (**777**) was deduced to have L stereochemistry for the histidine portion and D for the alanine portion.[617] When the acid hydrolysate of theonellamide F (**777**) was prepared under standard conditions (6N HCl, 110 °C, 16 h), a 1:1 mixture of the LD- and the LL-isomers was obtained.[617] Under milder hydrolysis conditions (6N HCl, 107 °C, 8 h), the ratio of the LD- and the LL-isomers was 3:1.[617] In the mild acid hydrolysate (6N HCl, 110 °C, 3 h) of theonellamides A (**772**), the LD- and the LL-isomers were detected in a ratio of 5:1 by HPLC analysis of the Marfey derivative, suggesting the stereochemistry of the Hisala residue in (**772**) to be identical with that in (**777**).

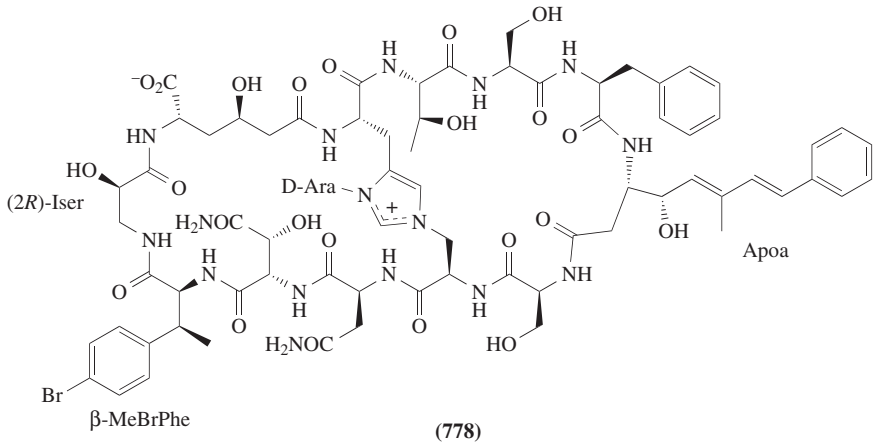


(**772**) R^1 = OH, R^2 = Me, R^3 = H, R^4 = β-D-Gal
(**775**) R^1 = H, R^2 = H, R^3 = Br, R^4 = β-L-Ara
(**776**) R^1 = H, R^2 = H, R^3 = Br, R^4 = β-D-Gal



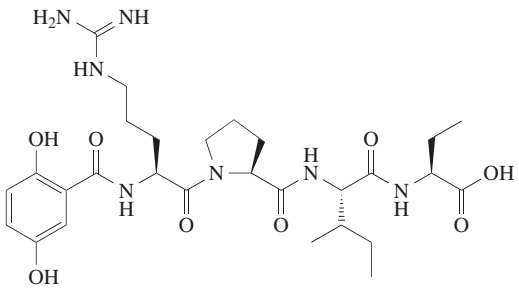
(**773**) R^1 = OH, R^2 = Me, R^3 = Br, R^4 = H
(**774**) R^1 = H, R^2 = H, R^3 = H, R^4 = Br
(**777**) R^1 = H, R^2 = H, R^3 = Br, R^4 = Br

However, there was still a possibility of an overlap of the DL- and the DD-isomer peaks with the LL-isomer peak in the Marfey analysis. In order to exclude this possibility, the LD- and the LL-isomers were derivatized with Marfey's reagent prepared from D-Ala (D-Marfey's reagent), which introduced opposite chiral centers in the molecule. The LD-isomer and the LL-isomer derivatized with Marfey's reagent prepared from D-Marfey's reagent were enantiomeric to, and therefore chromatographically equivalent to, the DL-isomer and the DD-isomer derivatized with the conventional Marfey's reagent. All four peaks were well separated and the major Hisala residue liberated from (**772**) by mild acid hydrolysis was determined unambiguously as the LD-isomer. Theonellamides A–E (**772**)–(**776**) were moderately cytotoxic against P388 murine leukemia cells with IC_{50} values of 5.0, 1.7, 2.5, 1.7, and 0.9 μg ml^{-1}, respectively. Bewley and Faulkner also isolated theonegramide (**778**), an antifungal glycopeptide consisting of D-arabinose joined to a bicycle dodecapeptide, which was structurally related to theonellamide F (**777**),[617] from the lithistid sponge *Theonella swinhoei* collected at Antolang, Negros Island, the Philippines.[642] Theonegramide (**778**) inhibited the growth of *Candida albicans* in the standard disk assay at a loading of 10 μg/disk.



(778)

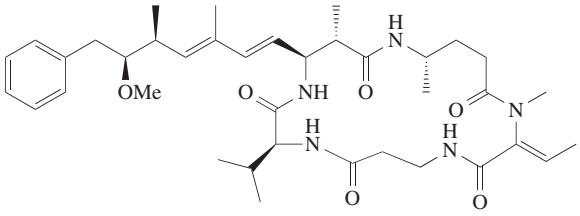
Nazumamide A (**779**), a thrombin-inhibiting linear tetrapeptide, was isolated from a marine sponge, *Theonella* sp. which contained cyclotheonamides.[618] The structure of (**779**) was determined by interpretation of 2D NMR data and by chemical degradation as 2,5-dihydroxybenzoyl-L-arginyl-L-prolyl-L-isoleucyl-L-α-aminobutyric acid.[643] Nazumamide A (**779**) inhibited thrombin with an IC_{50} of 2.8 μg ml^{-1}, but not trypsin at a concentration of 100 μg ml^{-1}. Its total synthesis was achieved in an efficient manner using diethyl phosphorocyanidate (DEPC) as a coupling reagent.[644]



(779)

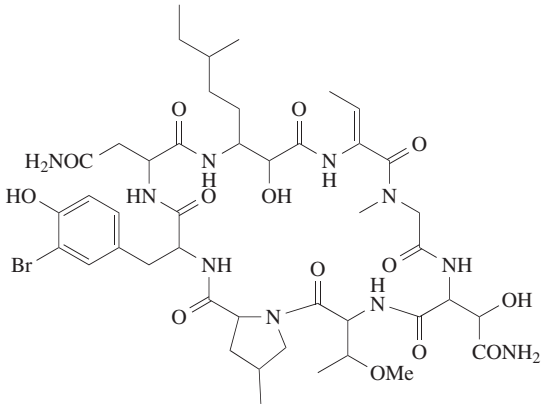
Motuporin (**780**), a cyclic pentapeptide, was isolated from the marine sponge *Theonella swinhoei* collected off Motupore Island, Papua New Guinea.[645] The structure of motuporin (**780**) was elucidated by spectroscopic analysis and chemical degradation. Motuporin (**780**) inhibited protein phosphatase-1 in a standard phosphorylase phosphatase assay at a concentration of <1 nmol, making it one of the most potent PP1 inhibitors, and it also displayed considerable *in vitro* cytotoxicity against murine leukemia (P388: IC_{50} 6 μg ml^{-1}), human lung (A549: IC_{50} 2.4 μg ml^{-1}), ovarian (HEY: IC_{50} 2.8 μg ml^{-1}), colon (LoVo: IC_{50} 2.3 μg ml^{-1}), breast (MCF7: IC_{50} 12.4 μg ml^{-1}),

and brain (U373MG: IC_{50} 2.4 μg ml^{-1}) cancer cell lines. Motuporin (**780**) was an analogue of nodularin, a hepatotoxic cyclic pentapeptide from the brackish-water blue-green alga *Nodularia spumigena*.[646] Motuporin (**780**) differed from nodularin by the replacement of a polar arginine residue with a nonpolar valine residue. The structural similarity between motuporin (**780**) and nodularin provided the suggestion that motuporin (**780**) was being produced by a blue-green alga.



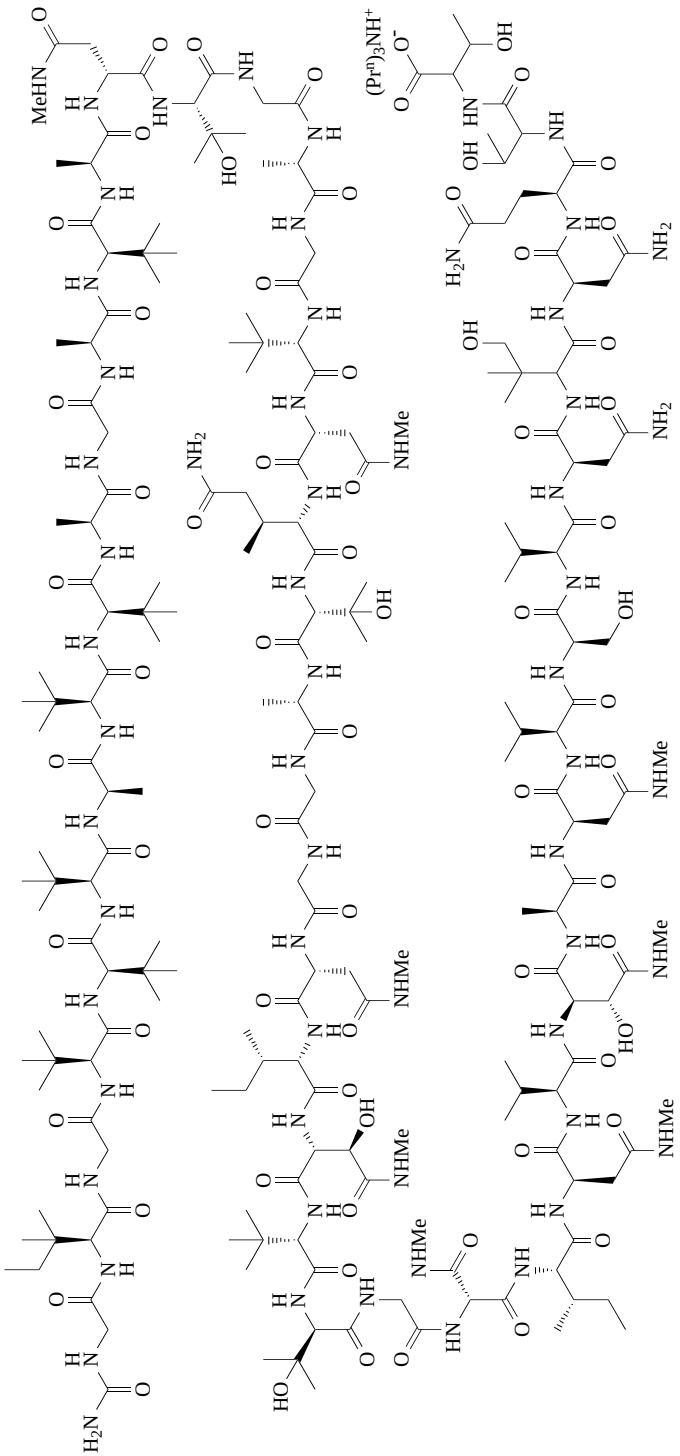
(780)

A sponge of the genus *Theonella*, collected near Perth, off Cape Vlamingh in Western Australia, contained a cyclic octapeptide, perthamide B (**781**).[647] The structure was elucidated by spectroscopic methods, revealing that (**781**) contained several unusual amino acid residues such as 3-amino-2-hydroxy-6-methyloctanoic acid, 2,3-dehydro-2-aminobutyric acid, *N*-methylglycine, *β*-hydroxy-asparagine, *O*-methylthreonine, *γ*-methylproline, and *m*-bromotyrosine. Perthamide B (**781**) weakly inhibited the binding of [^{125}I]interleukin-1.beta. to intact EL4.6.1 cells with an IC_{50} of 27.6 μM. However, the binding could not be differentiated from the toxicity of this compound.



(781)

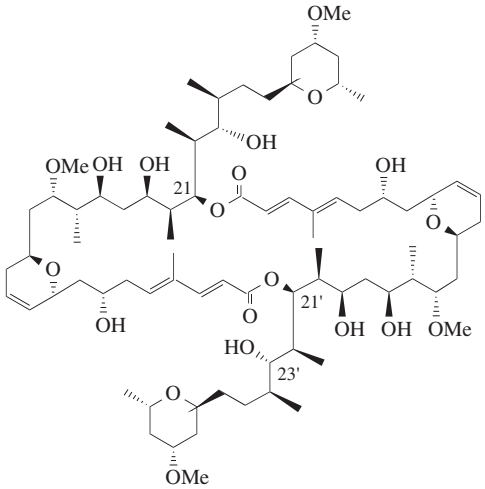
Fusetani and his research group isolated highly cytotoxic polypeptides, polytheonamides A–C, from the marine sponge *Theonella swinhoei*.[648] Interpretation of the 2D NMR data of the acid hydrolyzate of polytheonamide B (**782**) as well as amino acid analysis led to identification of Ala, Asp, α-Thr, Ser, Glu, Val, Gly, Ile, t-Leu, *β*-methylGlu, *β*-methylIle, *β*-hydroxyVal, and *β*-hydroxyAsp. The 2D NMR data of polytheonamide B (**782**) suggested the presence of *γ*-hydroxyl t-Leu, which was a new amino acid. The structure of polytheonamide B (**782**) was assigned to be linear 48-residue peptides with *N*-terminus blocked by a carbamoyl group, mainly by interpretation of spectral data.[649] Similarly polytheonamide A was analyzed by a spectroscopic method leading to the same amino acid sequence as (**782**). The chemical shift discrepancy of NH protons between polytheonamides A and B (**782**) was observed in residues 41–48, suggesting that the stereochemistry of amino acids in this region may differ between the two compounds. Polytheonamide A gave the same $(M+Pr_3N)^+$ ion in the FABMS at m/z 5033 as polytheonamide B (**782**). Polytheonamide C gave the $(M+Pr_3N)^+$ ion 14 mass units larger than (**782**). Interpretation of HOHAHA and NOESY data led to the amino acid sequence with Gln-46 residue in (**782**) being replaced by *β*-MeGln. Polytheonamides exhibited highly cytotoxic activity against L1210 with $IC_{50} < 4$ ng ml^{-1}.



(782)

The absolute configuration of each amino acid residue of polytheonamide B (**782**) was determined by chiral chromatography of fragment peptides obtained by partial acid hydrolysis.[650] Amino acid residues in polytheonamide B (**782**) have alternating D- and L-configuration. The NMR spectra in $CD_3OH/CDCl_3$ (1:1) indicated that polytheonamide B (**782**) adopted a certain secondary structure. Conformational analysis was carried out by distance geometry calculations, using the DADAS90 program, with NMR parameters obtained in $CD_3OH/CDCl_3$ (1:1), i.e., a total of 378 distance constraints (160 intraresidue, 96 sequential, 80 long-range NOEs, and 42 hydrogen bonds) and 48 dihedral angle constraints. The calculated structure fitted a right-handed parallel β-helix structure, which was proposed for the structure of a pore-forming peptide, gramicidin A.

Swinholide A (**783**) is a potent cytotoxic macrolide, first isolated from the Red Sea sponge *Theonella swinhoei* by Carmely and Kashman in 1985, and its structure was initially described as a monomeric 22-membered macrolide.[651] Swinholide A (**783**) was revealed to have a 44-membered dimeric dilactone structure. The absolute stereostructure of (**783**) was obtained by means of X-ray crystallographic analysis of a dimeric diketone structurally related to (**783**) and by chemical derivatizations.[652] The configurations of each asymmetric carbon in (**783**) were similar to those of scytophycin C (**784**) previously isolated from the cultured blue-green alga *Scytonema pseudo-hofmanni*.[653] The crystal structure further revealed the conformational characteristic of the 44-membered ring structure, which was probably related to the cytotoxic activity.[654] Swinholide A (**783**) (21,21′-diolide) exhibited high cytotoxic activity against L1210 (IC_{50} 0.03 μg ml^{-1}) and KB (IC_{50} 0.04 μg ml^{-1}) tumor cells, while the activity of isoswinholide A (21,23′-diolide) was significantly lowered (IC_{50} values, 0.12 μg ml^{-1} and 1.4 μg ml^{-1} for L1210 and KB cells, respectively). Acidic treatment of swinholide A (**783**) provided several isomeric macrolides having different sizes of the dilactone ring structure. The *in vitro* cytotoxicities and *in vivo* antitumour activities of these dimeric macrolides, together with two monomeric macrolides which were synthesized from (**783**) were examined from the structure–activity correlation viewpoint. Two monomeric macrolides, 21-olide (**785**) and 23-olide (**786**), were obtained by treatment of the monomeric acid (**787**), which was prepared from (**783**) through alkaline hydrolysis, with 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine in 41% and 24% yields, respectively. These compounds, however, did not show promising antitumor activity.[655]



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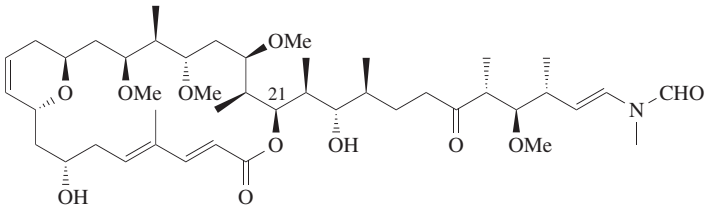

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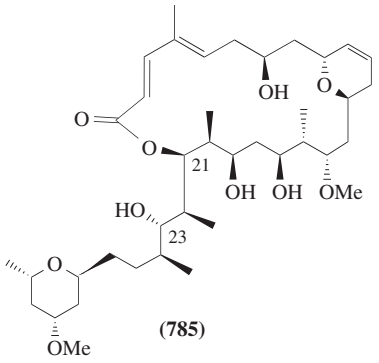

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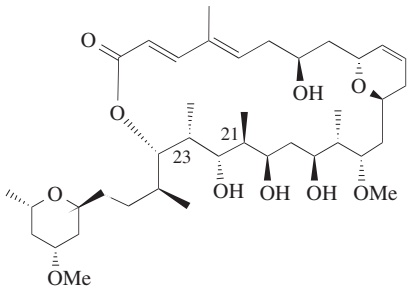

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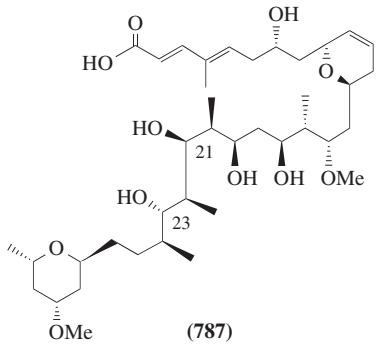
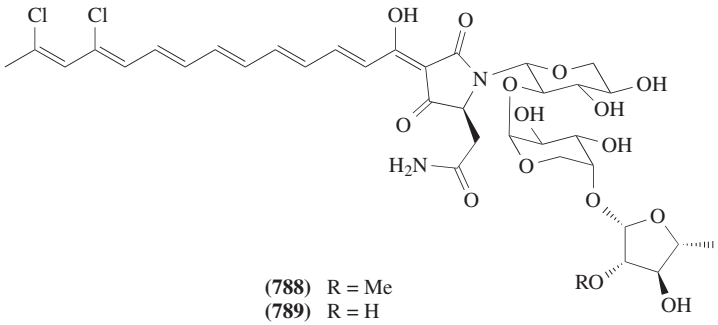


(787)

Thus, the marine sponge *Theonella swinhoei* yielded many important, bioactive natural products, most of which shared structural features with bacterial natural products. The presence of microbial symbionts in *T. swinhoei* was reported, and it was originally suggested that the cytotoxic macrolide swinholide A (**783**) and many of the bioactive cyclic peptides from *T. swinhoei* were all produced by symbiotic cyanobacteria. By transmission electron microscopy, four distinct cell populations were found to be consistently present in *T. swinhoei*: eukaryotic sponge cells, unicellular heterotrophic bacteria, unicellular cyanobacteria, and filamentous heterotrophic bacteria. Purification and chemical analyses of each cell type showed the macrolide swinholide A (**783**) to be limited to the mixed population of unicellular heterotrophic bacteria, and an antifungal cyclic peptide such as (**778**) occurred only in the filamentous heterotrophic bacteria. Contrary to prior speculation, no major metabolites were located in the cyanobacteria or sponge cells.[656]

The marine sponge *Theonella* sp., from which cyclotheonamides were isolated, contained orange pigments associated with cytotoxicity. The MeOH extract of the sponge was partitioned between water and ether, and the aqueous phase was extracted with Bu^{n}OH. The Bu^{n}OH phase was successively purified by Sephadex LH20 and reverse-phase chromatographies to yield aurantosides A and B (**788**) and (**789**), both as orange powders.[657] Aurantoside A (**788**) had a molecular formula of $C_{36}H_{46}N_2O_{15}Cl_2$ as revealed by FABMS, NMR spectra, and elemental analysis. Its gross structure was determined by the interpretation of 2D NMR spectra to be a tetramic acid glycoside: one side-chain was a dichlorinated conjugated heptaene, while the other was a methylenecarboxyamide; xylopyranose, arabinopyranose, and a 5-deoxypentofuranose, whose hydroxyl group on C-2 was methylated, were linked via nitrogen to the pyrrolidone ring, in order. Aurantoside B (**789**) gave almost superimposable NMR spectra to that of (**788**), except for the absence of the *O*-methyl group. Detailed analysis of spectral data indicated that the 2-*O*-methyl group of the 5-deoxypentose unit of aurantoside A (**788**) was replaced by a hydroxyl group in aurantoside B (**789**). Aurantosides A (**788**) and B (**789**) were cytotoxic against P388 and L1210 leukemia cells ((**788**), IC_{50} 1.8 μg ml^{-1} and 3.4 μg ml^{-1}, respectively; (**789**), IC_{50} 3.2 μg ml^{-1} and 3.3 μg ml^{-1}, respectively).



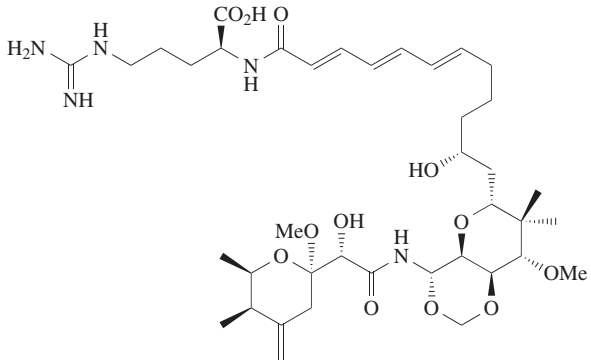
(**788**) R = Me
(**789**) R = H

Onnamide A (**790**) is a potent antiviral and highly cytotoxic compound, first isolated from a sponge of the genus *Theonella*.[658] Several other highly cytotoxic metabolites related to (**790**) were isolated from the sponges *Theonella*.[659,660] Theopederins A–E (**791**)–(**795**) were isolated from a marine sponge of the *Theonella* sp.[661] and their structures established mainly by extensive 2D NMR analyses as well a by comparison with spectral data of mycalamides A (**796**) and B (**797**).[662] Theopederins A–E (**791**)–(**795**) were highly cytotoxic against P388 murine leukemia cells with IC_{50} of 0.05, 0.1, 0.7, 1.0, and 9.0 ng ml^{-1}, respectively. Theopederins A and B (**791**) and (**792**) also showed promising antitumor activity against P388 (i.p.): T/C = 205% (0.1 mg kg^{-1} d^{-1}, treated on days 1, 2, and 4–6, i.p.), and T/C = 173% (0.4 mg kg^{-1} d^{-1}, treated on days 1, 2, and 4–6, i.p.), respectively. Theopederins A–C (**791**)–(**793**) might be generated by oxidative cleavage of a double bond of onnamide A (**790**),[658] the most abundant cytotoxic component of the water-soluble portion of the EtOH extract of the same sponge.

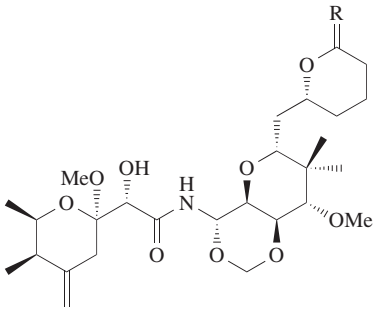
A novel tetrahydroprotoberberine alkaloid, theoneberine (**798**), was isolated from the Okinawan marine sponge of the species *Theonella*, collected off Ie Island, Okinawa, and its structure was elucidated on the basis of spectral and chemical means.[663] Theoneberine (**798**) was the first tetrahydroprotoberberine alkaloid isolated from marine organisms and also the first example as a naturally occurring tetrahydroprotoberberine alkaloid with substitution by bromine atoms. Biogenetically, theoneberine (**798**) seemed to be a unique isoquinoline alkaloid classified as a hybrid between 1-benzylisoquinolines and protoberberines, which was hitherto unknown from natural sources. Theoneberine (**798**) exhibited antimicrobial activity against Gram-positive bacteria (MIC values: *Staphylococcus aureus*, 16 μg ml^{-1}; *Sarcina lutea*, 2 μg ml^{-1}; *Bacillus subtilis*, 66 μg ml^{-1}; *Mycobacterium* sp. 607, 4 μg ml^{-1}), and was inactive against fungi or Gram-negative bacteria. Compound (**798**) also showed cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* with IC_{50} values of 2.9 μg ml^{-1} and ca. 10 μg ml^{-1}, respectively.

Theonezolide A (**799**), a novel macrolide, was isolated from the Okinawan marine sponge *Theonella* sp., collected off Ie Island, Okinawa, and the planar structure was elucidated on the basis of extensive spectroscopic analyses of (**799**) and its four ozonolysis products.[664] The 2D NMR techniques of gradient-enhanced HMBC and HSQC–HOHAHA along with the FABMS/MS experiment were applied and proved to be quite efficient for structural study of this long aliphatic molecule. Theonezolide A (**799**), $C_{79}H_{140}N_4O_{22}S_2$, was the first member of a new class of polyketide natural products consisting of two principal fatty acid chains with various functionalities such as sulfate ester, an oxazole, and a thiazole group, constituting a 37-membered macrocylic lactone ring bearing a long side-chain attached through an amide linkage. Theonezolides B (**800**) and C (**801**), two additional macrolides, were also isolated from the Okinawan marine sponge *Theonella* sp., collected of Ie Island, and their structures were elucidated on the basis of spectroscopic data as well as chemical degradation experiments. The absolute configurations of the terminal chiral center of theonezolides A (**799**), B (**800**), and C (**801**) were determined by synthesis of their ozonolysis products from L-alaninol.[665] Compounds (**799**)–(**801**) exhibited cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* (IC_{50} values for (**799**), both 0.75 μg ml^{-1}; for (**800**), 5.6 μg ml^{-1} and 11 μg ml^{-1}, respectively; and for (**801**), 0.3 μg ml^{-1} and 0.37 μg ml^{-1}, respectively).

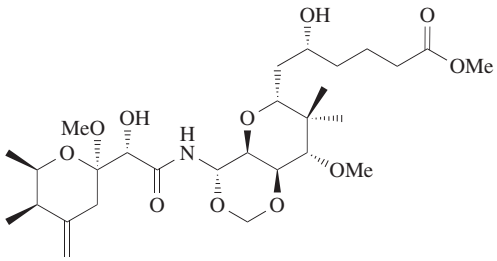
Theonezolide A (**799**) caused a marked platelet shape change at low concentrations (0.2–0.6 μM).[666] Increasing concentrations of (**799**) to 6 μM or more caused shape change followed by a small but sustained aggregation. In a Ca^{2+}-free solution, theonezolide A-induced aggregation was markedly inhibited, although the marked shape change was still observed. Aggregation stimulated by (**799**) increased in a linear fashion with increasing Ca^{2+} concentrations from 0.1 mM to 3.0 mM. Furthermore, theonezolide A (**799**) markedly enhanced $^{45}Ca^{2+}$ uptake into platelets. Aggregation induced by theonezolide A (**799**) was inhibited by Arg-Gly-Asp-Ser, an inhibitor of fibrinogen



(790)



(791) R = H, OH
(793) R = O



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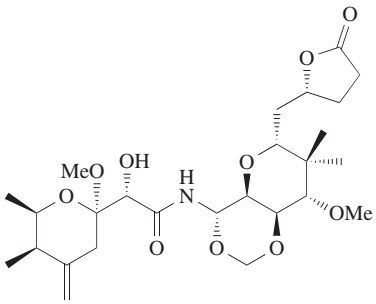

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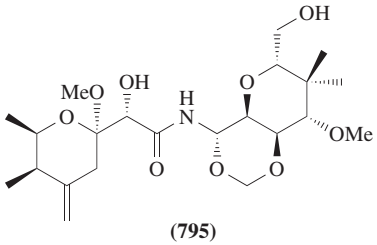
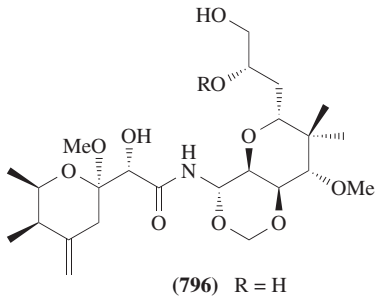
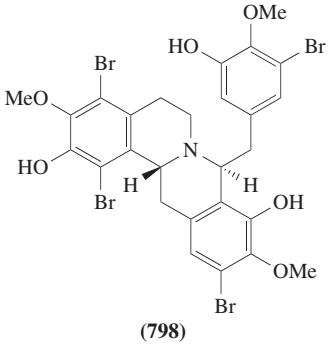


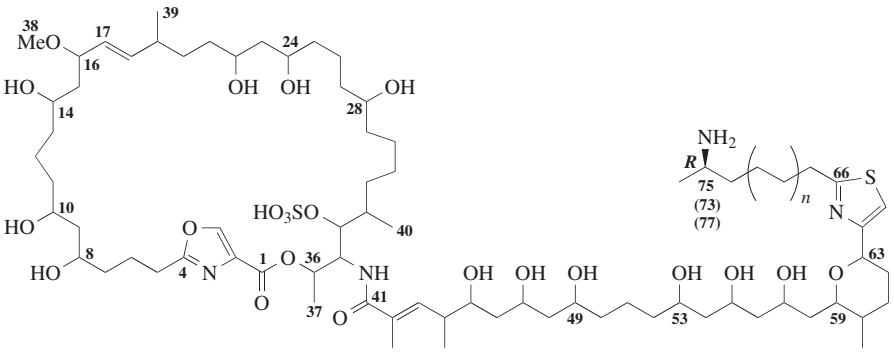
(795)



(796) R = H
(797) R = Me



(798)



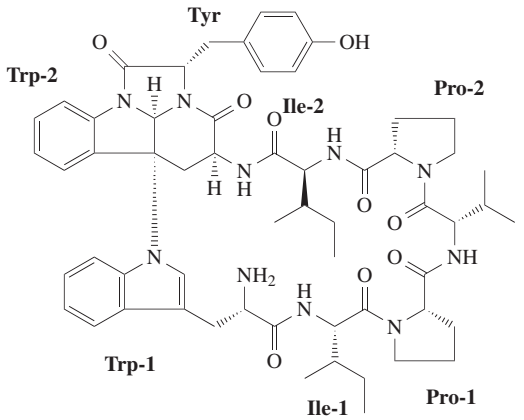
(**799**): $n = 3$, (**800**): $n = 2$, (**801**): $n = 4$

binding to glycoprotein IIb-IIa, H-7 and staurosporine, protein kinase C inhibitors, or genistein and tyrphostin A23, protein tyrosine kinase inhibitors, whereas shape change was blocked by genistein and tyrphostin A23. H-7 or staurosporine did not affect the theonezolide A-induced shape change. These results suggested that theonezolide A-induced platelet shape change was not dependent on external Ca^{2+}, whereas theonezolide A-induced aggregation was caused by an increase in Ca^{2+} permeability of the plasma membrane. It was also suggested that both aggregation and shape change induced by theonezolide A (**799**) were associated with protein phosphorylation by protein kinase C and tyrosine kinase.

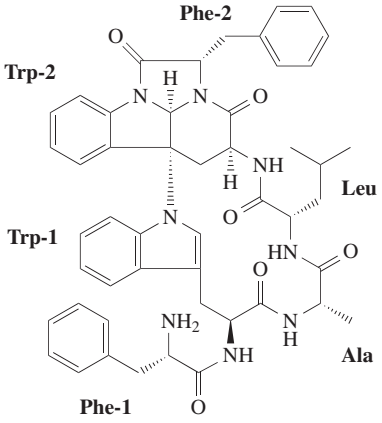
8.07.9.3.3 Others

Four cyclic peptides, kapakahines A (**802**) and B (**803**).[667] and stereoisomers C (**804**) and D (**805**), were isolated from the marine sponge *Cribrochalina olemda*, collected at Pohnpei, Micronesia. Their structures including complete stereochemistry were elucidated by spectral analysis and chemical degradation.[668] Kapakahine B (**803**) is a cyclic hexapeptide, while kapakahines A (**802**), C (**804**), and D (**805**) are octapeptides. The unique structural feature of these peptides was the lack of an amide linkage between two tryptophan residues. Instead the ring was closed by a bond from the indole nitrogen of Trp-1 to the β-carbon of Trp-2, and all four peptides contained a tryptophan-derived α-carboline which was part of a fused tetracyclic system that also included an imidazolone derived from phenylalanine or tyrosine. Kapakahines A (**802**), B (**803**), and C (**804**) showed moderate cytotoxicity against P388 murine leukemia cells at IC_{50} values of 5.4, 5.0, and 5.0 $\mu g\ ml^{-1}$, respectively. Kapakahine D (**805**) did not show cytotoxicity at a concentration of 10 $\mu g\ ml^{-1}$. Kapakahine A (**802**) was tested for inhibitory activities against several enzymes [thrombin, trypsin, plasmin, elastase, papain, angiotensin converting enzyme, and protein phosphatase 2A (PP2A)], but showed only 15% inhibition against PP2A at a concentration of 30 μM (32 $\mu g\ ml^{-1}$). Against other enzymes, (**802**) did not show any activity at a concentration of 100 $\mu g\ ml^{-1}$.

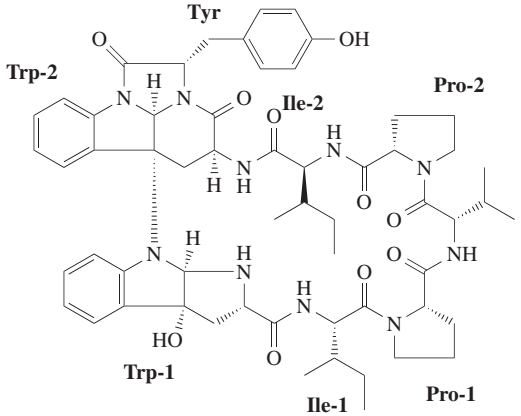
Two antifungal and cytostatic bis-oxazole macrolides, phorboxazoles A (**806**) and B (**807**), were isolated from the marine sponge, *Phorbas* sp., collected near Muiron Island, Western Australia.[669] Structural assignment was accomplished through extensive 2D NMR spectroscopy including COSY, RCT, HMQC, and HMBC. The complete relative stereochemistry about the macrolide ring and the solution conformation of (**806**) were established from analysis of ROESY experiments. Phorboxazoles A (**806**) and B (**807**) exhibited *in vitro* antifungal activity against *Candida albicans* at 0.1 μg/disk and extraordinary cytostatic activity against a variety of human solid tumor cell lines (mean 50% Growth Inhibition (GI_{50}) $< 7.9 \times 10^{-10}$ M in the National Cancer Institute's 60 tumor cell line panel). For example, phorboxazoles A (**806**) inhibited growth of colon tumor cells HCT-116 (GI_{50} 4.36×10^{-10} M) and HT29 (3.31×10^{-10} M). Although all attempts to obtain crystals of either compound (**806**) or (**807**) suitable for X-ray diffraction studies unfortunately failed, the absolute configurations of 14 out of the 15 stereocenters in both (**806**) and (**807**) were elucidated by a combination of techniques including synthesis of a suitable model compound for NMR spectroscopic comparisons.[670] The absolute configuration of C-43 in phorboxazole A (**806**) was established as *R* by correlation with (*R*)-dimethyl methoxysuccinate, while 43*R* was also suggested for phorboxazole B (**807**) by CD comparison, thus completing the entire stereochemical determination of the phorboxazoles (**806**) and (**807**).[671]



(802)



(803)



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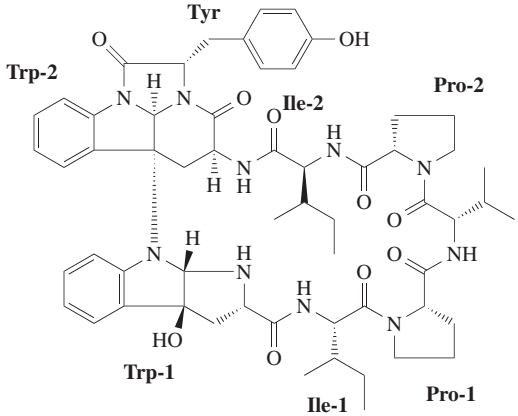
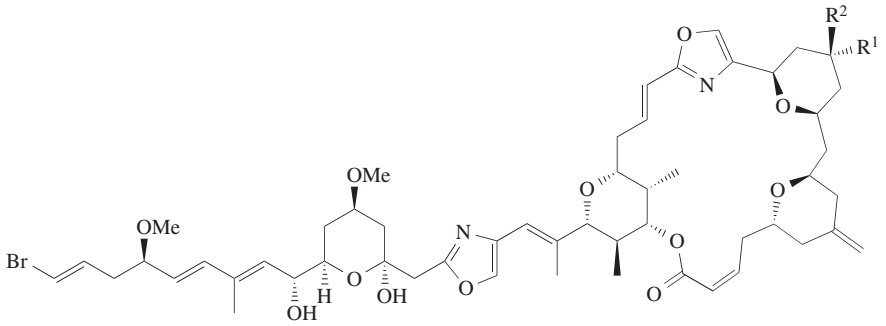
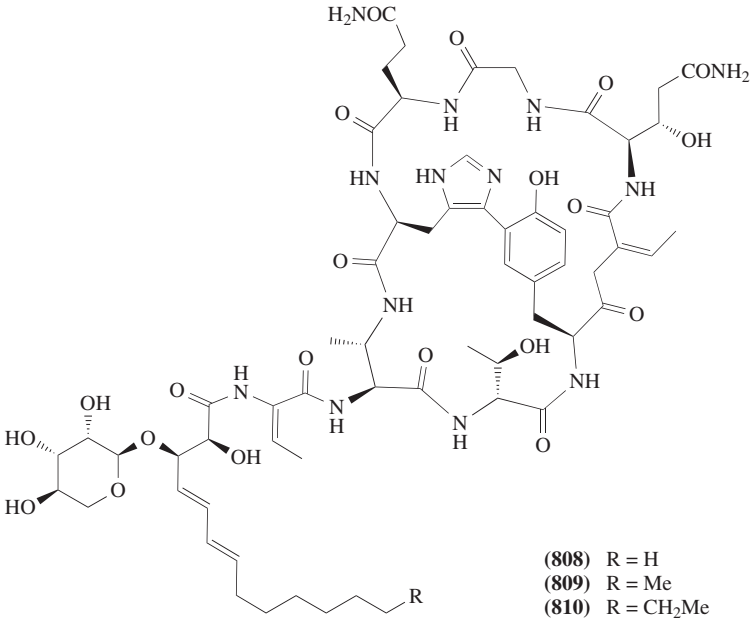


(805)



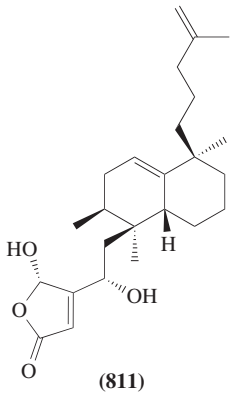
(806) R^1 = OH, R^2 = H
(807) R^1 = H, R^2 = OH

The lithistid sponge *Aciculites orientalis*, collected from the small island of Siquijor, located southeast of the large island of Negros, in the Philippines, contained three cyclic peptides, aciculitins A–C (**808**)–(**810**), that were identical except for homologous lipid residues.[672] The structure of the major peptide, aciculitin B (**809**), $C_{62}H_{88}N_{14}O_{21}$, was elucidated by interpretation of spectroscopic data. The absolute stereochemistries of the amino acid residues, the 2,3-dihydroxy-4,6-dienoic acid unit, and the sugar moiety were determined by combination of chiral GC analysis, chemical degradations, and synthesis of authentic samples. The aciculitins consisted of a bicyclic peptide that contained an unusual histidino-tyrosine bridge. Attached to the bicyclic peptide were C_{13}-C_{15} 2,3-dihydroxy-4,6-dienoic acids bearing D-lyxose at the 3-position. The aciculitins (**808**)–(**810**) inhibited the growth of *Candida albicans* at a loading of 2.5 μg/disk in the standard disk assay, and were cytotoxic toward the human colon tumor cell line HCT-116 with an IC_{50} of 0.5 μg ml^{-1}.



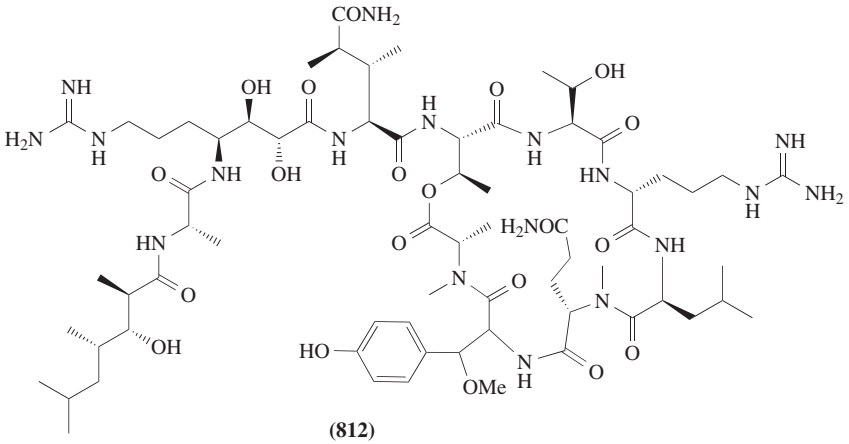
(808) R = H
(809) R = Me
(810) R = CH_2Me

Dysidiolide (**811**), a sesterterpene γ-hydroxybutenolide, was isolated from a sponge *Dysidea etheria* de Laubenfels, collected off Long Island, Bahamas at a depth of 18 m.[673] Its structure was determined by single-crystal X-ray diffraction. Dysidiolide (**811**) blocks the activity of a protein phosphatase called cdc25A. This enzyme catalyzes the removal of phosphate groups from a protein complex that signals cells to undergo mitotic division. Inhibitors of the enzyme arrest the cell cycle and are potentially useful in the treatment of cancer. The organization of the carbon skeleton of dysidiolide (**811**) was the first reported for the sesterterpene group of relatively rare natural products.



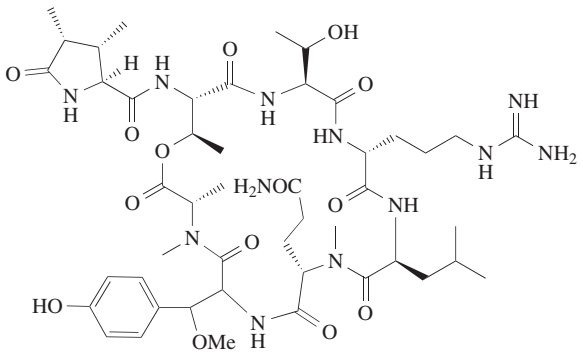
(811)

Callipeltin A (**812**) is a cyclic depsidecapeptide isolated from a shallow-water sponge of the genus *Callipelta* (order Lithistida), collected in the waters off the east coast of New Caledonia.[674] The structure of callipeltin A (**812**), which possesses the *N*-terminus blocked with a β-hydroxyl acid, and the *C*-terminus lactonized with a threonine residue, was determined by interpretation of spectral data, chemical degradation, and evaluation of the amino acids obtained by acid hydrolysis. Along with four common L-, one D-, and two *N*-methyl amino acids, it contained three new amino acid residues: β-methoxytyrosine (βOMeTyr), (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHE), and (3*S*,4*R*)-3,4-dimethyl-L-glutamine. Callipeltin A (**812**) was found to protect cells infected by human immunodeficiency (HIV) virus. The antiviral activity of callipeltin A (**812**) was measured on CEM4 lymphocytic cell lines infected with HIV-1 (Lai strain). In order to evaluate the antiviral activity of callipeltin A (**812**) the inhibition of cytopathic effects (CPE) induced by HIV-1 were studied, using MTT cell viability to determine the CD_{50} (50% cytotoxic dose) and ED_{50} (50% effective dose). At day six post-infection, callipeltin A (**812**) exhibited a CD_{50} of 0.29 $\mu g\ ml^{-1}$ and an ED_{50} of 0.01 $\mu g\ ml^{-1}$ giving a selectivity index (SI ratio CD_{50}/ED_{50}) of 29. The AZT reference had a CD_{50} of 50 μM and an ED_{50} of 30 nM.

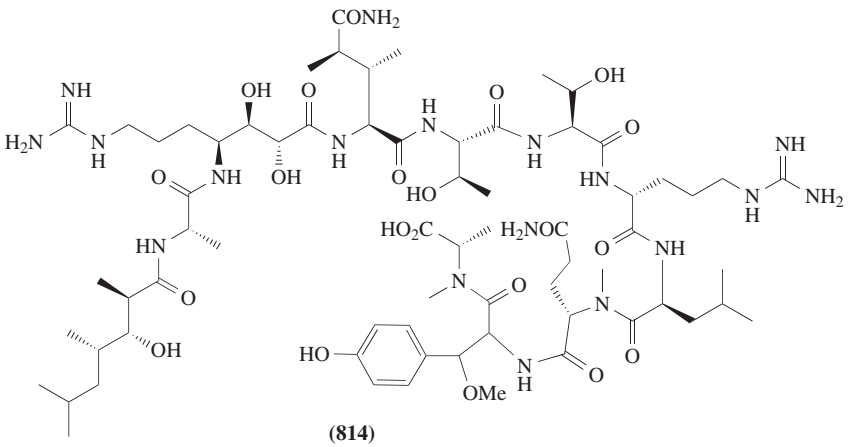


(812)

Following the characterization of callipeltin A (**812**), two other cytotoxic peptides, callipeltins B (**813**) and C (**814**), were isolated from the same New Caledonian sponge *Callipelta* sp.[675] Callipeltin B (**813**) possessed the same cyclic depsipeptidal structure as in callipeltin A (**812**) and differed from (**812**) by having the *N*-terminal 2,3-dimethylpyroglutamic acid unit instead of the tripeptide chain with the *N*-terminus blocked by a hydroxyl acid. Callipeltin C (**814**) was simply the acyclic callipeltin A. Callipeltins A–C (**812**)–(**814**) were cytotoxic against various human carcinoma cells *in vitro* and the activities of (**812**) and (**813**) exceeded significantly those of (**814**), thus suggesting the importance of the macrocycle for the bioactivity. Compounds (**812**) and (**813**) also exhibited antifungal activity against *Candida albicans* showing 30 mm and 9 mm inhibition at 100 μg/disk, respectively. Whereas callipeltin A (**812**) showed anti-HIV activity, callipeltins B (**813**) and C (**814**) proved to be inactive as antiviral compounds.

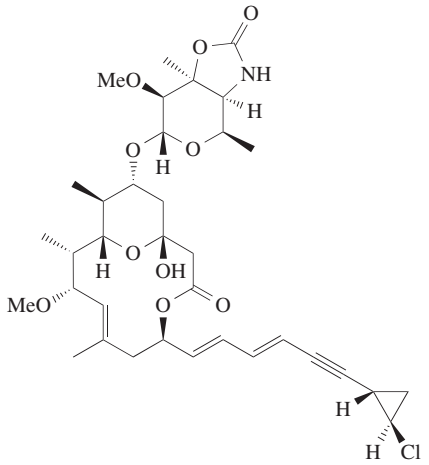


(813)



(814)

Further investigations on the dichloromethane extract from several collections of the same lithistid New Caledonian sponge *Callipelta* sp. (2.5 kg freeze-dried in total) resulted in the isolation of another cytotoxic component of a different class of natural products, named callipeltoside A (**815**) (3.5 mg yield; 1.4×10^{-4}%, dry weight).[676] Callipeltoside A (**815**) was a glycoside macrolide and its structural assignment was accomplished through extensive 2D NMR spectroscopy. The complete relative stereochemistry was proposed from the analysis of ROESY and NOE difference experiments. Callipeltoside A (**815**) represented the first member of a new class of marine-derived macrolides, containing unusual structural features including the previously unknown 4-amino-4,6-dideoxy-2-*O*-3-*C*-dimethyl-α-talopyranosyl-3,4-urethane unit (callipeltose), linked through an *a*-*O*-glycoside linkage to a hemiketal oxane ring, which was part of a 14-membered macrocycle lactone with a dienyne cyclopropane side-chain. Callipeltoside A (**815**) exhibited moderate cytotoxic activity with IC_{50} values against the NSCLC-N6 human bronchopulmonary non-small-cell lung carcinoma and P388 of 11.26 μg ml^{-1} and 15.26 μg ml^{-1}, respectively. Further, cell cycle analysis by flow cytometry assays of the NSCLC-N6 cell line treated with callipeltoside A (**815**) revealed a cell cycle-dependent effect, involving a dependent G1 blockage. These results were indicative of a blockage of NSCLC-N6 cell proliferation *in vitro* at the level of the G1 phase or by enzyme inhibition or inducing terminal cell differentiation. In the latter case callipeltoside A (**815**) would be an interesting mechanism-based lead.



(815)

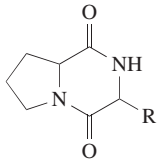
8.07.9.4 Microbial Metabolites

8.07.9.4.1 Bacteria

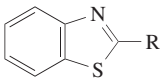
Following the discovery of over 6000 new compounds during investigations on marine natural products since the 1950s, marine microorganisms, including bacteria, fungi, and microalgae have come into focus as important new sources of biologically active compounds.[677,678] Bioactive metabolites of marine sponges have been extensively studied by marine natural products chemists (see Section 8.07.9.3). The unique metabolites of sponges are, however, often very minor constituents. It is often found that structurally similar compounds are obtained from sponges of different species and sometimes sponges of the same species contain quite different metabolites. These facts, as well as the structural characteristics of the compounds, strongly suggest that microorganisms living in or on sponges are responsible for the production of many bioactive compounds. Some of secondary metabolites which had been isolated from extracts of sponges were also obtained from the culture media of marine bacteria isolated from sponges. These are described below.

Cardellina and co-workers examined the microorganisms growing on or in sponges from Bermudan waters. From a specimen of the sponge *Tedania ignis* a bright orange-pigmented, Gram-positive bacterium was isolated and identified as a member of the genus *Micrococcus*. Culturing of the

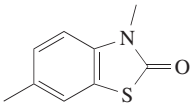
Micrococcus sp. was successfully carried out and from the culture media three diketopiperazines (**816**)–(**818**) were isolated by chromatographic techniques, including centrifugal countercurrent chromatography.[679] Compounds (**816**)–(**818**) proved to be identical to those previously isolated from extracts of the sponge *Tedania ignis*.[680] From the fermentation culture extracts of the same *Micrococcus* sp. four benzothiazoles (**819**)–(**822**) were obtained.[681] These benzothiazoles were previously known as volatile constituents of cranberries, an aroma constituent of tea leaves, a fermentation product of yeast, or synthesized compounds.



(**816**) R = $CH_2CH(Me)_2$
(**817**) R = $CH(Me)_2$
(**818**) R = Me

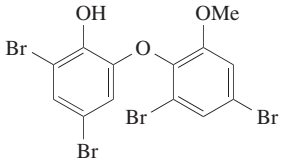


(**819**) R = SH
(**820**) R = Me
(**821**) R = OH



(**822**)

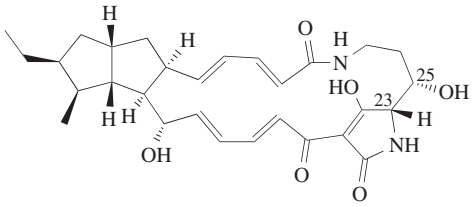
Elyakov *et al*. reported that a *Vibrio* sp. of bacteria isolated from the sponge *Dysidea* sp. collected at Eastern Samoa biosynthesized brominated diphenyl ethers on the basis of GC–MS analyses using 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol (**823**) as a standard. Compound (**823**) was previously obtained from extracts of the sponge *Dysidea* sp.[682]



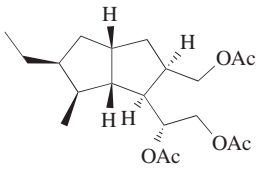
(**823**)

From a Japanese sponge *Halichondria okadai* a bacterium of a member of the genus *Alteromonas* was isolated and cultured in the laboratory. From the cultured mycelium of this bacterium Kobayashi and co-workers isolated a unique macrocylic lactam, alteramide A (**824**), possessing cytotoxic activity (IC_{50} values against P388, L1210, and KB cells, 0.1, 1.7, and 5.0 μg ml^{-1}, respectively).[683] The structure of compound (**824**) was elucidated by extensive analyses of the spectral data (e.g., ^{1}H-^{1}H COSY, HMQC, HMBC, NOESY, and ROESY) as well as chemical degradation into small fragments. Treatment of (**824**) with ozone followed by $NaBH_4$ reduction and acetylation afforded the bicyclo[3.3.0]octane derivative (**825**), while β-hydroxyornithine (**826**) was obtained from (**824**) through ozonolysis and oxidative workup with H_2O_2. The relative stereochemistry of the bicyclo[3.3.0]octane unit was established from NOESY data obtained for (**825**) and the absolute configurations of the C-23 and C-25 positions of (**824**) were defined through chiral HPLC examination of (**826**). Compound (**824**) possesses two diene groups which were vulnerable to an intramolecular photochemical [4+4] cycloaddition to give a hexacyclic compound (**827**). It should be noted that the structure of alteramide A (**824**) appears to be biogenetically related to those of an antibiotic ikarugamycin (**828**) produced by a terrestrial *Streptomyces* sp.[684] and an antifungal and cytotoxic metabolite discodermide (**829**) isolated from a Caribbean marine sponge *Discodermia dissoluta*.[685] Since compound (**824**) was obtained from a bacterium isolated from a marine sponge, discodermide (**829**) was strongly suggested to be of microbial origin.

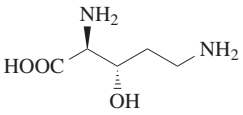
An antibiotic indole trimer named trisindoline (**830**) was isolated from the culture of a bacterium of *Vibrio* sp. by Kitagawa and co-workers.[686] This bacterium was separated from the Okinawan marine sponge *Hyrtios altum*. The structure of trisindoline (**830**) was verified by synthesis of (**830**) from oxindole (**831**) through two steps, (i) copper(II) bromide in ethyl acetate under reflux for 3 h, and (ii) indole and silver carbonate in tetrahydrofuran at 25 °C for 1.5 h, giving 47% overall yield. Trisindoline (**830**) showed antibiotic activities (16, 17, and 10 mm diameter growth inhibitions for *E. coli*, *B. subtilis*, and *S. aureus*, respectively, at 10 μg/disk (i.e. = 8 mm)).



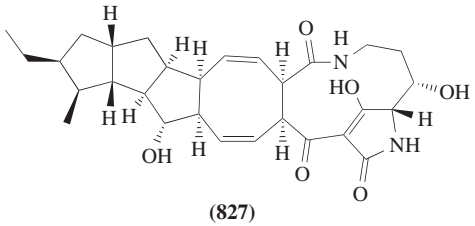
(824)



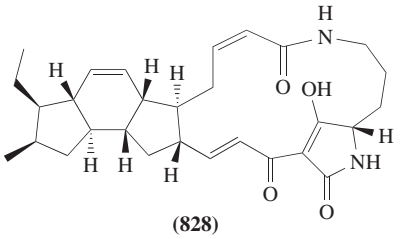
(825)



(826)



(827)



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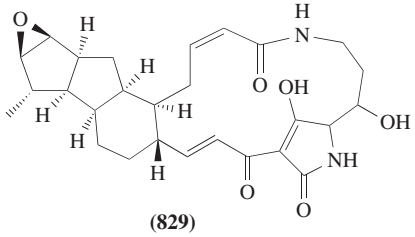
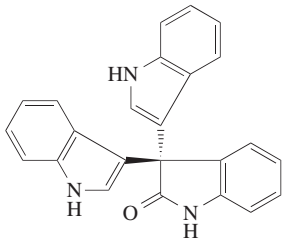


(829)



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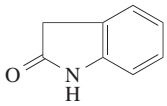
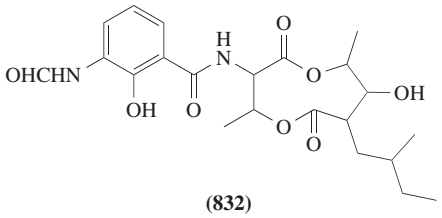


(831)

Imamura *et al*. isolated two new antimycin antibiotics, named urauchimycins A (**832**) and B (**833**), from a fermentation broth of a *Streptomyces* sp., which was obtained from an unidentified sponge collected at Urauchi-cove, Iriomote, Japan.[687] They were the first antimycin antibiotics which possess a branched side-chain moiety. They exhibited inhibitory activity against morphological differentiation of *Candida albicans*.



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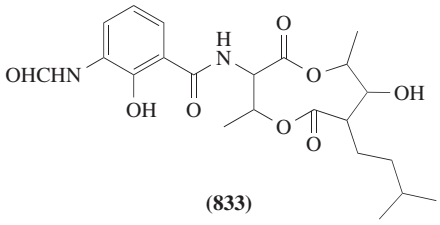
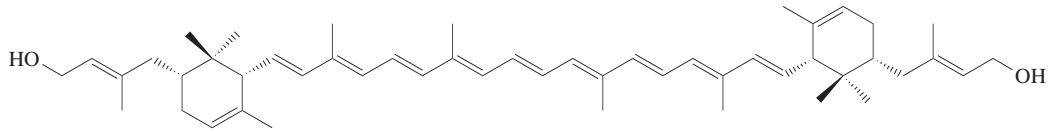


(833)

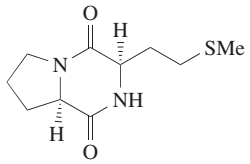
A number of interesting carotenoids including aromatic carotenoids have been isolated from marine sponges. Carotenoids are noted as quenchers and/or scavengers of active oxygen species.

The origins of carotenoids remain unknown, especially whether sponges can modify dietary carotenoids into suitable structures through enzymatic bioconversion or whether the precursors and/or carotenoids themselves are biosynthesized by some symbiotic or co-existing microorganisms before being transferred to the host sponge and stored. To study the origins of the carotenoids in marine sponges, Miki *et al*. investigated a marine bacterium *Pseudomonas* sp. strain number KK10206C, which was associated with a marine sponge *Halichondria okadai*, to obtain a novel C_{50}-carotenoid, okadaxanthin (**834**), through a visible absorption spectrum-guided isolation procedure.[688] Its structure, 2,2′-bis(4-hydroxy-2-methyl-2-butenyl)-ε,ε-carotene, was elucidated mainly by spectroscopic methods. Okadaxanthin (**834**) turned out to be a potent singlet oxygen quencher, approximately 10 times as strong as α-tocopherol.



(834)

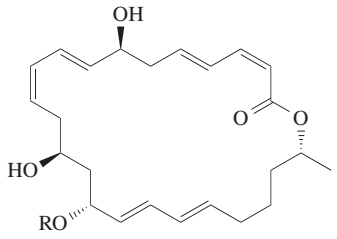
Baker and co-workers isolated a new diketopiperazine, cyclo-(L-proline-L-methionine) (**835**), along with five known diketopiperazines and two known phenazine alkaloid antibiotics from a strain of *Pseudomonas aeruginosa* obtained from a sponge *Isodictya setifera* Topsent (family Esperiopsidae), which was collected from Hut Point and Danger Slopes on Ross Island, Antarctica. Diketopiperazines including (**835**) were found to be inactive as antibiotics or cytotoxins.[689]

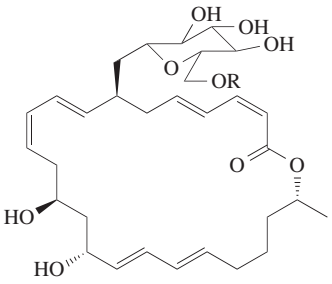


(835)

Marine bacteria obtained from other than sponges were also investigated as follows. Fenical and co-workers isolated eight new secondary metabolites, macrolactins A–F (**836**)–(**841**), and macrolactinic and isomacrolactinic acids (**842**) and (**843**), of an unprecedented C_{24} linear acetogenin origin, from the culture broth of an apparently taxonomically unclassifiable marine bacterium.[690] This bacterium was obtained from a deep-sea core, collected at a depth of 980 m in the North Pacific, and was a motile, Gram-positive, oxidase and catalase positive, unicellular bacterium with a strong salt requirement for growth. Macrolactin A (**836**), the parent aglycone, showed selective antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* at concentrations of 5 μg/disk and 20 μg/disk, respectively, by using standard agar plate-assay disk methods. Macrolactin A (**836**) also inhibited B16-F10 murine melanoma cell replication with *in vitro* IC_{50} values of 3.5 μg ml^{-1}, and (**836**) was a potent inhibitor of *Herpes simplex* type I virus (strain LL), as well as type II virus (strain G) with IC_{50} values of 5.0 μg ml^{-1} and 8.3 μg ml^{-1}, respectively. Further macrolactin A (**836**) protected T-lymphoblast cells against human HIV viral replication. Unfortunately, fermentation of this deep-sea bacterium was unreliable, and macrolactin A (**836**) was no longer available in significant yield. The structures of macrolactins were determined by a combination of spectroscopic techniques that included extensive use of proton NMR spectroscopy. The complete relative and absolute stereochemistries of macrolactines were determined by a combination of ^{13}C-acetonide analysis, degradation, and chemical correlations in collaboration with Rychnovsky's research group.[691]

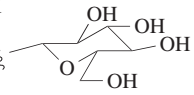
A marine-derived actinomycete of the genus *Streptomyces* was isolated from the surface of an unidentified gorgonian of the genus *Pacifigorgia* by Fenical and co-workers.[692] From the culture broth of the *Streptomyces* sp. two closely related novel compounds, octalactins A (**844**) and B (**845**), were isolated and their structures, including relative stereochemistries, were firmly established, with (**844**) being defined by single-crystal X-ray crystallographic analysis. These metabolites contain unusual saturated eight-membered lactone functionalities and the lactone ring has a boat-chair conformation with a *cis* lactone in the solid state, which was consistent with the result of the





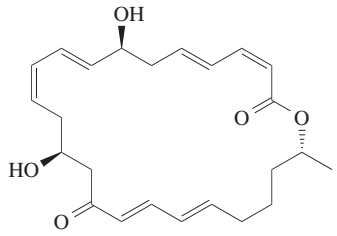
(836) R = H

(838) R =

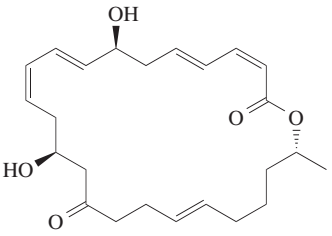


(837) R = H

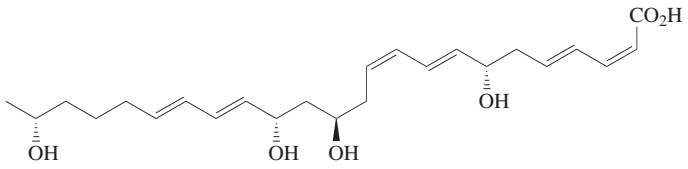
(839) R = $COCH_2CH_2CO_2H$



(840)



(841)



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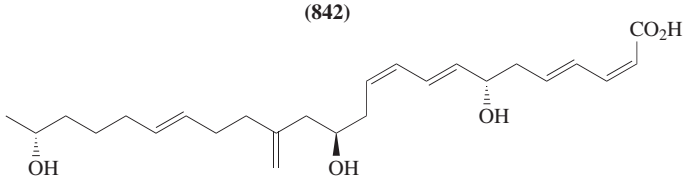
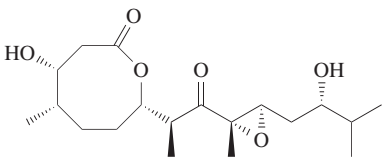
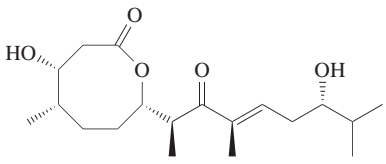


(843)

molecular mechanics calculation. Octalactin A (**844**) exhibited strong cytotoxic activity toward B16-F10 murine melanoma and HCT-116 human colon tumor cell lines with IC_{50} values of 0.0072 μg ml^{-1} and 0.5 μg ml^{-1}, respectively, while quite surprisingly octalactin B (**845**) was completely inactive in the cytotoxicity assay.



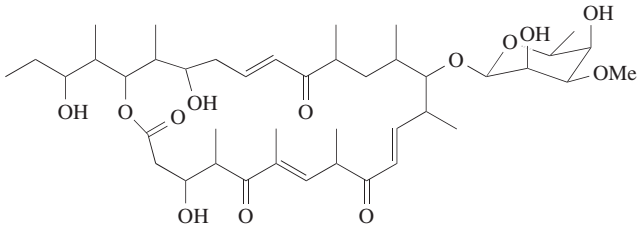
(844)



(845)

Fenical's research group reported the isolation of several more unique secondary metabolites from marine bacteria. From the culture of an unidentified marine bacterium of the order Actinomycetales, which was obtained by serial dilution and plating methods from a marine sediment sample collected

in the shallow waters of Bodega Bay, California, a novel macrolide, maduralide (**846**), possessing a 24-membered lactone ring was isolated.[693] The 6-deoxy-3-*O*-methyl talopyranoside in maduralide (**846**) was unprecedented among macrolide glycosides. Maduralide (**846**) showed weak antibiotic activity against *Bacillus subtilis*. A new γ-lactone (**847**), (1′*R*,2*S*,4*S*)-2-(1-hydroxy-6-methylheptyl)-4-hydroxymethyl-butanolide, was isolated from the culture broth of a marine actinomycete, which was isolated from a sediment sample collected at 24 m depth in the Bahamas Islands.[694] Physiological functions for butanolide (**847**) were not demonstrated. Furthermore, four new alkaloid esters of the rare phenazine class (**848**)–(**851**) were isolated from the fermentation extract of a filamentous bacterium of an unknown *Streptomyces* sp., which was isolated from the shallow sediments in Bodega Bay.[695] The sugar ester functionalities of (**848**)–(**851**) were uncommon, and the natural quinovose in (**848**)–(**851**) was found to possess the rare L configuration from the sign of the Cotton effect in the CD spectrum, although D-quinovose was a common hexose found in the saponin glycosides from marine sea cucumbers. These phenazine esters were shown to exhibit modest broad-spectrum activity against numerous Gram-positive and Gram-negative bacteria. Phenazine (**848**) showed its most potent activity against *Hemophilus influenzae* with Minimum Inhibitory Concentration (MIC) values of 1 $\mu g\ ml^{-1}$ and also inhibited *Clostridium perfringens* (MIC = 4 $\mu g\ ml^{-1}$). Phenazine (**851**) was more active overall, showing inhibitory activities against *E. coli* (4 $\mu g\ ml^{-1}$), *Salmonella enteritidis* (4 $\mu g\ ml^{-1}$), and *Clostridium perfringens* (4 $\mu g\ ml^{-1}$). These compounds were not appreciably cytotoxic against murine and human cancer cells tested *in vitro*.



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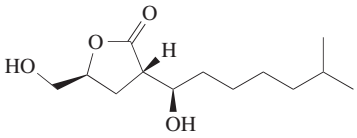

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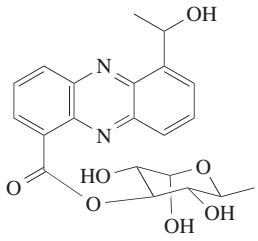
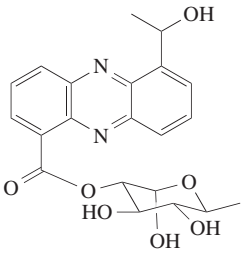


(848)



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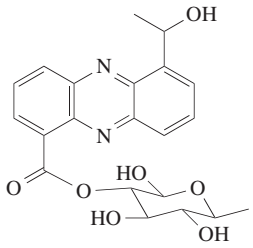

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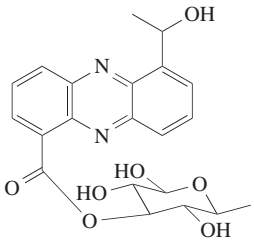
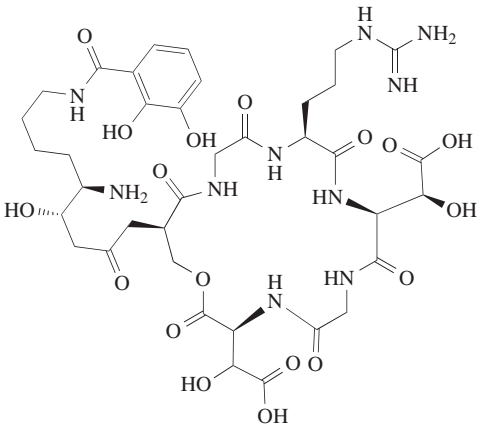


(851)

Several bioactive peptides have been discovered from marine bacteria. Butler and co-workers isolated siderophores having peptide structures, named alterobactins A (**852**) and B (**853**), from an open-ocean bacterium *Alteromonas luteoviolacea*, which was found in oligotrophic and coastal waters.[696] A siderophore is a low-molecular-mass compound with a high affinity for ferric ion which is secreted by microorganisms in response to low-iron environments. Virtually all microorganisms require iron for growth. The paucity of iron in surface ocean water has spurred a lively debate concerning iron limitation of primary productivity, yet little is known about the molecular mech-

anisms used by marine microorganisms to sequester iron. Alterobactin A (**852**) had an exceptionally high affinity constant for ferric iron, suggesting that at least some marine microorganisms may have developed higher-affinity iron chelators as part of an efficient iron-uptake mechanism which is more effective than that of their terrestrial counterparts.



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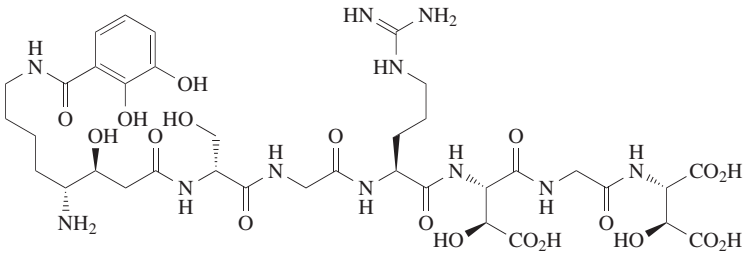
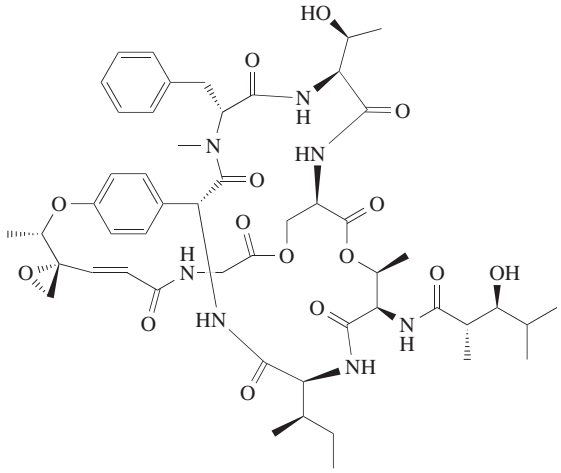


(853)

Fenical's research group isolated antiinflammatory depsipeptides, salinamides A (**854**) and B (**855**), from a marine Streptomycete, which was isolated from the surface of the jellyfish *Cassiopeia xamachana* collected in the Florida Keys.[697] Fermentation in seawater-based media, followed by vacuum-flash chromatography, and reversed-phase HPLC, yielded only salinamide A (**854**) as approximately 9% of the dry extract. A subsequent fermentation yielded almost equal portions of salinamides A (**854**) and B (**855**); the latter was a crystalline compound, and its structure was established by single-crystal X-ray diffraction analysis. The X-ray analysis determined only the relative stereostructure, and chiral GC analysis of the hydrolytic fragments revealed the absolute stereochemistry (D-Thr and L-allo-Thr). The core of salinamide B (**855**) was a bicyclic hexadepsipeptide with two ester links involving serine and threonine residues as well as an aromatic ether link. The connectivity of salinamide B (**855**) severely limited the flexibility of the central core—a feature that complicated the NMR analysis by increasing relaxation times—and a CPK model showed a tightly packed molecular interior. Upon treatment with HCl, salinamide A (**854**) was converted into a chlorohydrin that was identical in all respects to salinamide B (**855**). Salinamides A (**854**) and B (**855**) exhibited moderate antibiotic activity against Gram-positive bacteria. The most potent *in vitro* activity was against *Streptococcus pneumoniae* and *Staphylococcus pyrogenes* with MIC values of 4 μg ml^{-1} for salinamide A (**854**) and 4 μg ml^{-1} and 2 μg ml^{-1} for salinamide B (**855**). More importantly, salinamides A (**854**) and B (**855**) showed potent topical antiinflammatory activity using the phorbol ester-induced mouse ear edema assay. Salinamide A (**854**) showed 84% inhibition of edema and salinamide B (**855**) showed 83% inhibition at the standard testing dose of 50 μg/ear.



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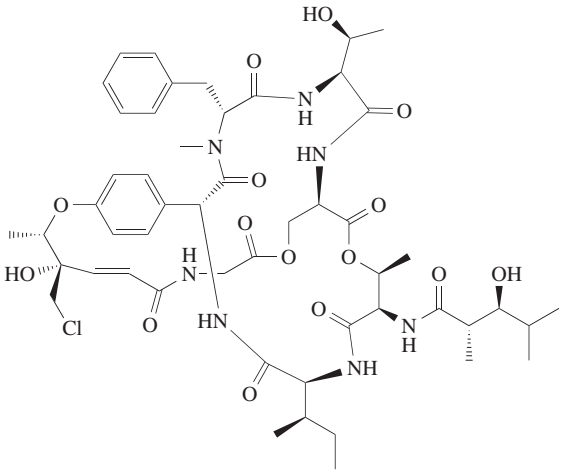
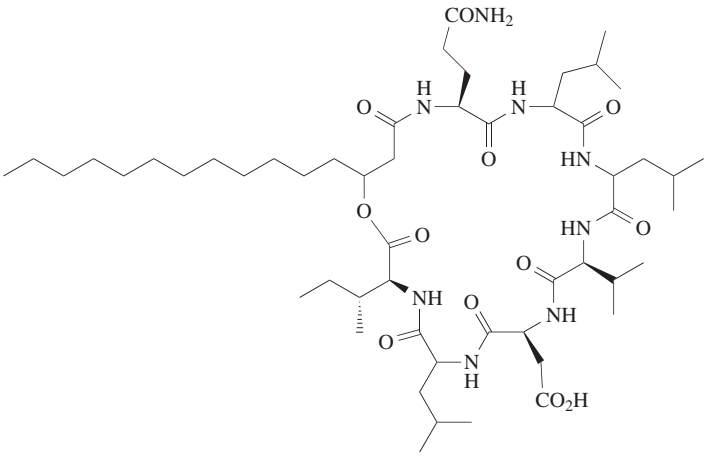


(855)

A cyclic acylpeptide, halobacillin (**856**), was isolated from cultures of a *Bacillus* species, obtained from a marine sediment core taken at 124 m near the Guaymas Basin, Mexico.[698] Halobacillin (**856**) was the first acylpeptide of the iturin class produced by a marine isolate. The iturins are a class of cyclic acylpeptides produced exclusively by several *Bacillus* species, and are characterized as polar cyclic heptapeptides with lipophilic β-acyloxy or β-amino fatty acid components. Halobacillin (**856**) exhibited moderate human cancer cell cytotoxicity, but in contrast to the iturins, no antifungal or antibiotic activity.

A known metabolite, andrimid (**857**), and three new related metabolites, moiramides A (**858**), B (**859**), and C (**860**), were isolated by Andersen and co-workers from solid agar cultures of a marine isolate of the bacterium *Pseudomonas fluorescens*, obtained from tissues of an unidentified tunicate collected at Prince of Wales Island in Moira Sound, Alaska.[699] Andrimid (**857**) was first isolated from cultures of an *Enterobacter* sp. that was an intracellular symbiont of the brown planthopper *Nilaparvata lugens*, and it was found to exhibit potent activity against *Xanthomonas campestris* pv. *oryzae*, the pathogen responsible for causing bacterial blight in rice plants.[700] Andrimid (**857**) and moiramide B (**859**) were found to exhibit potent *in vitro* inhibition of methicillin-resistant *Staphylococcus aureus* with MIC values of 2 $\mu g\ ml^{-1}$ and 0.5 $\mu g\ ml^{-1}$, respectively. Stable isotope incorporation experiments were carried out to elucidate the biogenesis of the acylsuccinimide fragment of andrimid (**857**) that was essential for antimicrobial activity, demonstrating that the acylsuccinimide fragment was derived from a combination of acetate and amino acid building blocks. It was proposed that the biosynthesis proceeded through a dipeptide-like intermediate formed from γ-amino-β-keto acids that were in turn formed from valine and glycine homologated with acetate, presumably via malonyl-CoA.



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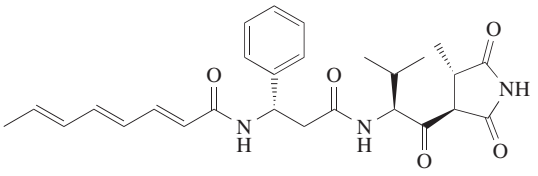

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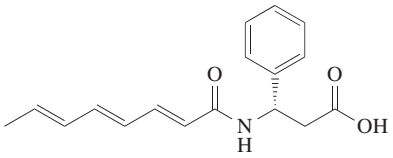

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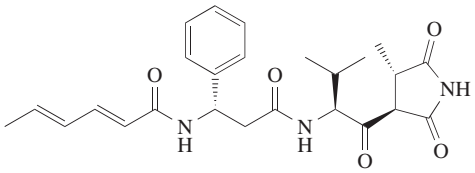

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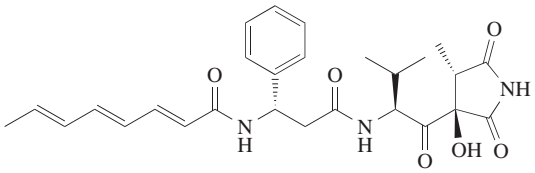
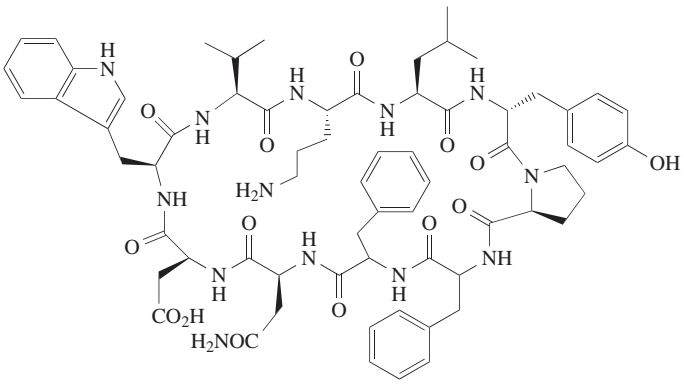


(860)

Andersen and co-workers also isolated a cyclic decapeptide antibiotic, loloatin B (**861**), from the culture of a *Bacillus* sp. obtained from the tissues of an unidentified tube worm collected at 15 m depth off of Loloata Island, Papua New Guinea.[701] Loloatin B (**861**) inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* sp. (VRE), and penicillin-resistant *Streptococcus pneumoniae* with MICs of 1–2 μg ml^{-1}. VRE first appeared in the early 1990s and they are rapidly spreading across North America. It was reported that an outbreak of VRE had a 73% mortality rate and there are no effective antibiotics currently available for such organisms.

Davidson and Schumacher isolated two new caprolactams, caprolactins A (**862**) and B (**863**), from the liquid culture of an unidentified Gram-positive bacterium obtained from a deep-ocean sediment sample.[702] Caprolactins A (**862**) and B (**863**), obtained as an inseparable mixture, were composed of cyclic-L-lysine linked to 7-methyloactanoic acid and 6-methyloctanoic acid, respectively. The structures were proposed using spectroscopic methods and confirmed by synthesis. Both caprolactins A (**862**) and B (**863**) were cytotoxic towards human epidermoid carcinoma KB cells and human colorectal adenocarcinoma LoVo cells, and exhibited antiviral activity towards *Herpes simplex* type II virus.



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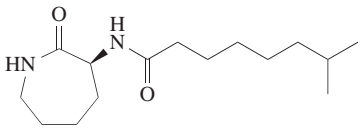

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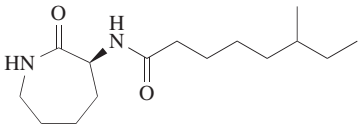
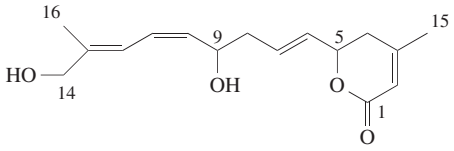


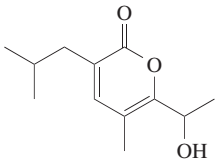
(863)

Several pyrones and related compounds have been reported as marine bacterial metabolites. Oncorhyncolide (**864**) was isolated from liquid shake cultures of the bacterial isolate, which was obtained from a surface seawater sample taken near a chinook salmon (*Oncorhyncus tshawytscha*) net-pen farming operation.[703] This bacterial isolate was an oxidase-positive, Gram-negative bacillus that did not closely resemble any previously described species. Oncorhyncolide (**864**) was a biologically inactive but chemically novel metabolite. Although oncorhyncolide (**864**) had a relatively simple structure, it was not possible to predict the exact nature of its biogenetic origin with any degree of certainty. Stable isotope incorporation studies were therefore undertaken to show that all of the carbons, including the branching methyls at C-15 and C-16 in oncorhynolide (**864**) were derived from acetate.[704]



(864)

Four new α-pyrones, elijopyrones A–D (**865**)–(**868**), were isolated from a cultured marine actinomycete, which was obtained from a sediment collected from the San Elijo Lagoon, Cardiff, California.[705] The structures of elijopyrones A–D (**865**)–(**868**) were determined by comprehensive spectral analyses.



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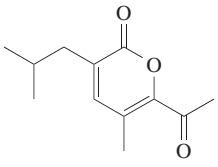

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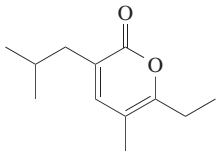

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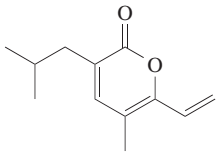
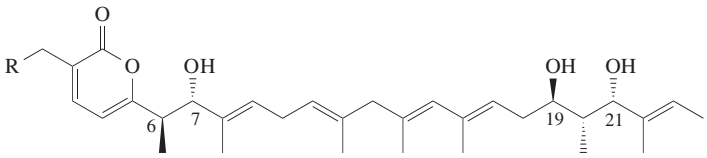


(868)

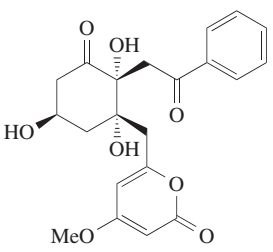
Three cytotoxic α-pyrones, lagunapyrones A–C (**869**)–(**871**), were produced in fermentation by a marine bacterium, which was an unidentified actinomycete isolated from sediment collected in the Agua Hedionda Lagoon in Carlsbad, California.[706] The structure was assigned on the basis of comprehensive spectroscopic analyses, and transformation of lagunapyrone B (**870**) into its [1′,3′-$^{13}C_2$]-labeled acetonide allowed the relative stereochemistry of the flexible 1,3-diol moiety to be

determined as 19*R**, 20*R**, and 21*S**. Assignment of the relative stereochemistry at C-6 and C-7 was accomplished as 6*R**, 7*S** by computer analysis of the vicinal proton coupling constants, and by comparison of these values with two isomeric synthetic analogues. Since the C-6, C-7 stereochemistry could not be related to the relative stereochemistry assigned at C-19 through C-21, the alternative 6*S**, 7*R**, 19*R**, 20*R**, and 21*S** configuration may also be assigned. The carbon skeleton of the lagunapyrones was not previously observed. Biosynthetically, these compounds could be derived by the condensation of acetate and/or propionate units, or by a combination including methylation of the carbon chain through the methionine pathway. Lagunapyrone B (**870**) showed modest *in vitro* cytotoxicity, $ED_{50} = 3.5\ \mu g\ ml^{-1}$, against the human colon cancer cell line HCT-116. A structurally similar metabolite, leptomycin, isolated from a *Streptomyces* sp. inhibits the proliferation of *Schizosaccharomyces pombe* in both the G1 and the G2 phases of the cell cycle.[707]



(869) R = H
(870) R = CH_2Me
(871) R = CH_2CH_2Me

Four new α-pyrone-containing metabolites, wailupemycins A–C (**872**)–(**874**) and 3-pi-5-deoxyenterocin (**875**) were isolated together with known compounds enterocin (**876**) and 5-deoxyenterocin (**877**) from a *Streptomyces* sp., which was obtained from shallow water marine sediments collected at Wailupe beach park on the southeast shore of Oahu, Hawaii.[708] Compounds (**872**)–(**875**) were tested for antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli in vitro* using the paper disk diffusion method. Compound (**875**) exhibited activity against only *S. aureus* (18 mm zone of inhibition at 1 mg/6 mm disk), while compound (**872**) was inhibitory to only *E. coli* (15 mm zone at 0.1 mg/6 mm disk). Compounds (**873**) and (**874**) were inactive against all three test organisms at 0.1 mm/disk.



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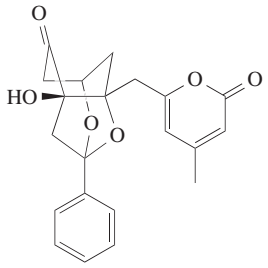

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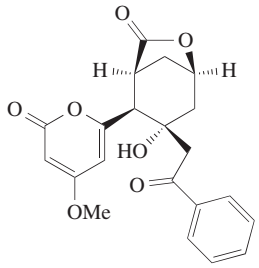

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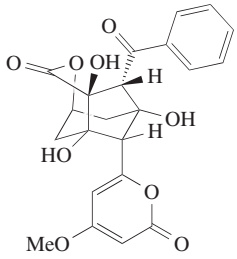

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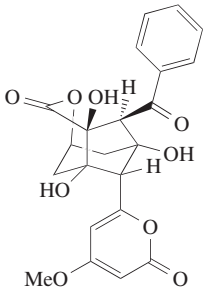

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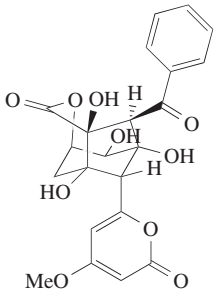
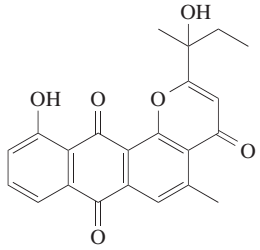


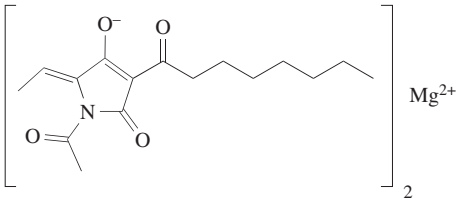
(877)

A new pluramycin-type metabolite, γ-indomycinone (**878**), was isolated together with some previously known metabolites from the culture broth of an actinomycete identified as *Streptomyces* sp. obtained from a deep-ocean sediment sample.[709] Pluramycin antibiotics are most commonly isolated from terrestrial *Streptomyces* sp., and contain an anthraquinone-γ-pyrone nucleus. γ-Indomycinone (**878**) bore a 1-hydroxy-1-methylpropyl side-chain attached to the anthraquinone-γ-pyrone nucleus. Although the crude EtOAc-extract of the entire culture broth of this bacterium was potently cytotoxic against the KB cell line with an MIC value of $\leqslant 0.001$ μg ml^{-1}, the cytotoxicity of the new compound (**878**) was not described, but the rubiflavin antibiotic complex, which was related to (**878**), was shown to bind to DNA, inhibiting DNA synthesis.



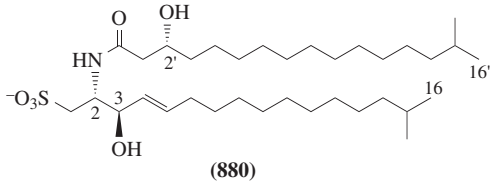
(878)

Magnesidin, a magnesium-containing antibiotic, was isolated from a marine bacterium *Pseudomonas magnesiorubra*, obtained from the surface of macro alga *Caulerpa peltate*, and was described to consist of two analogous components and magnesium.[710,711] Imamura *et al*. isolated magnesidin A (**879**), a component of magnesidin, from halophilic bacterium *Vibrio gazogenes*, obtained from marine mud, and its structure including stereochemistry was elucidated by mainly NMR data.[712] Magnesidin A (**879**) as well as its magnesium-free sample were active against microalga *Prorocentrum micans* at the concentration of 1 μg ml^{-1} and weakly active against *Bacillus subtilis*.



(879)

Kobayashi *et al*. investigated extracts of cultured marine bacterium *Flavobacterium* sp., which was isolated from the bivalve *Cristaria plicata* collected in Ishikari Bay, Hokkaido, and succeeded in isolating two new sphingolipids, flavocristamides A (**880**) and B (**881**),[713] possessing a sulfonic acid group. The structures of compounds (**880**) and (**881**) along with the stereochemistry of all chiral centers were elucidated on the basis of extensive spectroscopic analyses including FAB MS/MS measurements as well as degradation experiments such as acid hydrolysis and Lemieux oxidation. Both (**880**) and (**881**) exhibited marked inhibitory activity against DNA polymerase α. Ceramide-1-sulfonic acids were previously obtained from marine diatom *Nitzschia alba*,[714] and studies on the synthesis of ceramide-1-sulfonic acids were also reported.[715] An identical compound to flavocristamide B (**881**), named sulfobactin A, was isolated independently by Kamiyama *et al*. almost at the same time from the culture broth of terrestrial bacterium *Chryseobacterium* sp. (*Flavobacterium* sp.) as a von Willebrand factor (vWF) receptor antagonist.[716,717] Sulfobactin A (=flavocristamide B (**881**)) inhibited the binding of vWF to its receptor with an IC_{50} value of 0.47 μM, and it also inhibited ristocetin-induced agglutination in human platelets fixed with paraformaldehyde with an IC_{50} value of 0.58 μM.



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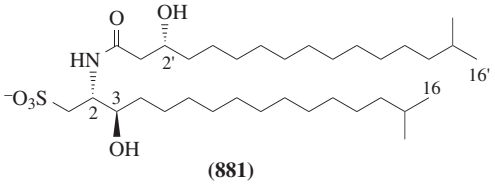
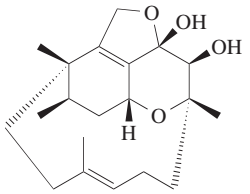


(881)

8.07.9.4.2 Fungi

Chemical studies on the bioactive metabolites of fungal isolates from marine environments have been limited. Marine fungi, however, can be expected as a potential source of new bioactive substances from the following studies. Researchers of the Sankyo group isolated the novel platelet activating factor (PAF) antagonists, phomactins A (**882**),[718] B (**883**), B_1 (**884**), B_2 (**885**), C (**886**), D (**887**),[719] E (**888**), F (**889**), and G (**890**)[720] from the cultured broth of a marine deuteromycetes, *Phoma* sp., which lived in the shell of a crab *Chionoecetes opilio* collected off the coast of Fukui Prefecture, Japan. Phomactins are unique diterpenoids and their structures, including the absolute configurations, were established by the X-ray analyses performed on a mono-*p*-bromobenzoyl derivative of phomactin A (**882**) as well as a diketone derived from phomactin B (**883**). Phomactins B_1 (**884**) and B_2 (**885**) were chemically correlated with phomactin B (**883**). Compounds (**882**)–(**890**) exhibited PAF antagonistic activities. In particular, phomactin D (**887**) potently inhibited the binding of PAF to its receptors and PAF-induced platelet aggregation with IC_{50} values of 0.12 μM and 0.80 μM, respectively, while other compounds antagonized PAF action at higher concentrations. Phomactin A (**882**), however, exhibited no effect on adenosine diphosphate-, arachidonic acid-, and collagen-induced platelet aggregation. Phomactin A (**882**) was thus considered to be a new type of specific PAF antagonist. Chu *et al*. also reported several PAF antagonists with the same skeletal framework as phomactins.[721,722]



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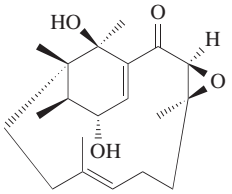

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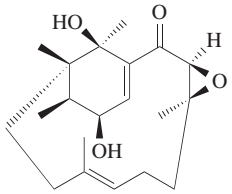

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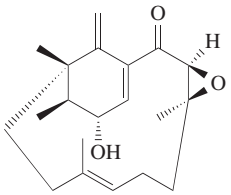

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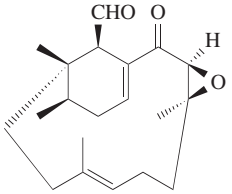

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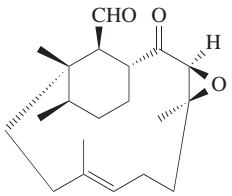

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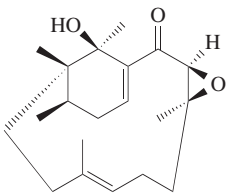

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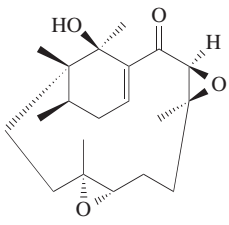

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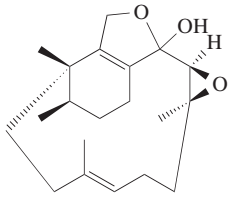
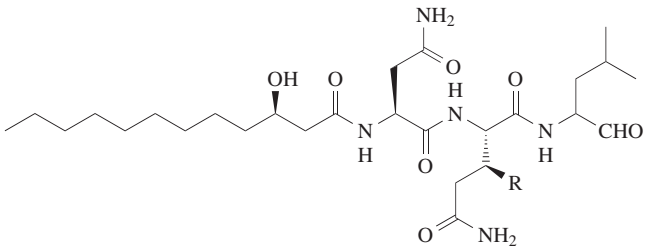


(890)

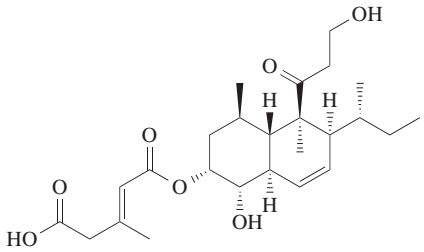
Two lipophilic tripeptides, fellutamides A (**891**) and B (**892**), have been isolated by Kobayashi's research group from the cultured mycelium of a fungus *Penicillium fellutanum* Biourge which was isolated from the gastrointestine of the marine fish *Apogon endekataenia* Bleeker, collected off Manazuru beach, Kanagawa Prefecture, Japan.[723] The structures of fellutamides were elucidated by applying several types of 2D NMR techniques in combination with FAB MS/MS data. The

stereochemistries of amino acid residues were determined by chiral HPLC and GC analyses and the absolute configuration of 3-hydroxydodecanoic acid obtained through hydrolysis was assigned by comparison of the optical rotation with those of standard samples. Fellutamides A (**891**) and B (**892**) were potently cytotoxic against murine leukemia P388 (IC_{50} 0.2 μg ml^{-1} and 0.1 μg ml^{-1}, respectively) and L1210 (IC_{50} 0.8 μg ml^{-1} and 0.7 μg ml^{-1}, respectively) cells, and human epidermoid carcinoma KB (IC_{50} 0.5 μg ml^{-1} and 0.7 μg ml^{-1}, respectively) cells *in vitro*. The fungus *Penicillium fellutanum* is not a marine-specific one, since the same species have been isolated from terrestrial sources. The peptides (**891**) and (**892**) which are structurally related to the leupeptines[724] exhibited stimulating activity on nerve growth factor (NGF) synthesis.[725]



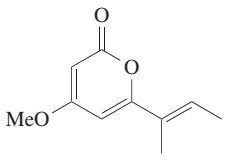
(891) R = OH
(892) R = H

Kitagawa and co-workers isolated a new polyketide compound, trichoharzin (**893**), from a culture of the imperfect fungus *Trichoderma harzianum*, which was separated from the marine sponge *Micale cecilia*, collected at Amami Island, Kagoshima Prefecture, Japan.[726] The absolute stereochemistry of trichoharzin (**893**) was determined by spectral data and CD spectrum of its dibenzoate derivative. Trichoharzin (**893**) was a new polyketide constructed with an alkylated decalin skeleton and esterified with 3-methylglutaconic acid, a rare acyl moiety. It exhibited inhibitory activity against morphological differentiation of *Candida albicans*.



(893)

Crews and co-workers also reported studies on marine fungal metabolites. The salt-water culture of an unidentified fungus separated from an Indo-Pacific marine sponge of the genus *Stylotella* sp., collected in the Somosomo Strait near Taveuni, Fiji, was revealed to yield new tetraketide natural products, demethyl nectriapyrone A (**894**) and B (**895**).[727] These α-pyrone-containing compounds are analogous to nectriapyrone (**896**), previously reported from a terrestrial fungus, and also to the α-pyrones obtained from marine bacteria (see Section 8.07.9.4.1).



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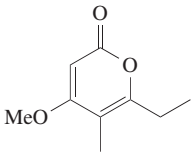

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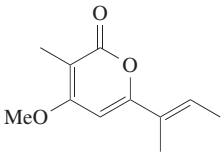
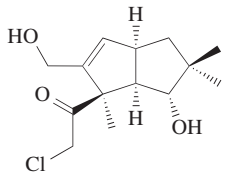


(896)

Three new chlorinated cyclic sesquiterpenes, chloriolins A–C (**897**)–(**899**), were isolated from an unidentified fungus which was initially separated from the Indo-Pacific marine sponge *Jaspis* aff. *johnstoni* and then cultured on marine media.[728] 2D NMR experiments, synthetic transformations, and X-ray crystallography were used to establish the structures of these compounds. The sesquiterpenes (**897**)–(**899**) were, however, inactive in the National Cancer Institute (NCI) disease-oriented screening program.



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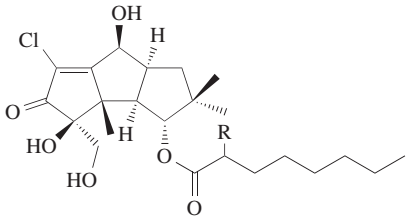
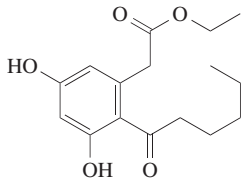


(898) R = OH
(899) R = H

From the salt-water culture of an unidentified fungus separated from inside the encrusting sponge *Spirastrella vagabunda*, collected from the Togian Islands in central Sulawesi, Indonesia, a new polyketide natural product, 14,15-secocurvularin (**900**), was isolated by bioassay guided fractionation, and its structure was elucidated by spectral studies.[729] 14,15-Secocurvularin (**900**) exhibited mild antibiotic activity against *Bacillus subtilis*. Interestingly, curvularin (**901**) had been reported to be isolated from four different fungi (*Curvularia*, *Cochliobolus*, *Penicillium*, and *Alternaria*).



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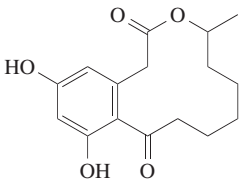
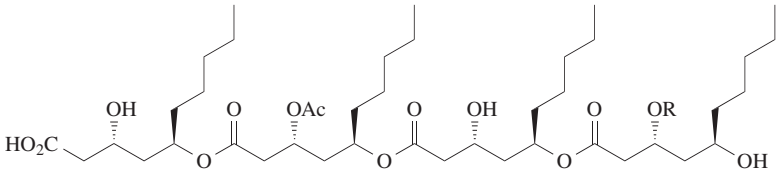


(901)

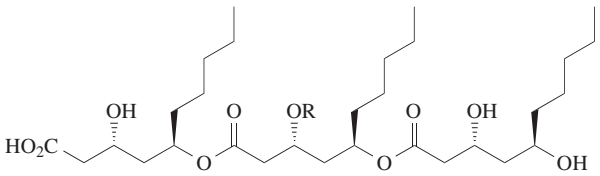
Chen *et al*. isolated three antimicroalgal substances, halymecins A–C (**902**)–(**904**), from the fungus, *Fusarium* sp. of an alga (*Halymenia dilatata*) isolate collected at Palau, by using centrifuged partition chromatography. Another fungus, *Acremonium* sp. of an alga isolate collected at Aburatsubo, Japan, contained two related metabolites, halymecins D (**905**) and E (**906**). Halymecins A–E (**902**)–(**906**) exhibited inhibition activity against diatom *Skeletonema costatum* at 10 μg ml^{-1}. Halymecins A (**902**) also showed antimicroalgal activity against blue-green alga *Oscillatoria amphibia* (500 μg ml^{-1}), green alga *Brachiomonas submarina* (67.5 μg ml^{-1}), and dinoflagellate *Prorocentrum micans* (67.5 μg ml^{-1}).[730] A structurally related antibacterial compound, exophilin A (**907**), was isolated by Nippon Suisan's research group in the culture of the marine microorganism *Exopiala pisciphila*, which was separated from a marine sponge *Mycale adhaerens*.[731] The absolute chemical structure of exophilin A (**907**) was elucidated as a trimer of (3*R*,5*R*)-3,5-dihydroxydecanoic acid by spectroscopic methods and analysis of a degradation product. Exophilin A (**907**) showed antimicrobial activity against Gram-positive bacteria. The marine microorganism *Exopiala pisciphila* is a member of the so-called "black yeasts," a group presenting taxonomic problems, because of their developmental plasticity and the limited number of morphological characteristics available for classification.

A new tricyclic sesquiterpene, isoculmorin (**908**), was isolated by Alam *et al*. from the marine fungus *Kallichroma tethys*, which was cultured in a seawater medium, and the structure was determined by X-ray crystallography.[732]

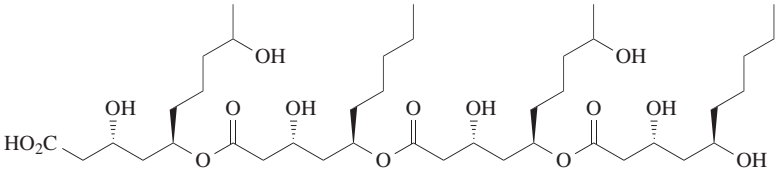
Numata *et al*. have reported a series of studies on bioactive metabolites of fungi separated from marine algae or fish. Communesins A (**909**) and B (**910**) were isolated from the mycelium of a strain of *Penicillium* sp. stuck on the marine alga *Enteromorpha intestinalis*, and (**909**) and (**910**) exhibited moderate to potent cytotoxic activity in the P-388 lymphocytic leukemia in cell culture with ED_{50} values of 3.5 μg ml^{-1} and 0.45 μg ml^{-1}, respectively.[733]



(902) R = H
(903) R = β-D-Mannosyl



(904) R = Ac
(907) R = H



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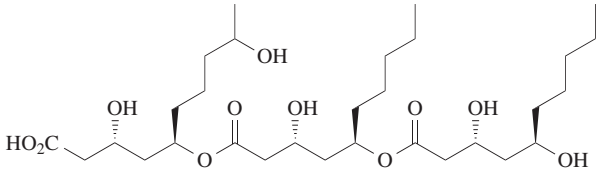

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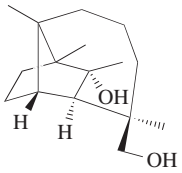

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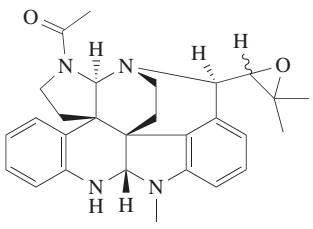

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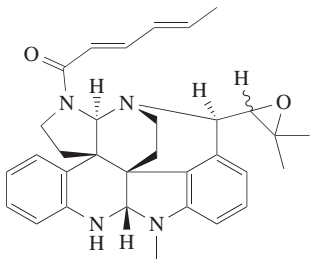
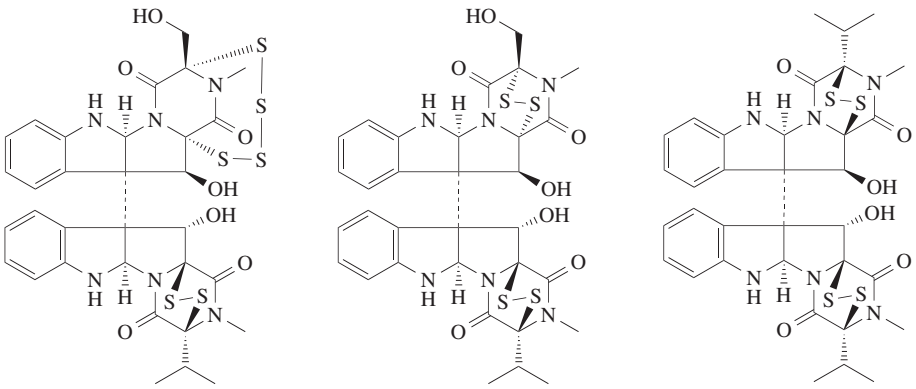


(910)

Leptosin A (**911**) and its analogues leptosins B, C, D, E, F, G, G_1, G_2, H, I, J, K, K_1, and K_2 were isolated from a strain of *Leptosphaeria* sp. attached to the marine alga *Sargassum tortile*.[734–737] They were potently cytotoxic against cultured P388 cells ((**911**): ED_{50} 1.85 ng ml^{-1}) and leptosins A (**911**) and C (**912**) exhibited significant antitumor activity against Sarcoma 180 ascites (T/C 260% and 293%, respectively) at a dose of 0.5 mg kg^{-1} and 0.25 mg kg^{-1}, respectively. The structure of leptosin K (**913**) with a different configuration from that of leptosin A (**911**) or C (**912**) was determined by X-ray analysis. Leptosin K (**913**) was revealed to exist in a mixture of four conformers by X-ray and NOE studies.[737]



(**911**) (**912**) (**913**)

Seven quinazoline alkaloids, fumiquinazolines A–G (**914**)–(**920**) were isolated from the cultured mycelium of a strain of *Aspergillus fumigatus* which existed in the gastrointestinal tract of the salt-water fish *Pseudolabrus japonicus*.[738,739] Their structures were based on spectral and chemical evidences as well as X-ray diffraction analysis of (**919**). The absolute stereostructure of (**919**) was revealed by the production of L-(+)-alanine through its acid hydrolysis. All these compounds (**914**)–(**920**) exhibited moderate cytotoxic activity against cultured P388 lymphocytic leukemia cells.

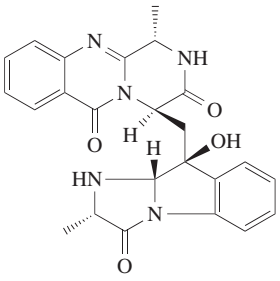
Halichomycin (**921**), a new class of cytotoxic compounds, was isolated from a strain of *Streptomyces hygroscopicus* which was obtained from the gastrointestinal tract of the marine fish *Halichoeres bleekeri*.[740] Halichomycin (**921**) exhibited cytotoxicity (ED_{50} 0.13 μg ml^{-1}) in the P388 lymphocytic leukemia test system in cell culture. The relative stereochemistry of (**921**) was established by NOESY experiments. Although Numata and co-workers described this compound as a macrolide, the biosynthetic provenance and path of compound (**921**) appeared to be strange.

From the same fungal strain of *Penicillium* sp., which was originally separated from the marine alga *Enteromorpha intestinalis*, as that which produced communesins A (**909**) and B (**910**), a new class of cytochalasans, named penochalasins A (**922**), B (**923**), and C (**924**) were isolated and their stereostructures were established on the basis of NMR spectral analyses and chemical transformations.[741] Different conformations of penochalasin A (**922**) in $CDCl_3$ and pyridine-d_5 were determined by analysis of NMR data and molecular modeling. All three compounds exhibited cytotoxicity against cultured P388 cells (ED_{50} 0.3–0.5 μg ml^{-1}).

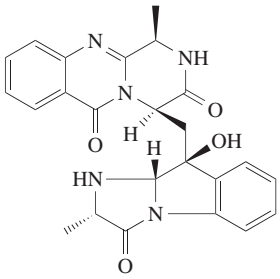
Furthermore, the same *Penicillium* sp. also produced four new cytotoxic metabolites, penostatins A–D (**925**)–(**928**), and their stereostructures were established on the basis of spectral analyses.[742] Compounds (**925**)–(**927**) exhibited significant cytotoxicity against cultured P388 cells (ED_{50} 0.8–1.2 μg ml^{-1}), while the cytotoxicity of (**928**) was weak (ED_{50} 11.5 μg ml^{-1}).

8.07.9.4.3 Blue-green algae

Blue-green algae or cyanobacteria (cyanophytes) have been demonstrated as a source of interesting natural products of significant bioactivities, in particular, by a series of leading studies by Moore *et al.*[743] His research group isolated debromoaplysiatoxin (**929**)[744] and lyngbyatoxin (**930**)[745] from the filamentous blue-green alga *Lyngbya majuscula* in the 1970s. These natural products had structural similarities to those isolated from sea hare *Aplysia* (aplysiatoxins) and antibiotics from *Streptomyces* (teleocidins), respectively. Moore further studied the metabolites of blue-green alga *Lyngbya*



(914)



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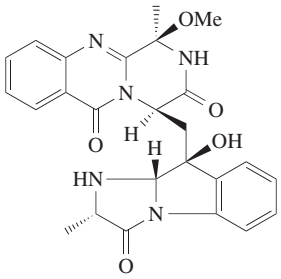

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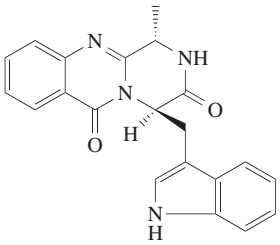

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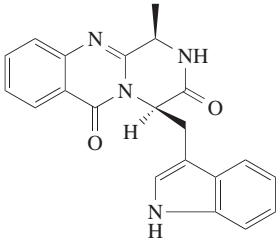

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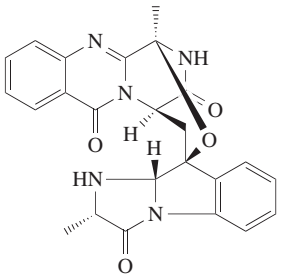

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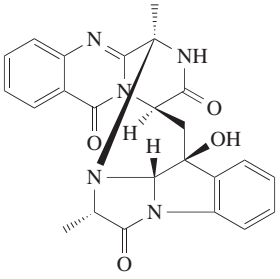

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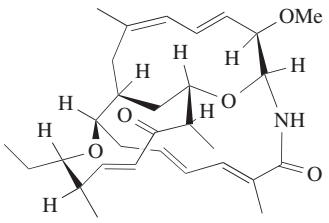

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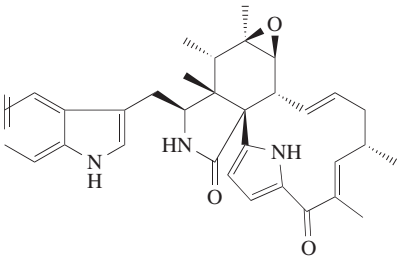

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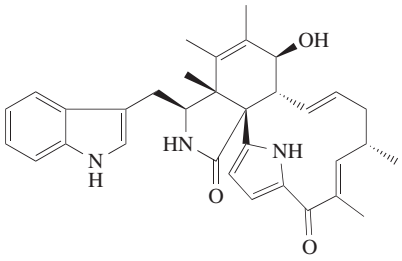

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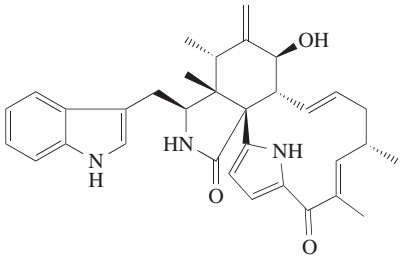

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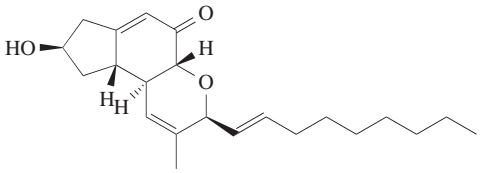

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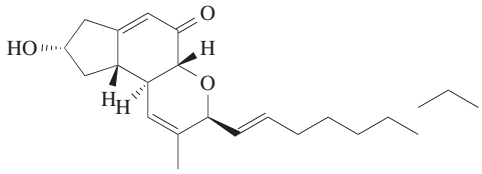

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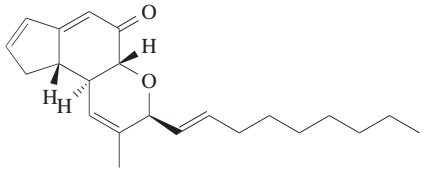

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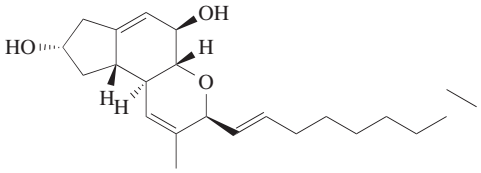

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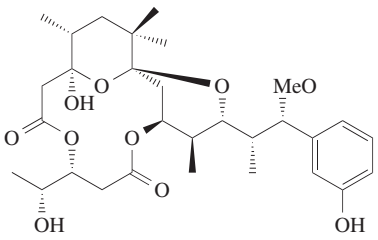

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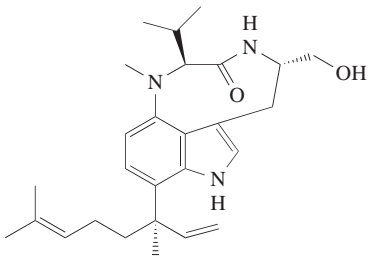
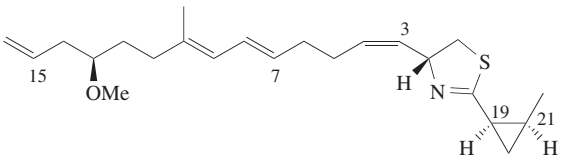


(930)

majuscula in the 1970s and 1980s and isolated a number of unique natural products such as majusculamides, malyngolide, malyngamides, malyngic acid, pukeleimides, and others.[746]

In 1994, Gerwick *et al.* studied the organic extract of *Lyngbya majuscula*, a Caribbean marine cyanobacterium collected from Curaçao, Netherland Antilles. The extract was found to be strongly cytotoxic against a Vero cell line and also highly toxic to brine shrimp ($LC_{50} = 25$ ng ml^{-1}). Using a brine shrimp assay to guide fractionation, a unique metabolite, named curacin A (**931**), was isolated as 8–10% of the crude extract and was found to be responsible for the potent brine shrimp toxicity ($LC_{50} = 3$ ng ml^{-1}) as well as mammalian cell antiproliferative activity ($IC_{50} = 6.8$ ng ml^{-1} in the Chinese hamster Aux B1 cell line).[747] The structure containing unique thiazoline and cyclopropane moieties was deduced by spectroscopic analyses, and the complete relative and absolute configuration of curacin A (**931**) was defined by comparison of products obtained from chemical degradation of the natural product with the same substance prepared by synthesis.[748] Curacin A (**931**) was partially hydrogenated with Wilkinson's catalyst to yield a mixture of 15,16-dihydro and 3,4,15,16-tetrahydro derivatives. This mixture was ozonized and the ozonide was reduced with excess dimethyl sulfide to yield 5-methoxyoctan-2-one (**932**). On the other hand, ozonolysis of curacin A (**931**) ($-78\,^{\circ}C$, $CHCl_3$, 2 min), followed by oxidative workup (H_2O_2, $45\,^{\circ}C$, 16 h), and then reaction with excess CH_2N_2 in Et_2O gave a methyl sulfonate derivative (**933**). Optically active (**932**) and (**933**) were obtained by enantioselective syntheses. Thus, curacin A (**931**) was shown to have 2*R*, 13*R*, 19*R*, and 21*S* absolute configuration.



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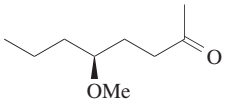

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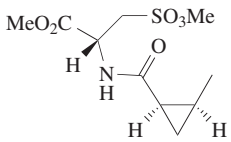
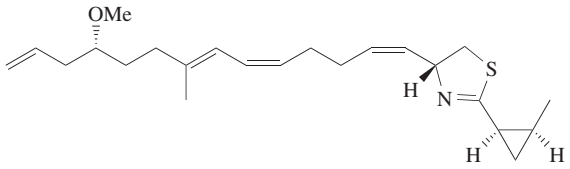


(933)

Curacin A (**931**) was examined in the NCI cell line screen, and its differential cytotoxicity pattern was evaluated by the COMPARE algorithm, indicating that curacin A (**931**) was an antitubulin agent. Pure curacin A (**931**) was revealed to be an antimitotic agent (IC_{50} values in three tumor cell lines, ranging from 7 nM to 200 nM) that inhibited microtubule assembly and the binding of colchicine to tubulin. Curacin A (**931**) probably binds in the colchicine site because it competitively inhibited the binding of [^{3}H]colchicine to tubulin with an apparent K_i value of 0.6 μM and stimulated tubulin-dependent GTP hydrolysis, as do most other colchicine-site agents. The binding of curacin A (**931**) to tubulin resembled the binding reactions of combretastatin A-4 and podophyllotoxin in contrast to that of colchicine in that it occurred as extensively on ice as at higher temperatures. However, once bound, the dissociation rate of curacin A (**931**) from tubulin was very slow, most closely resembling that observed with colchicinoids (thiocolchicine was the drug examined) than the faster dissociation that occurs with combretastatin A-4 and podophyllotoxin. Because the molecular structure of curacin A (**931**) is so different from that of previously described colchicine-site drugs (e.g., there is no aromatic moiety, and there are only two conjugated double bonds in its linear hydrocarbon chain), the activities of natural isomers, curacins B (**934**) and C (**935**),[749] and synthetic derivatives were also examined. Only modest enhancement or reduction of activity was observed with a variety of structural changes.[750]

Gerwick and co-workers further studied the metabolites of the blue-green alga *L. majuscula* and isolated several bioactive substances. From a Curaçao collection of *L. majuscula*, a new lipopeptide, malyngmide H (**936**), was isolated, guided by ichthyotoxic activity against goldfish, together with



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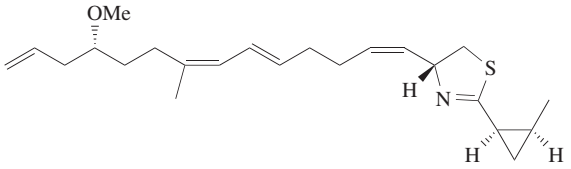
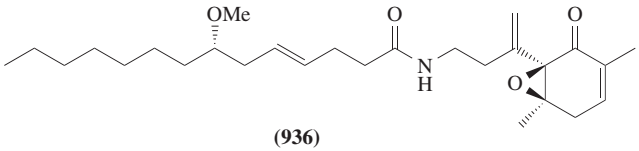


(935)

the corresponding free acid, 7-methoxytetradec-4*E*-enoic acid (**937**).[751] The absolute stereochemistry of the cyclohexenone moiety of malyngamide H (**936**) was deduced by a combination of 2D NOESY and exciton chirality CD spectroscopy. Malyngmide H (**936**) showed ichthyotoxic effect ($LC_{50} = 5$ μg ml^{-1}, $EC_{50} = 2$ μg ml^{-1}), but it was not active in brine shrimp lethality or molluskicidal assays. Malyngamide H (**936**) may be part of the natural defense of this cyanobacterium. An ichthyotoxic amide of 7(*S*)-methoxytetradec-4(*E*)-enoic acid, malyngamide I (**938**), was isolated from *L. majuscula* collected from shallow water at Uken, Okinawa.[752] Malyngamide I (**938**) was moderately toxic towards brine shrimp (*Artemia salina*, LD_{50} ca. 35 μg ml^{-1}) and goldfish (*Carassius auratus*, $LD_{50} < 10$ μg ml^{-1}). Data from the acetate derivative (**939**) of this amide were used for the probable structure



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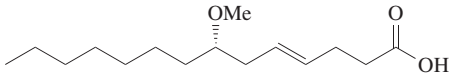

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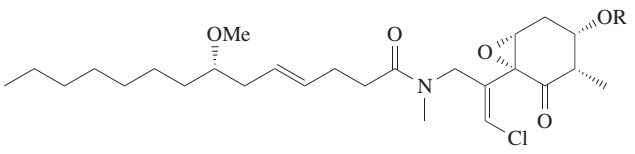
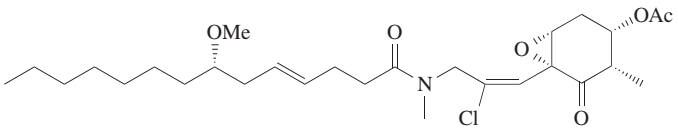


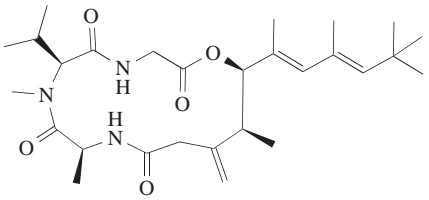
(938) R = H
(939) R = Ac



(940)

revision of stylocheilamide (**940**),[753] a previously reported metabolite from the sea hare *Stylocheilus longicauda*. The structure of stylocheilamide should likely be revised from (**940**) to (**939**), being in keeping with the established structures of the other chlorine-containing malyngamides and also consistent ecologically with the fact that *Stylocheilus longicauda* is believed to have a dietary origin from *L. majuscula*.

The lipid extract of a Curaçao collection of *L. majuscula*, which contained curacin A (**931**), exhibited several bioactivities including potent brine shrimp toxicity (*Artemia salina*, $LC_{50} = 25$ μg ml^{-1}), goldfish ichthyotoxicity (*Carassius auratus*, $LC_{50} = 25$ μg ml^{-1}), and molluskicidal activity (*Biomphalaria glabrata*, $LC_{100} < 100$ μg ml^{-1}). The brine shrimp toxicity was traced to curacin A (**931**), and part of the ichthyotoxicity was ascribed to malyngamide H (**936**). Fractionation using fish and snail bioassays led to the isolation of two other distinct classes of natural products from this organism, i.e., the most ichthyotoxic compound, antillatoxin (**941**) ($LD_{50} = 0.05$ μg ml^{-1}), and a strongly molluskicidal agent, barbaramide (**942**) ($LD_{100} = 10$ μg ml^{-1}). Antillatoxin (**941**)[754] was isolated in small yield as an amorphous powder (1.3 mg, 0.07% of extract) and its structure was elucidated on the basis of spectroscopic data to be composed of a lipid portion and three amino acid residues, glycine, *N*-methylvaline, and alanine. The stereochemistries of the *N*-methylvaline and alanine residues were both assigned to be the L configuration from chiral phase TLC analysis. The stereochemistry at the C-4 and C-5 positions in the lipid portion was examined using a combination of molecular modeling, NOESY data, *J* values, and CD spectroscopy. Modeling of antillatoxin's structure was accomplished using a dynamic simulated annealing protocol with the program XPLOR. The calculated structure of the 4*S*,5*R* stereoisomer was most consistently accounted for by the NMR and CD spectral data, thus assigning the absolute stereochemistry of antillatoxin (**941**) as 4*S*, 5*R*, 2′*S*, 5′*S*. On the other hand, barbaramide (**942**)[755] was a novel lipopeptide containing a trichloromethyl group and the methyl enol ether of a β-keto amide.



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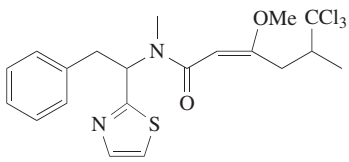
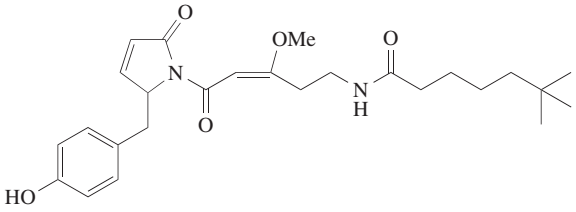


(942)

The trichloromethyl portion of barbamide (**942**) closely resembled the trichloromethyl portion of dysidin, a polychlorinated amino acid derivative found in the sponge *Dysidea herbaceae*.[756] Microscopic investigations of *Dysidea* showed it to be rich in symbiotic filamentous cyanobacteria. A flow-cytometric separation of the symbiont *Oscillatoria spongeliae* from the sponge cells suggested that the polychlorinated amino acid derivatives were associated with the cyanobacterial filaments.[757] The finding of structurally similar components in the marine cyanobacterium *L. majuscula* provided further support for the cyanobacterial origin of these metabolites. It was interesting that the amine portion of barbamide (**942**) was the *N*-methyl equivalent of dolaphenine (*N*-methyldolaphenine), a structural component of the antineoplastic peptide dolastatin 10 isolated from the sea hare *Dolabella auricularia*.[758] Hence, it is conceivable that dolastatin 10, or at least a portion of it, also arises from cyanobacterial metabolism. Sea hare are well known to incorporate unique secondary metabolites from their algal diets. The crude lipid extract of a Curaçao collection of *L. majuscula* exhibited different biological properties including brine shrimp toxicity, goldfish ichthyotoxicity, and molluskicidal activity. Fractionation guided by these different biological assays led to isolation of four distinctly different classes of natural products, each of which was selective in its range of activity. The molluskicidal activity was due to barbamide (**942**), while the ichthyotoxicity was due to two components, antillatoxin (**941**) and malyngamide H (**936**). Finally, the brine shrimp toxicity was traced to a potent new antimitotic agent, curacin A (**931**). The apparently selective activity of each of these compounds to only one class of animal led to the speculation that these four kinds of compounds represent separate adaptations by this cyanobacterium to these different classes of predators.

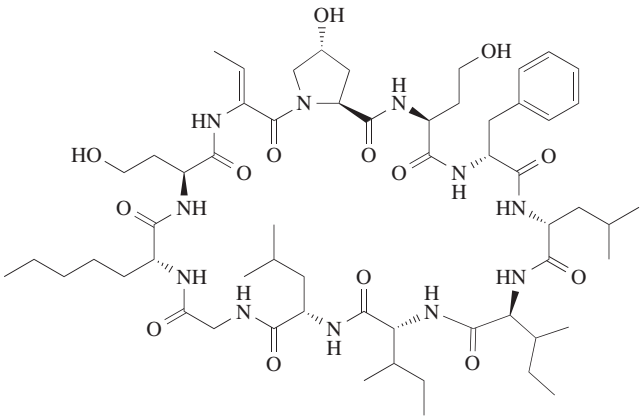
In May 1994, a simultaneous blue-green algal bloom and a massive die-off of pelagic larval rabbitfishes (*Siganus argenteus* and *S. spinus*) occurred at Ypao beach, Guam. From this microbial assemblage which was composed of *Schizothrix calcicola* and *Lynbya majuscula*, a new herbivore antifeedant metabolite, ypaoamide (**943**), was isolated and its structure was determined spectro-

scopically by Paul and co-workers.[759] Isolated cells of the *L. majuscula* were found to produce ypaoamide (**943**) in laboratory culture based on GC–EIMS analysis. Ypaoamide (**943**) had structural similarities to malyngamides or majusculamides, suggesting that common biosynthetic pathways may be employed by different chemotypes of *L. majuscula*. The *t*-butyl lipid side-chain contained in ypaoamide (**943**) appeared unusual with little biosynthetic precedent other than in antillatoxin (**941**).



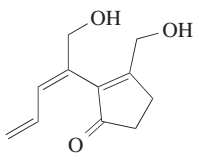
(943)

Gerwick *et al*. studied a suite of cytotoxic and antimicrobial cyclic peptides produced by the Caribbean cyanobacterium *Hormothamnion enteromorphoides*, which grows abundantly in the shallow coastal waters off northern Puerto Rico. The structure of the most lipophilic and abundant of these peptides, hormothamnin A (**944**), was determined by interpretation of physical data, principally high field NMR and FAB MS, in combination with chemical derivatization and degradations. Hormothamnin A (**944**) is a cyclic undecapeptide consisting of several unusual amino acid residues containing D-amino acids.[760]



(944)

Nagle and Gerwick also isolated a series of cytotoxic metabolites, nakienones A–C (**945**)–(**947**) and nakitriol (**948**), from dead and necrotic branches of stony coral (*Acropora* sp.) which were completely covered with a gray-black mat of cyanobacteria (*Synechocystis* sp.) in the waters off Yonaine at Nakijin Village, Okinawa.[761] Nakienone A (**945**) was cytotoxic against KB and HCT 116 cell lines, with LD_{50} values of ca. 5 μg ml^{-1} and 20 μg ml^{-1}, respectively. Nakienone C (**947**) was generated from nakienone A (**945**) while obtaining the NMR spectra of (**126**) in $CDCl_3$ solution, presumably through an acid catalyzed rearrangement. Nakienone A (**945**) and C (**947**) were structurally similar to the didemnenones A–D,[762] isolated from *Didemnum* and *Tridemnum* sp., ascidians known to contain unicellular algal symbionts. The tunicate symbiont *Prochloron* is a prochlorophyte which is remarkably similar to the unicellular cyanobacterium *Synechocystis*.



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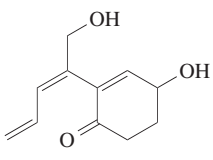

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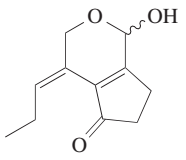

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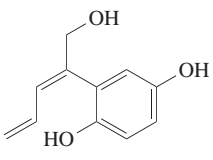
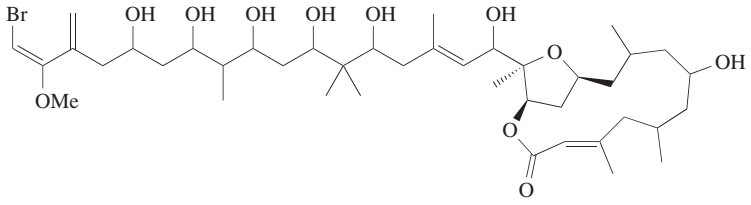


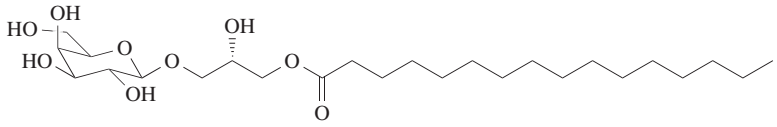
(948)

From a marine reddish blue-green alga *Oscillatoria* sp., Murakami *et al*. isolated a macrolide, oscillariolide (**949**), possessing inhibitory activity of development of fertilized echinoderm eggs.[763] The blue-green alga was collected from Gokashowan-Bay, Mie Prefecture, Japan, and cultured in 1000 l tanks. Oscillariolide (**949**) had a unique 14-membered macrocyclic lactone with a side-chain bearing 1,3-polyol and brominated diene moieties.



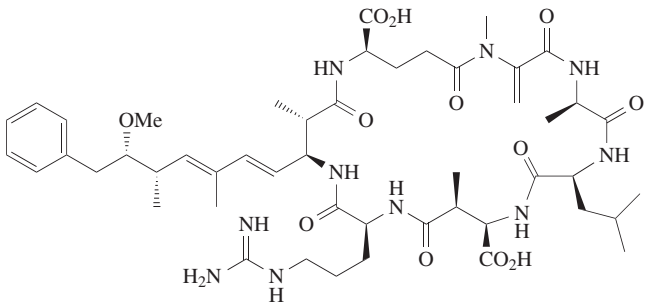
(949)

Ohizumi and co-workers isolated (2*S*)-1-*O*-palmitoyl-3-*O*-*β*-D-galactopyranosylglycerol (**950**) from the cultured cells of the marine Cyanophyceae *Oscillatoria rosea*, supplied by the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Environmental Agency, Japan).[764] The monogalactosylacylglycerol (**950**) caused a concentration-dependent inhibition of platelet aggregation induced by U46619, a thromboxane A_2 analogue, with an IC_{50} value of 6.0×10^{-5} M. Compound (**950**) (100 μg ml^{-1}) only markedly inhibited platelet aggregation induced by U46619 and did not inhibit that induced by thrombin or ionomycin.



(950)

Although only a limited number of studies have been reported on the secondary metabolites of marine blue-green algae, those of freshwater or terrestrial origins have been extensively investigated by several research groups. In particular, potent cyclic heptapeptide hepatotoxins termed microcystins and related peptides, produced by freshwater blue-green algae of the genera of *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria*, have been thoroughly studied, and more than 40 microcystins have been reported.[765] A number of cyclic or linear peptides related to microcystins have also been investigated from freshwater blue-green algae.[766–772] Microcystin LR (**951**) is found most often among the microcystins, and microcystins show strong inhibitory activity against protein phosphatases PP1 and PP2A and are also reported to be tumor promoters.[773–775]

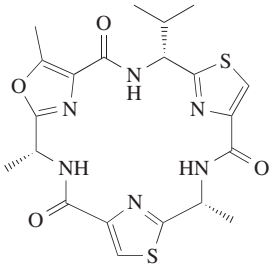


(951)

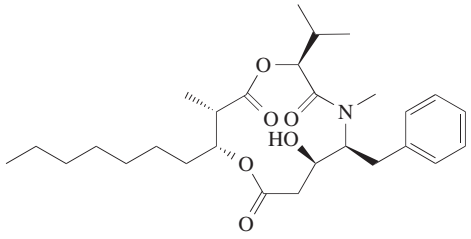
Murakami and co-workers described a series of studies on freshwater cyanobacterial peptides isolated as protease inhibitors including those against thrombin,[776], trypsin,[777] plasmin,[778] elastase,[779] chymotrypsin,[780] and angiotensin-converting enzyme.[781]

Since the early 1980s, Moore and co-workers have been continuously studying laboratory-cultured freshwater cyanobacterial natural products.[743,782] These cyanobacterial secondary metabolites have chemically unique structures, belonging to various biosynthetic classifications such as cyclic peptides (e.g. dendroamide A (**952**))[783] or depsipeptides (e.g., hapalosin (**953**)),[784] macrolides (e.g., tolytoxin

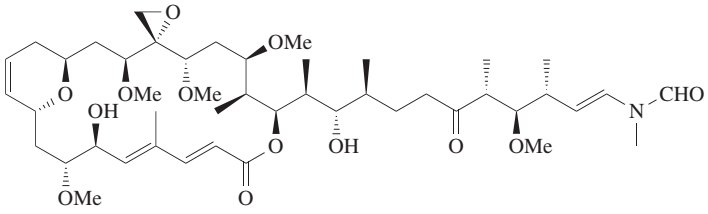
(**954**)),[785] isotactic polymethoxy-1-alkenes (e.g., mirabilene isonitrile A (**955**)),[786] cyclophanes (e.g., cylindrocyclophane A (**956**)),[787] porphyrins (e.g., tolyporphin (**957**)),[788] nucleosides (e.g., tubericidin (**958**)),[789] oxazole and/or thiazole-containing polythiazoline alkaloids (e.g., tantazole B (**959**)),[790,791] indole alkaloids with an isonitrile or isothiocyanate-containing isoprenoid moiety (e.g., welwitindolinone A isonitrile (**960**)),[792] chlorinated *N*-acylpyrrolinones (e.g., mirabimide E (**961**)),[793] and guanidium compounds (e.g., anatoxina(s) (**962**),[794] cylindrospermopsin (**963**)).[795] These unique compounds exhibit a wide variety of potentially useful bioactivities including cytotoxic, antifungal, antiviral, hepatotoxic, anticholinesterase, and multidrug resistance reversal effects.



(**952**)



(**953**)



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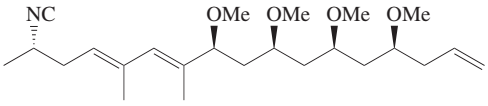
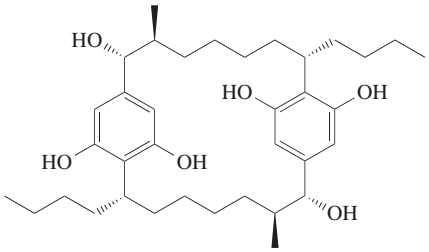


(**955**)



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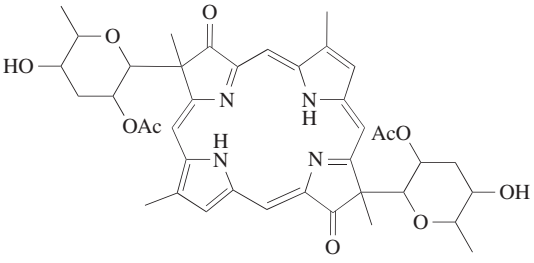
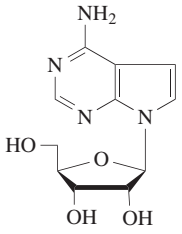
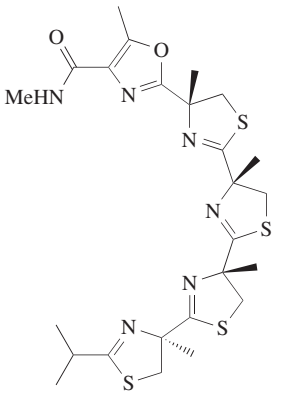


(**957**)



(**958**)

Thus, blue-green algae (cyanobacteria) have been recognized as a rich source of structurally novel and biologically active natural products. It has been pointed out that most of these unique natural



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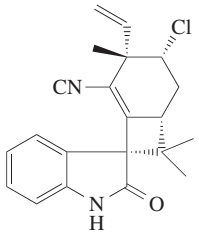

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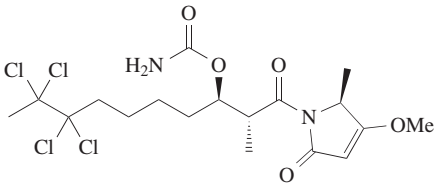

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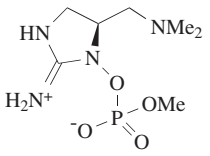

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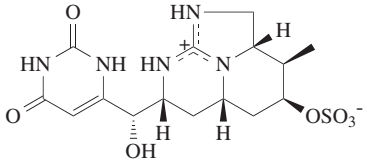
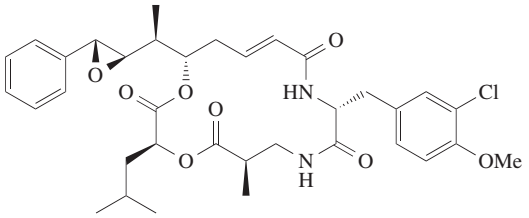


(963)

products will probably only be useful as biochemical research tools.[796] A few, however, have the potential for development into useful commercial products.[796] For example, cryptophycin-1 (**964**), a novel inhibitor of microtubule assembly from *Nostoc* sp. GSV 224, shows impressive activity against a broad spectrum of solid tumors implanted in mice, including multidrug-resistant ones. This cyclic depsipeptide (**964**) had previously been isolated from *Nostoc* sp. ATCC 53787 as an antifungal agent and its gross structure determined.[797] Moore and co-workers established the relative and absolute stereochemistry of cryptophycin-1 (**964**) using a combination of chemical and spectral techniques.[798] Several minor cryptophycins were also isolated from GSV 224 and their total structures were determined. The convergent total synthesis of cryptophycin-3 (**965**) was achieved and the chloro-*O*-methyltyrosine unit in cryptophycin-1 (**964**) was revised to have the D-configuration.[799] Using a modified isolation procedure devoid of MeOH, a further 18 cyclic cryptophycins were isolated from *Nostoc* sp. GSV 224 as minor constituents.[800] Acyclic cryptophycins were not found, indicating that acyclic cryptophycin analogues are artifacts of isolation as a consequence of using MeOH. The relative stereochemistry of cryptophycin-3 (**965**) was further rigorously established by X-ray crystallography. NOE studies show that the preferred conformations of most cryptophycins in solution differ from the conformation of cryptophycin-3 (**965**) in the crystal state. Although cryptophycin-1 (**964**) was relatively stable at pH 7, both in ionic and nonionic media, the ester bond linking L-leuicic acid and (*R*)-3-amino-2-methylpropanoic acid units was fairly labile to solvolysis and mild base hydrolysis. Structure–activity relationship studies indicated that the intact macrolide ring, the epoxide group, the chloro and *O*-methyl groups in the chloro-*O*-methyl-D-tyrosine unit, and the methyl group in the (*R*)-3-amino-2-methylpropanoic acid unit were needed for the *in vivo* activity of cryptophycin-1 (**964**).



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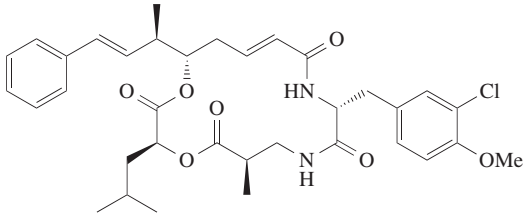

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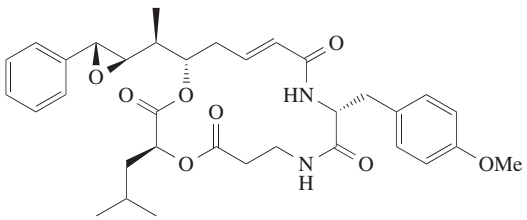
The mechanism of action of cryptophycin demonstrated that this compound potently disrupts the microtubule structure in cultured cells, and cryptophycin appeared to be a poor substrate for the drug-efflux pump P-glycoprotein in contrast with the *Vinca* alkaloids.[801] The site of cryptophycin interaction with tubulin was examined to reveal that crytophycin blocked the formation of vinblastine-tubulin paracrystals in intact cells and suppressed vinblastine-induced tubulin aggregation *in vitro*. Cryptophycin inhibited the binding of [^{3}H]vinblastine and the hydrolysis of [γ-^{32}P]GTP by isolated tubulin, but did not block the binding of colchicine. These results indicated that cryptophycin disrupts the *Vinca* alkaloid site of tubulin.[802,803]

Kitagawa and co-workers isolated a potent cytotoxic depsipeptide, arenastatin A (**966**) from the Okinawan marine sponge *Dysidea arenaria*,[804] and its structure including all absolute configurations was established by NMR and synthetic studies.[805] Interestingly, the chemical structure of arenastatin A (**966**) was very similar to that reported for cryptophycins, suggesting the participation of a presumably symbiotic cyanobacterium in the biosynthesis of arenastatin A (**966**) in the marine sponge. The total synthesis of arenastatin A (**966**) was achieved[806] and stereoisomers of (**966**) with different configurations at the epoxide moiety, a secondary methyl group on C-6, and/or the *O*-methyltyrosine unit were also prepared by applying improved methods to show that only (**966**) was potently cytotoxic (IC_{50} 5 pg ml^{-1} against KB cells) and other stereoisomers did not show cytotoxicity at concentrations below 0.1 μg ml^{-1}.[807]

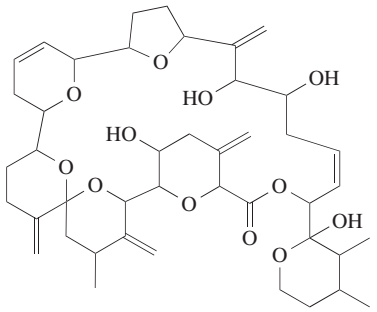


(966)

8.07.9.4.4 Dinoflagellates and other microalgae

In 1968 Sharma *et al*. isolated goniodomin from a marine dinoflagellate *Goniodoma* sp. and proposed that it contained five hydroxyl groups, a lactone ring, four ether linkages, and a dihydro-

geranyl side-chain by functional group analysis.[808] In paper disk-agar plate tests, goniodomin (0.5–500 μg ml^{-1}) strongly inhibited *Cryptococcus neoformans*, *Trichophyton mentagrophytes*, and other fungi, but had little or no activity against bacteria. Twenty years later, Murakami *et al*. isolated an antifungal polyether macrolide, named goniodomin A (**967**), from *Goniodoma pseudogoniaulax* collected in a rock pool.[809] Its structure was elucidated by spectral data.

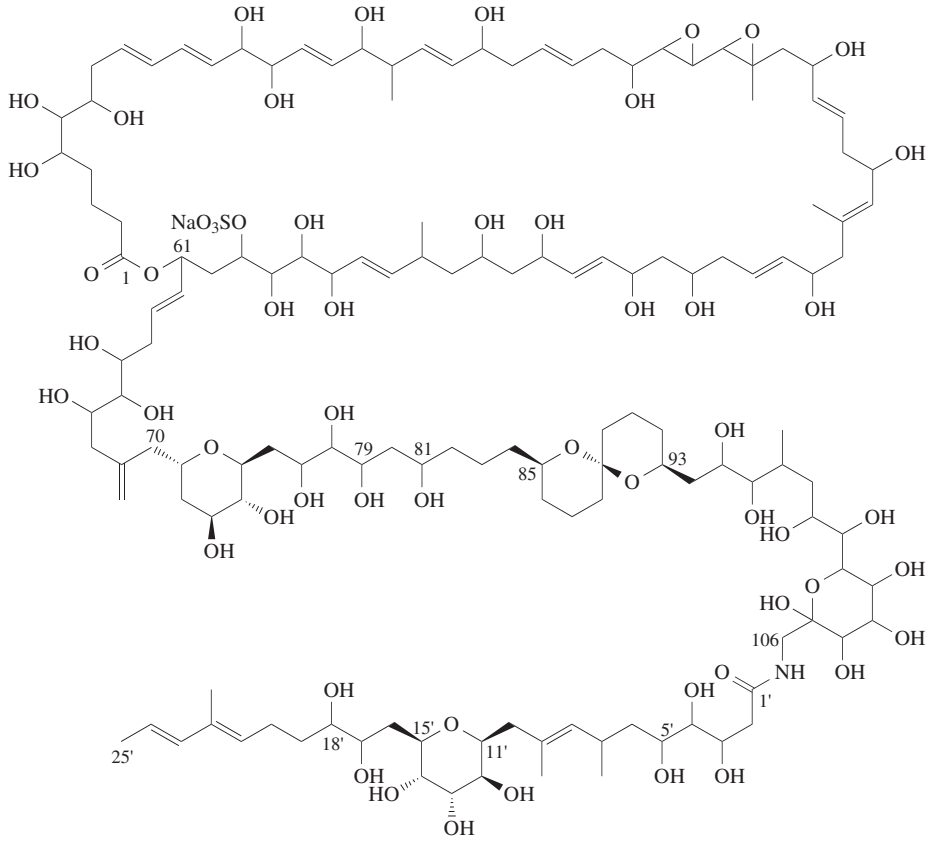


(967)

Goniodomin A (**967**) showed antifungal activity against *Mortierella ramannianus* at a concentration of 0.5 μg ml^{-1}, and inhibited the cell division of fertilized sea urchin eggs at 0.05 μg ml^{-1}. In addition, goniodomin A (**967**) administered i.p. at 0.6 mg kg^{-1} to P388 leukemia cell-inoculated mice prolonged the survival time. The i.p. LD_{50} values of (**967**) in male ICR mice were 1.2 mg kg^{-1} and 0.7 mg kg^{-1} at 24 h and 48 h, respectively. Morphological changes in the liver and thymus of male ICR mice were induced by (**967**).[810] Furthermore, Ohizumi and co-workers showed that goniodomin A (**967**) induces modulation of actomyosin ATPase activity mediated through conformational change of actin.[811] The effect of (**967**) was dependent on the concentration of actin, but not of myosin. The actomyosin ATPase activity was increased by pretreatment of actin (but not of myosin) with (**967**). Goniodomin A (**967**) induced a sustained and concentration-dependent increase in the fluorescence intensity (excitation wavelength, 277 nm; emission wavelength, 329 nm) of actin. The maximum response was obtained with concentrations of (**967**) in the 10^{-5} to 10^{-4} M range in the presence of 5 μM F-actin. However, the ATPase activity and fluorescence intensity of myosin were not changed by (**967**) at concentrations from 10^{-8} to 10^{-5} M. Interestingly, goniodomin A (**967**) induced a remarkable but transient increase in the fluorescence intensity of actomyosin in a concentration-dependent manner, with a peak at 3×10^{-7} M. This profile was quite similar to that found in the stimulation of the actomyosin ATPase activity induced by (**967**). To investigate further the effect of (**967**), actin was labeled with *N*-(1-pyrenyl)iodoacetamide. Goniodomin A (**967**) at 10^{-6} M had no effect on the fluorescence intensity of pyrenyl-actin (excitation wavelength, 365 nm; emission wavelength, 407 nm), but increasing concentrations of (**967**) to 3×10^{-6} M remarkably decreased its intensity. This effect was potentiated by heavy meromyosin. Actin molecules treated with (**967**) were completely sedimented by mild centrifugation (for 15 min at 12 000 *g*). Electron microscopic observations suggested that actin filaments associated with each other to form a gel in the presence of 3×10^{-6} M goniodomin A (**967**). The conformational change of actin molecules, resulting from stoichiometric binding of (**967**) to actin monomers in filaments, may modify the interaction between actin and myosin.

New polyhydroxypolyenes with potent vasoconstrictive activity, zooxanthellatoxin A (**968**) and B (**969**), were isolated from a cultured zooxanthella, *Symbiodinium* sp., isolated from the flatworm *Amphiscolops* sp. collected at Okinawa.[812] These compounds caused sustained contractions of isolated rabbit aorta at concentrations above 7×10^{-7} M; this effect was abolished in Ca^{2+}-free solution or in the presence of verapamil. Both compounds had relatively large molecular weights, containing a large number of oxygen atoms and alkenic carbons, thus differing from two other vasoconstrictive marine toxins, maitotoxin and palytoxin (see Sections 8.07.8.4.2 and 8.07.8.5.1), containing more alkenes than palytoxin, and fewer ethereal rings than maitotoxin. The structures of zooxanthellatoxin A (**968**) and B (**969**) were studied by chemical degradations (e.g., periodate oxidation and $NaBH_4$ reduction).[813,814] Zooxanthellatoxin A (**968**) gave a seco-acid upon treatment with a weak base and further hydrolyzed to afford a terminal carboxylic acid segment of the amide structure. Comparison of the DQF–COSY, TOCSY, HMQC, and HMBC spectra of (**968**) in CD_3OD or CD_3OD-C_5D_5N with those of its degradation products revealed a novel 62-membered

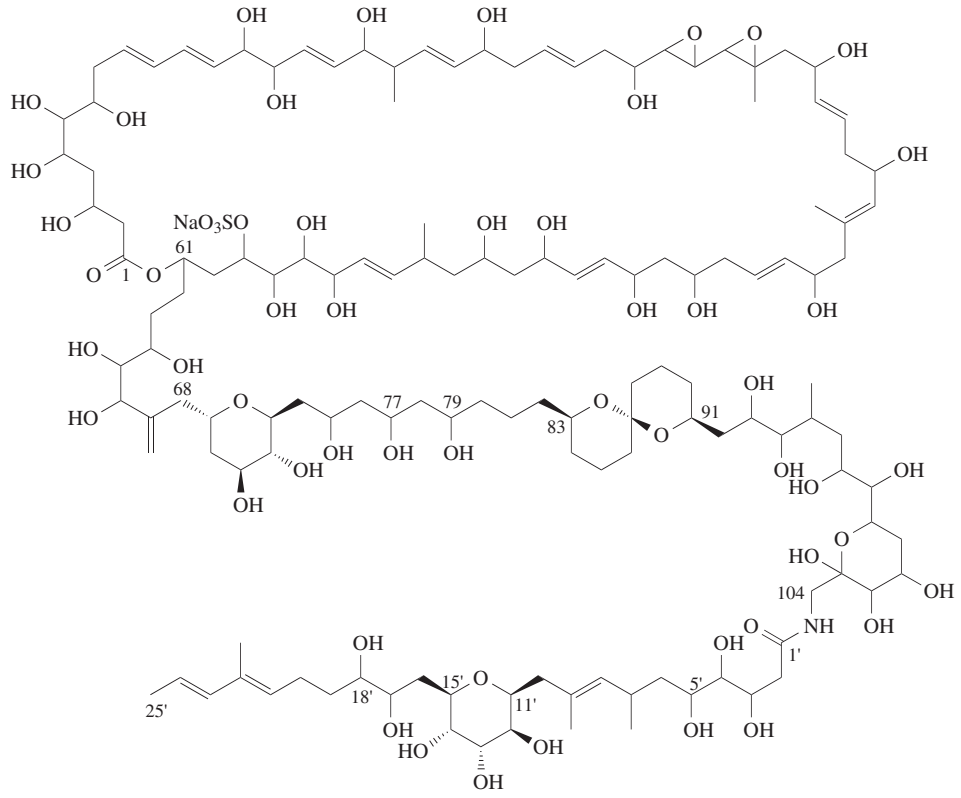
lactone structure for (**968**).[815] Compound (**968**) contained several characteristic functionalities including a bisepoxide, a sulfate ester, an amide, two conjugated dienes, and many allylic alcohols. The structure of zooxanthellatoxin B (**969**) was also determined to be a 62-membered lactone by comparing spectral data and degradation products with those of zooxanthellatoxin A (**968**).[816]



(**968**)

Two degradation products, (**970**) and (**971**), were synthesized and the absolute stereochemistry of the chiral centers contained in them (C-11′, C-12′, C-13′, C-14′, C-15′, C-17′, and C-18′; C-81, C-85, and C-93) were determined.[817,818] Ohizumi and co-workers revealed that zooxanthellatoxin A (**968**) caused aggregation in rabbit washed platelets in a concentration-dependent manner (1–4 μM), accompanied by an increase in cytosolic Ca^{2+} concentration. It was also suggested that (**968**) elicits Ca^{2+}-influx from platelet plasma membranes and the resulting increase in cytosolic Ca^{2+} concentration subsequently stimulates the secondary release of thromboxane A2 from platelets.[819]

Marine dinoflagellates have proven to be a rich source of unique natural products as described above (see also Sections 8.07.8.3–8.07.8.5). Among other dinoflagellates, the genus *Amphidinium* attracts much attention because of the production of bioactive substances such as amphidinolides (Section 8.07.9.4.5) and amphidinols. In 1991 Yasumoto and co-workers isolated a polyhydroxy-polyene antifungal substance, named amphidinol (**972**) (synonymous to amphidinol 1) from the cultures of the dinoflagellate *Amphidinium klebsii* mainly by gel permeation chromatography.[820] The dinoflagellate *Amphidinium* sp. was collected at Ishigaki Island, Japan. Antifungal activity (6 μg/disk) of amphidinol (**972**) against *Aspergillus niger* was three times more potent than amphotericin B; the hemolytic activity was 120 times that of standard saponin. UV and IR spectra, respectively, indicated the presence of a conjugated triene and a sulfate ester in the molecule. The latter moiety was further confirmed by determination of SO_4^{2-} after solvolysis. FAB-MS indicated a molecular weight of 1488 ($C_{73}H_{125}O_{27}SNa$). The major part of the structure was deduced by a combined use of 2D NMR, such as ^1H-^1H COSY, ^1H-^1H HOHAHA, phase-sensitive NOESY, and phase-sensitive C-H COSY. To enhance ^{13}C for NMR measurements, $NaH^{13}CO_3$ was added to the culture media, which yielded (**972**) with 3.5% isotope abundance. Hetero-COSY experiments, including phase-sensitive C-H



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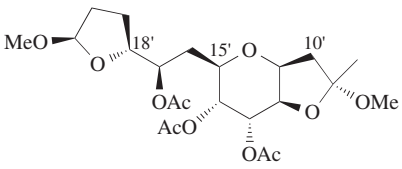

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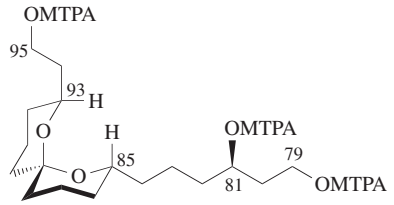
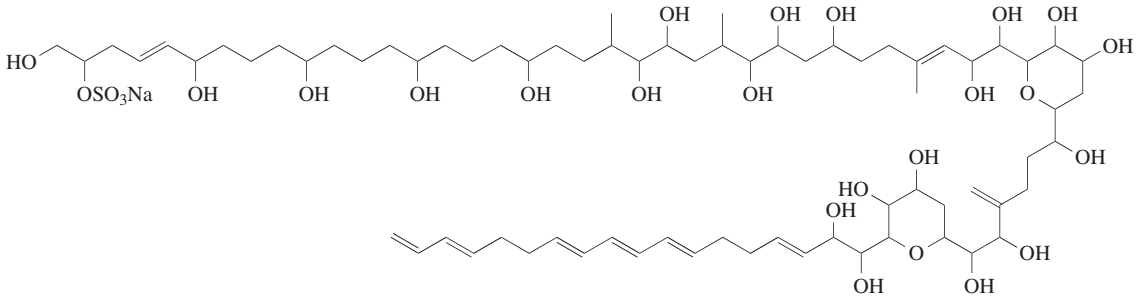


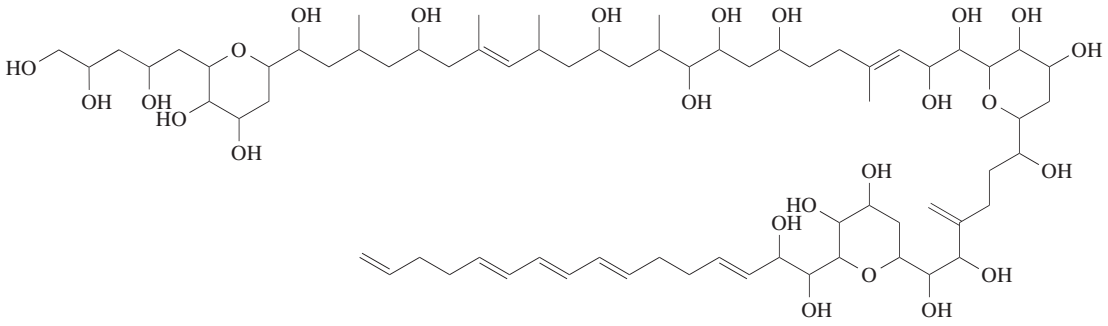
(971)

COSY and C-H HOHAHA, were successfully conducted with <3 mg of (**972**) (2 μmol). Tandem mass spectroscopy effectively clarified the structure in polyhydroxylated and polyene regions. Amphidinol (**972**) was the first member of a new class of polyhydroxy-polyenes. Notable structural features include a C_{69} chain with four C_1 branches, 21 hydroxyls, nine alkenes, and a sulfate ester.



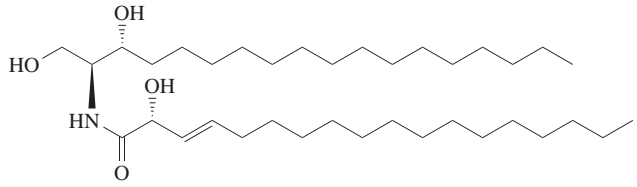
(972)

Another polyene-polyhydroxy compound, amphidinol 2 (**973**), was isolated as a potent hemolytic and antifungal agent from another cultured strain of dinoflagellate *Amphidinium klebsii* by Tachibana and co-workers.[821] The structure elucidated by spectroscopic methods turned out to be partly analogous to amphidinol 1 (**972**).



(973)

Kobayashi *et al*. studied a symbiotic dinoflagellate of the genus *Symbiodinium* isolated from the inside of gill cells of the Okinawan bivalve *Fragum* sp. From the toluene-soluble fraction of the extract of the harvested cells a sphingosine derivative, symbioramide (**974**),[822] was isolated by using silica gel column chromatographies. The structure of symbioramide (**974**) was elucidated on the basis of spectral and chemical means. The ^{1}H and ^{13}C NMR data suggested that compound (**974**) belongs to the ceramides. Acid hydrolysis of (**974**) afforded methyl 2-hydroxyoctadec-3*E*-enoate and 2*S*-amino-3*R*-hydroxyoctadecan-1-ol. The absolute configurations at the C-2 and C-3 positions of the sphingosine part of (**974**) were thus determined.

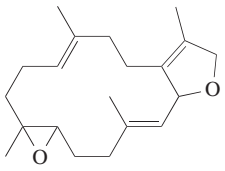


(974)

Symbioramide (**974**) exhibited weak cytotoxic activity against L1210 cells *in vitro* with an IC_{50} value of 9.5 μg ml^{-1}. Additionally, compound (**974**) was found to be a sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activator. The Ca^{2+}-ATPase in the SR membrane plays a key role in muscle relaxation by energizing Ca^{2+}-pumping from the cytoplasm into the lumen of SR. Symbioramide (**974**) at 10^{-4} M activated SR Ca^{2+}-ATPase activity by 30%. This is the first example of an SR Ca^{2+}-ATPase activator of marine origin. Symbioramide (**974**) may serve as a valuable chemical tool for studying the regulatory mechanisms of SR Ca^{2+}-pumping systems. The α-hydroxy-β,γ-dehydro fatty acid contained in symbioramide (**974**) is rare from natural sources. Previously ceramides of α-hydroxyl fatty acids were obtained from the sponge *Dysidea etheria*.[823] The ceramides isolated from the sponge may be of microbial origin since sponges are known to possess symbiotic microorganisms. The absolute configuration of the C-2 position of the 2-hydroxyoctadec-3*E*-enoic acid moiety of symbioramide (**974**), which was previously unassigned, was established as *R* based on the total synthesis of symbioramide (**974**) achieved by Nakagawa *et al*.[824] Mori and Uenishi also studied the synthesis of the ceramide (**974**), and they found the interesting phenomenon that the optical rotation of (**974**) was influenced by the temperature of the sample solution in a cell for rotation measurements: $[\alpha]_D^{19} = +3.6^\circ$, $[\alpha]_D^{23} = +0.76^\circ$, $[\alpha]_D^{28} = -1.5^\circ$, and $[\alpha]_D^{35} = -5.5^\circ$ ($c = 0.31$ in $CHCl_3$).[825]

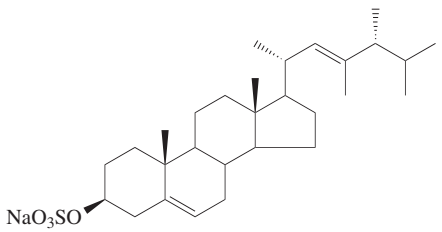
Soft corals are known to possess symbiotic microalgae. Kobayashi *et al*. isolated a diterpene, 16-deoxysarcophine (**975**),[826] with potent Ca-antagonistic activity from the Okinawan soft coral *Sarcophyton* sp. and established the structure by X-ray analysis. This was the first example of a Ca-antagonist from marine sources and of a nonalkaloid compound with such activity. Symbiotic microalgae of the soft coral *Sarcophyton* sp. were examined and a dinoflagellate belonging to the

genus *Symbiodinium* was isolated from the polyps of the soft coral. 16-Deoxysarcophine (**975**) was detected by HPLC analysis of the extracts of the dinoflagellate *Symbiodinium* sp.



(975)

The Haptophyceae are microscopic, unicellular algae, which are widely distributed in the oceans and often constitute a major proportion of marine phytoplankton. Kobayashi *et al*. encountered a haptophyte of the genus *Hymenomonas*, which was isolated from an unidentified cylindrical stony coral collected at Sesoko Island, Okinawa. The extract of the harvested cells was partitioned between toluene and water and the aqueous phase was further extracted with chloroform. The toluene-soluble fraction was subjected to separation using Sephadex LH-20 and silica gel column chromatographies to give a sterol sulfate, hymenosulfate (**976**),[827] with potent Ca^{2+}-releasing activity in SR. The structure of (**976**) was elucidated by conversion of (**976**) into known (24*R*)-23,24-dimethylcholesta-(22*E*)-5,22-dien-3β-ol through acid hydrolysis. The presence of a sulfate group was confirmed by ion chromatography of sulfate ions liberated by hydrolysis. Hymenosulfate (**976**) is the first sterol sulfate isolated from marine microalgae. The major components of the toluene-soluble fraction of the extract of this haptophyte were glycolipids, mono- and digalactosyldiacylglycerols ((**977**) and (**978**), respectively), while the chloroform layer contained mainly octadecatetraenoic acid (**979**) along with a small amount of monogalactosylmonoacylglycerol (**980**). In the SR, the Ca^{2+}-releasing activity of (**976**) was 10 times more potent than that of caffeine, a well-known Ca^{2+}-releaser. The glycolipids (**977**), (**978**), and (**980**) exhibited inhibition of Na^+, K^+-ATPase activity with an IC_{50} value of 2×10^{-5} M each.



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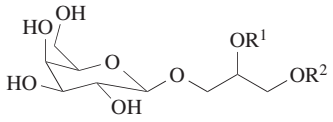
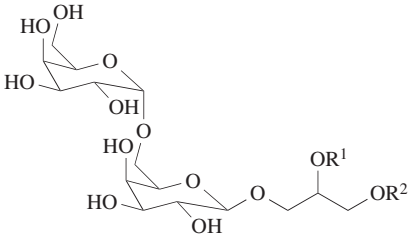
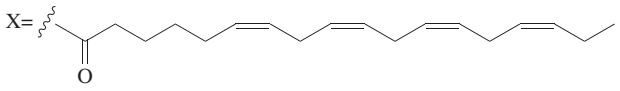


(977) $R^1 = R^2 = X$

(980) $R^1 = H$, $R^2 = X$

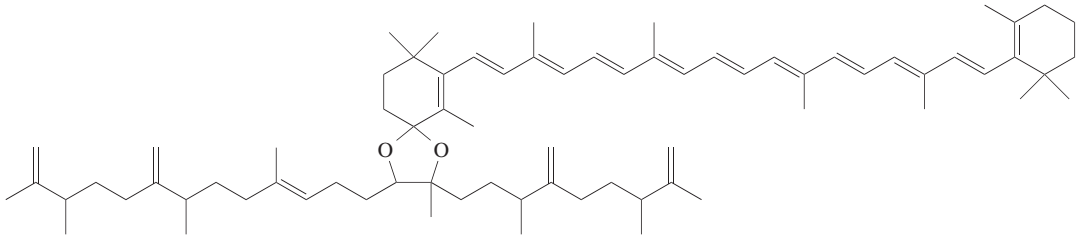


(978) $R^1 = R^2 = X$



(979) X-OH

From a cultured green colonial microalga, *Botryococcus braunii* Berkeley, Okada *et al*. isolated a new member of the natural carotenoids consisting of ketocarotenoid and its squalene analogue, botryoxanthin A (**981**).[828] The structure was determined by 2D NMR data. Botryoxanthin A (**981**) free from chlorophylls and intracellular carotenoids such as lutein could be extracted without breaking the cell wall. This implied that botryoxanthin A (**981**) may exist in the extracellular matrix like other secondary carotenoids and contribute to the color expression of the algal colonies. The presence of vicinally located C_1-branches (methyl and exomethylene) in the squalene moiety appears to be interesting in connection with the structures of microalgal metabolites like amphidinolides, as described in the following section.

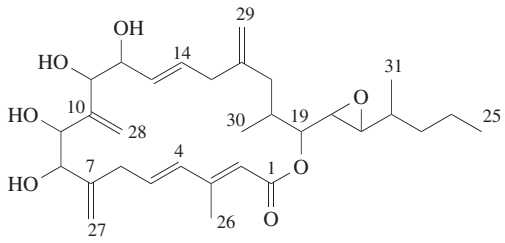


(981)

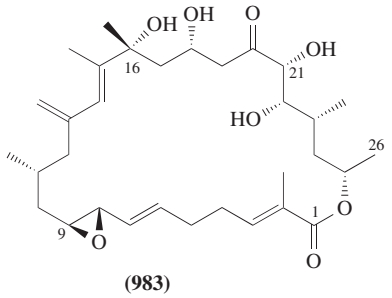
8.07.9.4.5 Amphidinolides

This section describes mainly the work carried out by the authors' research group on a series of unique macrolides, named amphidinolide, isolated from cultured marine dinoflagellate of the genus *Amphidinium*. During the course of studies on the search for bioactive substances from Okinawan marine organisms, the authors initiated a project on bioactive natural products of symbiotic marine microalgae in the mid 1980s. With help from previous studies by Yamasu who systematically collected symbiotic microalgae from marine invertebrates of Okinawan coastal waters,[829] large-scale cultures of the microalgae were carried out.

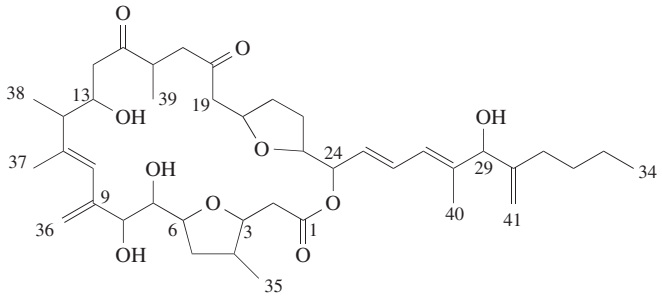
The first microalga utilized for research was a dinoflagellate belonging to the genus *Amphidinium* (strain number Y-5, 25 μm in length and 20 μm in width) isolated from the inner tissue of the host, a flatworm of the genus *Amphiscolops* Graff, 1905 (500 μm in length and 220 μm in width, green color), which was living on algae or seaweeds such as *Enteromorpha and Jania* spp. and collected at Chatan beach, Okinawa Island.[829] The unialgal cultures of *Amphidinium* sp. (Y-5) were grown in 31 glass bottles containing 21 of seawater medium enriched with Provasoli's ES supplement.[830,831] Cultures were incubated statically at 25 °C in an apparatus where illumination from a fluorescent light source was supplied in a cycle of 16 h light and 8 h darkness. After 2 weeks the culture was harvested by suction of the supernatant media with an aspirator, followed by centrifugation, to yield harvested cells ranging from 0.3 to 0.5 g l^{-1} of culture. The harvested cells were extracted with methanol/toluene (3:1) followed by partitioning between toluene and water. The toluene-soluble fraction was subjected to repeated silica-gel flash chromatography followed by reversed-phase HPLC resulting in isolation of four cytotoxic macrolides, amphidinolides A (**982**),[832] B (**983**),[833] C (**984**),[834] and D (**985**).[835]



(982)

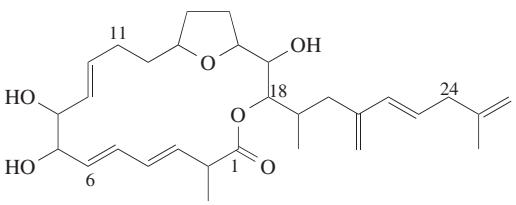


(983)
(985) C-21 epimer

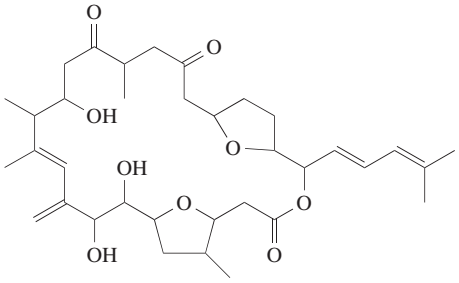


(984)

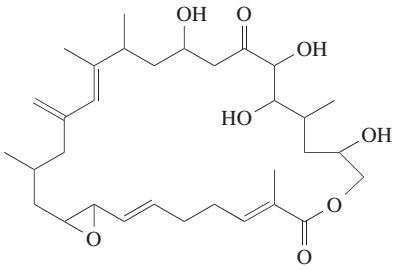
Three other species of dinoflagellates of the genus *Amphidinium* (strain numbers Y-5′, Y-25, and Y-26) were also investigated. Their host animals were also Okinawan flatworms [Y-5′: *Amphiscolops* sp. (different from the host of Y-5); Y-25: *Amphiscolops breviviridis*; Y-26: *Amphiscolops magniviridis*]. From extracts of the cultured cells of these strains of *Amphidinium* spp. four other cytotoxic macrolides, amphidinolides E (**986**),[836] F (**987**),[837] G (**988**), and H (**989**),[838] were isolated. In addition to potent cytotoxic activity, amphidinolides B (**983**) and C (**984**) were shown to activate rabbit skeletal muscle actomyosin ATPase activity.[839] The chemical structures of these macrolides were elucidated mainly on the basis of extensive spectroscopic studies including several types of 2D NMR experiments (e.g., ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, HMBC, HMQC, and NOESY spectra).



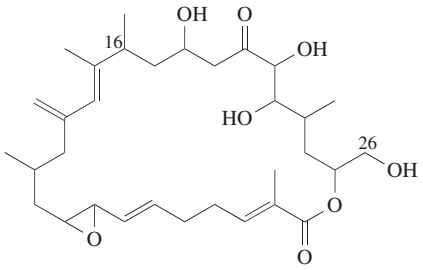
(986)



(987)

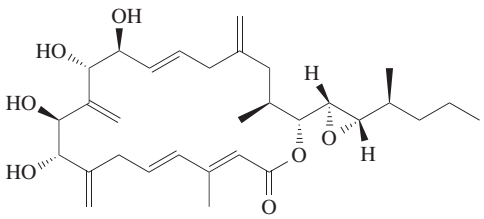


(988)



(989)

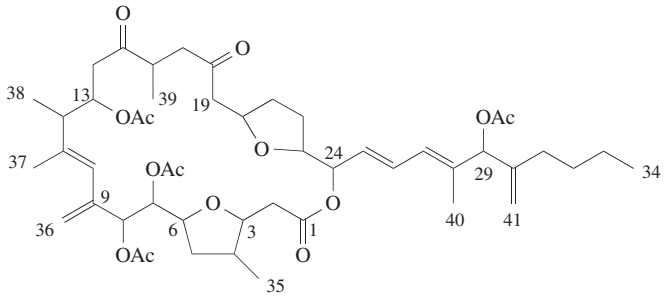
Amphidinolide A (**982**), $C_{31}H_{46}O_7$, was suggested to possess an $\alpha,\beta,\gamma,\delta$-dienoate chromophore from its UV absorption spectrum (λ_{max} 265 nm). Its ^{1}H and ^{13}C NMR data revealed the presence of one epoxide and three exomethylene groups. Detailed analysis of the ^{1}H-^{1}H COSY spectrum allowed assignment of all proton signals and clearly established the proton-connectivities, starting with H-2 through to H-25, leading to a 20-membered macrolide structure. It was ambiguous whether a secondary methyl group was located at C-22 or C-23 due to overlapping of the signals for protons on C-22–C-24. It was, however, placed on C-22 because the ^{13}C chemical shifts of the C-23–C-25 signals suggested the presence of an *n*-propyl group instead of an ethyl group.[840] Efforts to elucidate the stereochemistry of amphidinolide A (**982**) were made by analyzing the phase-sensitive NOESY spectra of this 20-membered macrolide with nine chiral centers. The authors have proposed a possible stereostructure of amphidinolide A as (**990**) containing the relative configurations of the nine chiral centers of (**982**) and most sufficiently satisfying the NOESY data obtained in several solvent systems.



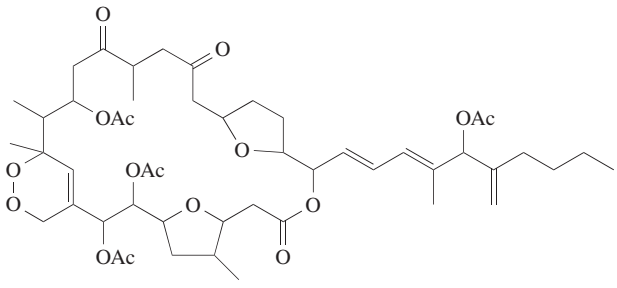
(990)

Amphidinolide B (**983**), $C_{32}H_{50}O_8$, possessed an α-methyl-α,β-unsaturated ester moiety, which was shown by the UV absorption (λ_{max} 222 nm) as well as the ^{13}C NMR chemical shifts. Its ^1H and ^{13}C NMR data also suggested the presence of one exomethylene, one epoxide, and one isolated ketone group. The ^1H-^1H COSY spectrum indicated the proton connectivities of three fragments (C-1–C-15, C-17–C-19, and C-21–C-26). Since the ^1H NMR chemical shifts for α positions to an sp^3 quaternary carbon (C-16) and ketone group (C-20) could be discriminated and NOE were observed between the methyl protons on C-15 and C-16, these three fragments were shown to be connected through C-16 and C-20, leading to a total structure consisting of a 26-membered macrocyclic lactone ring.

Amphidinolide C (**984**), $C_{41}H_{62}O_{10}$, exhibited a UV absorption maximum at λ_{max} 240 nm, implying the presence of a diene chromophore. Interpretation of the ^1H and ^{13}C NMR data suggested the presence of two tetrahydrofuran rings, two exomethylenes, and two isolated ketone groups. Extensive 2D NMR experiments were carried out on the tetraacetate (**991**) prepared from (**984**). Three partial structures (C-2–C-14, C-16–C-17, and C-19–C-34) were elucidated by analysis of the ^1H-^1H COSY and double relayed coherence transfer (RCT2) spectra. All protonated carbons were clearly assigned by ^1H-^{13}C COSY via one-bond couplings. The geometries of the double bonds were determined from NOE data. The connection of three partial structures through carbonyl groups (C-1, C-15, and C-18) was established by analyzing the ^1H-^{13}C long-range (two- and three-bond) couplings detected in the HMBC spectrum to give rise to a complete structure. The diene moiety (C-36, 9, 10, and 11) of the tetraacetate (**991**) was slowly oxidized by air to afford a [4+2] cycloaddition product (**992**), the spectral data of which provided additional proof for the structure of amphidinolide C (**984**).



(**991**)

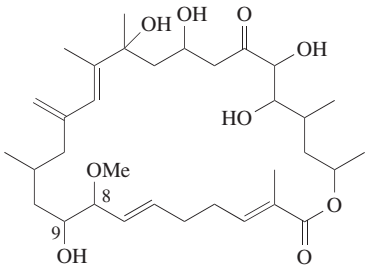


(**992**)

Amphidinolide E (**986**), $C_{30}H_{44}O_6$, showed a UV absorption maximum at λ_{max} 230 nm corresponding to a diene chromophore. The presence of two exomethylenes and one tetrahydrofuran ring was inferred by analyzing the ^1H and ^{13}C NMR data. Its ^1H-^1H COSY data afforded information on the proton-connectivities for three partial structures (C-2–C-14, C-15–C-19, and C-20–C-27). The geometries of the four disubstituted double bonds were deduced on the basis of the ^1H-^1H coupling constants determined by a *J*-resolved 2D NMR experiment. The partial structures were

revealed to be linked to each other by ^{1}H chemical shift data together with the relayed-correlations observed through an RCT-COSY spectrum to construct a 19-membered macrocyclic lactone ring with an alkyl side-chain. Spectral investigations of amphidinolides D (**985**), G (**988**), and H (**989**), whose molecular formulas are all $C_{32}H_{50}O_8$, revealed that they are structurally related closely to amphidinolide B (**983**): amphidinolides B (**983**) and D (**985**) are stereoisomers at C-21; amphidinolides G (**988**) and H (**989**) are regioisomers of the lactone-terminal positions (C-25 or C-26); amphidinolides B (**983**) and H (**989**) are only different in the position of one hydroxyl group (C-16 or C-26).

Amphidinolide D (**985**), an epimer of amphidinolide B (**983**) at the C-21 position, was, however, about 100 times less cytotoxic than the latter. During the isolation process of the extract of the strain Y-5, 1 mol of MeOH was added on the C-8/C-9 epoxide of amphidinolide B (**983**) to generate compound (**993**), the IC_{50} value of which was 0.081 μg ml^{-1} against the L1210 cell, being considerably weaker than that of (**983**) (1/600). These results implied that the stereochemistry at C-21 and the presence of an epoxide at the C-8/C-9 position are quite important for the cytotoxic activity of these compounds, presumably due to the significant change of the molecular conformation.



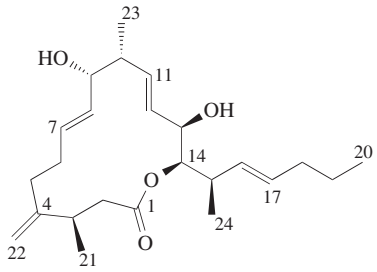
(993)

Amphidinolide F (**987**), $C_{35}H_{52}O_9$, proved to be analogous to amphidinolide C (**984**). The structural difference between amphidinolides F (**987**) and C (**984**) was found in the length of the alkyl side-chain, the former possibly being a biogenetic precursor of the latter. The cytotoxicities of amphidinolides C (**984**) and F (**987**) were also very different, the former being about 250 times as strong as the latter. The length of the side-chain may significantly affect the potency of cytotoxic activity.

After obtaining the series of macrolides, amphidinolides A–H (**982**)–(**989**), the mass-culturing of the dinoflagellates *Amphidinium* sp. (strain numbers Y-5 and Y-25) was continued.[841] Previous studies had revealed that fractionation by silica gel chromatography of the toluene-soluble portion of the extracts of these microalgae afforded several fractions exhibiting extremely potent cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro*, with the inhibition values at 10 μg ml^{-1} being more than 90%. These inhibition values cannot be fully accounted for by estimating from the IC_{50} values of previously isolated amphidinolides. Thus, further investigations continued to search for other cytotoxic components of these dinoflagellates. As a result, several novel cytotoxic macrolides with various ring numbers, amphidinolides J (**994**), K (**995**), M (**996**), N (**997**), O (**998**), P (**999**), and Q (**1000**), were isolated, together with a new linear metabolite amphidinin A (**1001**) from strain Y-5, while a new 27-membered macrolide, amphidinolide L (**1002**), was isolated from strain Y-25.

In 1993 the authors succeeded in the isolation and structure elucidation of amphidinolide J (**994**),[842] a novel 15-membered macrolide, and determined its absolute stereochemistry by combination of degradation experiments and synthesis of optically active compounds. Further cultivation of the dinoflagellate *Amphidinium* sp. (strain Y-5) was carried out at 25 °C for 2 weeks in a seawater medium enriched with Erd-Schreiber (ES) nutrients. The harvested algal cells (920 g, wet weight, from 3300 l of culture) were extracted with MeOH/toluene (3:1) and the extracts were partitioned between toluene and water. The toluene-soluble fraction was subjected to a silica gel column ($CHCl_3$/MeOH, 95:5) followed by gel filtration on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1). Subsequent separation by reversed-phase HPLC (ODS, 88% MeOH) afforded amphidinolide J (**994**) (0.0002% yield, wet weight) as a colorless oil.

The planar structure of amphidinolide J (**994**), $C_{24}H_{38}O_4$, was studied by detailed analyses of its ^{1}H and ^{13}C NMR data aided with 2D NMR experiments (^{1}H-^{1}H COSY, HSQC, HMBC, and



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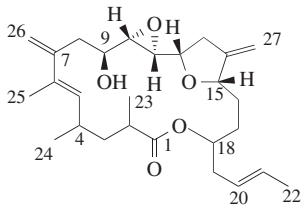
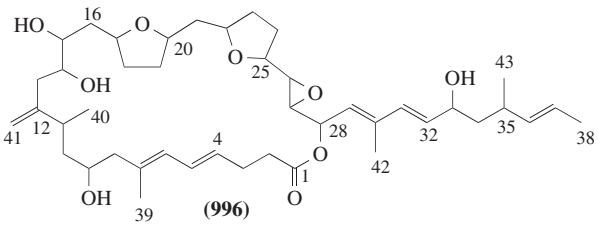
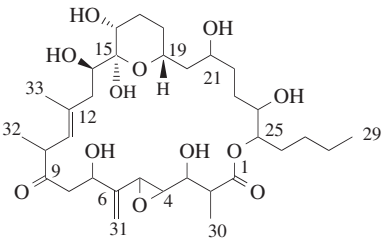


(995)



(996)



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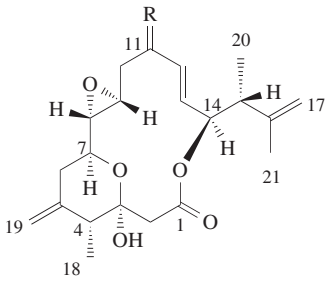
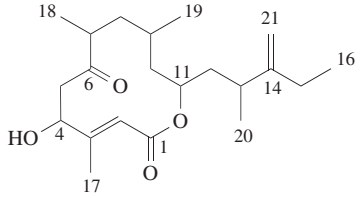
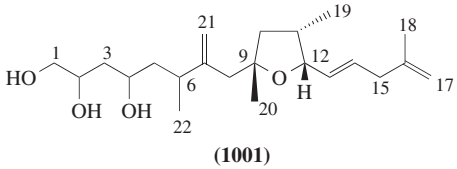


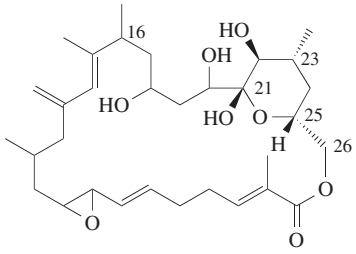
(998) R = O
(999) R = CH_2



(1000)

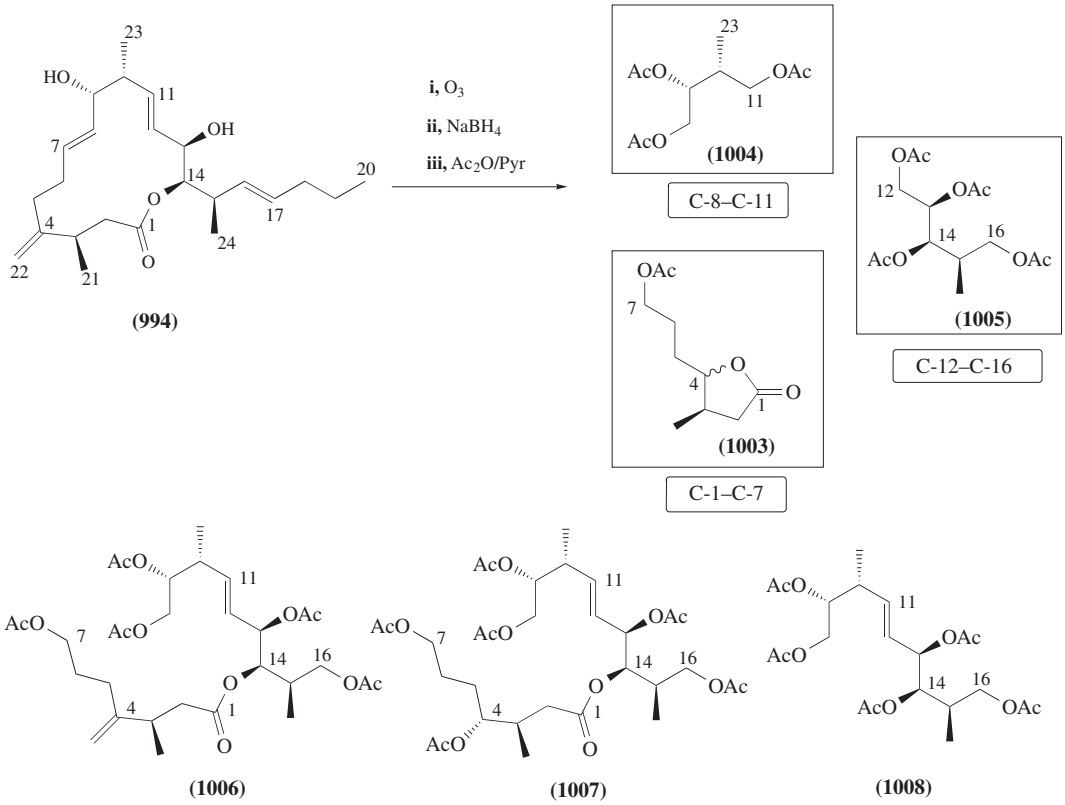


(1001)



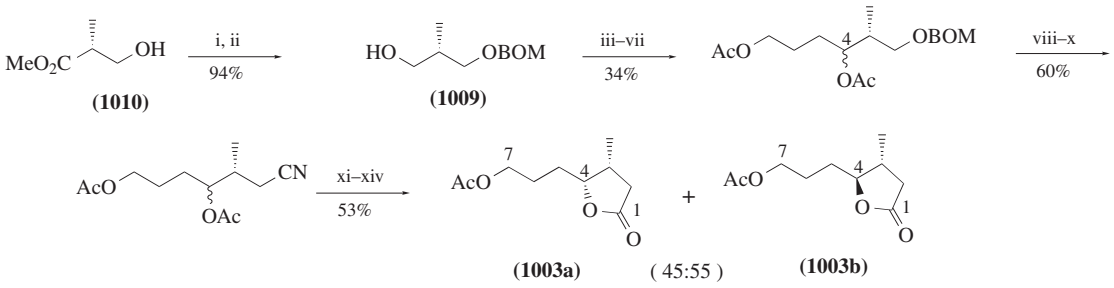
(1002)

NOESY), thereby leading to a gross structure of (**994**) consisting of a 15-membered lactone ring with three disubstituted *E*-alkenes ($J_{7,8}$ = 15.0 Hz, $J_{11,12}$ = 15.8 Hz, and $J_{16,17}$ = 15.0 Hz). This gross structure was further confirmed by the structures of the degradation products (**1003**)–(**1008**) obtained by the following ozonolysis experiments. Treatment of (**994**) with ozone (−78 °C, 1 min) followed by $NaBH_4$ reduction and acetylation (Scheme 30) afforded a complex mixture, from which the normal and reverse-phase HPLC separations were carefully carried out to obtain degradation products (**1003**)–(**1005**), corresponding to the C-1–C-7, C-8–C-11, and C-12–C-16 moieties of (**994**), respectively. In addition, partial-degradation products (**1006**)–(**1008**) were also obtained and their structures provided further evidence for the proposed planar structure of (**994**). For the unambiguous determination of the absolute configurations of the six chiral centers of (**994**), the fragments (**1003**)–(**1005**) together with all their possible diastereomers were prepared in optically active forms.



Scheme 30

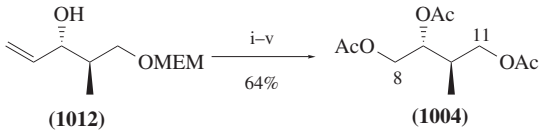
The C-1–C-7 fragment (**1003**) was synthesized as shown in Scheme 31, starting from mono-protected 2(*S*)-methylpropane-1,3-diol (**1009**), which was readily supplied from (−)-methyl 3-hydroxy-2(*R*)-methylpropionate (**1010**). The Grignard addition to the corresponding aldehyde from (**1009**) afforded a diastereomeric mixture at C-4 in the ratio of 45:55, which was separated in the final step by silica gel HPLC. The 3,4-*syn* (**1003a**) and 3,4-*anti* (**1003b**) isomers thus obtained were completely identical with those from natural specimens including the signs of optical rotations [synthetic, (**1003a**): $[\alpha]_D$ +17° (*c* 1.0, $CHCl_3$); (**1003b**): $[\alpha]_D$ −22° (*c* 1.0, $CHCl_3$); natural, (**1003a**): $[\alpha]_D$ +17° (*c* 0.06, $CHCl_3$); (**1003b**): $[\alpha]_D$ −34° (*c* 0.2, $CHCl_3$)] to establish the 3*R*-configuration for (**994**).



i, BOMCl, Pr^i_2NEt, CH_2Cl_2, room temperature, 44 h; ii, LAH, ether, room temperature, 30 min; iii, DMSO, $(COCl)_2$, CH_2Cl_2, –78 °C, 30 min, then Et_3N, 0 °C, 30 min; iv, CH_2=$CHCH_2CH_2MgBr$, ether, 50 °C, 40 min; v, O_3, MeOH, –78 °C, 2.5 h; vi, $NaBH_4$, MeOH, 0 °C, 1 h; vii, Ac_2O, pyridine, room temperature, 12 h; viii, H_2, Raney Ni (W-2), EtOH, room temperature, 48 h; ix, TsCl, pyridine, room temperature, 44 h; x, NaCN, DMSO, 85-90 °C, 2 h; xi, NaOH, H_2O_2, EtOH, 65 °C, 1.5 h, then 90 °C, 7 h; xii, 2M HCl, room temperature; xiii, Ac_2O, pyridine, room temperature, 11 h; xiv, HPLC separation

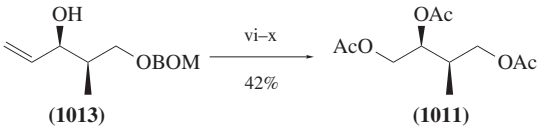
Scheme 31

The C-8–C-11 fragment (**1004**) and its *syn*-isomer (**1010**) were readily prepared ((i) reductive ozonolysis, (ii) deprotection, and (iii) acetylation; Scheme 32) from allyl alcohols ((**1012**) and (**1013**), respectively), which were obtained from (**1010**) via modifications of literature procedures.[843] The spectral data of the C-8–C-11 fragment obtained by degradation of (**994**) were identical with those of the *anti*-isomer (**1004**) and their optical data (synthetic, $[\alpha]_D$ $+5.0^\circ$ (*c* 1.0, $CHCl_3$); natural; $[\alpha]_D$ $+2.8^\circ$ (*c* 0.22, $CHCl_3$)) revealed the 9*R*,10*R*-configurations for (**994**).



(**1012**) (**1004**)

i, O_3, MeOH, –78 °C, 5 min; ii, $NaBH_4$, MeOH, 0 °C, 1 h; iii, Ac_2O, pyridine, room temperature, 38 h; iv, 4M HCl, THF, 50 °C, 2 h; v, Ac_2O, pyridine, room temperature, 20 h

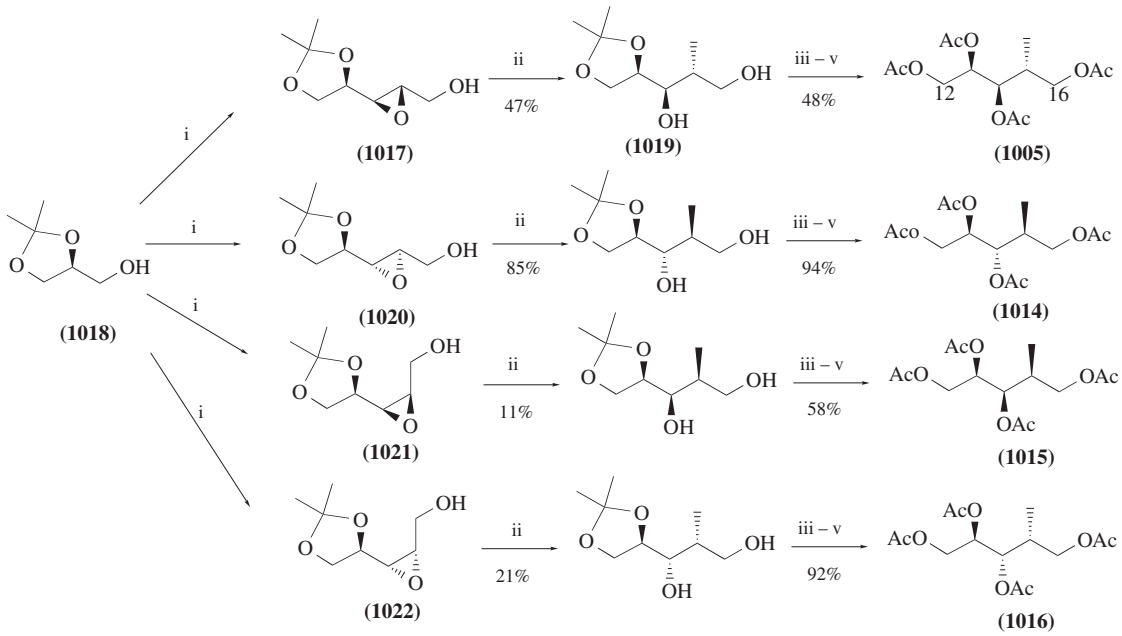


(**1013**) (**1011**)

vi, O_3, MeOH, –78 °C, 1 min; vii, $NaBH_4$, MeOH, 0 °C, 45 min; viii, Ac_2O, pyridine, room temperature, 17 h; ix, H_2, 10% Pd-C, MeOH, room temperature, 11h; x, Ac_2O, pyridine, room temperature, 20 h

Scheme 32

Preparations of the C-12–C-16 fragment (**1005**) and its diastereomers (**1014**)–(**1016**) were achieved by applying Kishi's methods for pentose synthesis[844] (Scheme 33). The epoxy alcohol (**1017**), obtained from D-glyceraldehyde acetonide (**1018**), was treated with lithium dimethylcuprate to give 1,3-diol (**1019**) together with undesired 1,2-diol in the ratio of 1:1, which was separated in the final step by silica gel HPLC (hexane/EtOAc, 2:1). The diastereomers (**1014**)–(**1016**) were also obtained by similar procedures from the corresponding epoxy alcohols ((**1020**)–(**1022**), respectively). The C-12–C-16 fragment derived from (**994**) was identical with the *syn-anti* isomer (**1005**) including the sign of optical rotation (synthetic, $[\alpha]_D$ $+41^\circ$ (*c* 1.0, $CHCl_3$); natural $[\alpha]_D$ $+44^\circ$ (*c* 0.23, $CHCl_3$)),

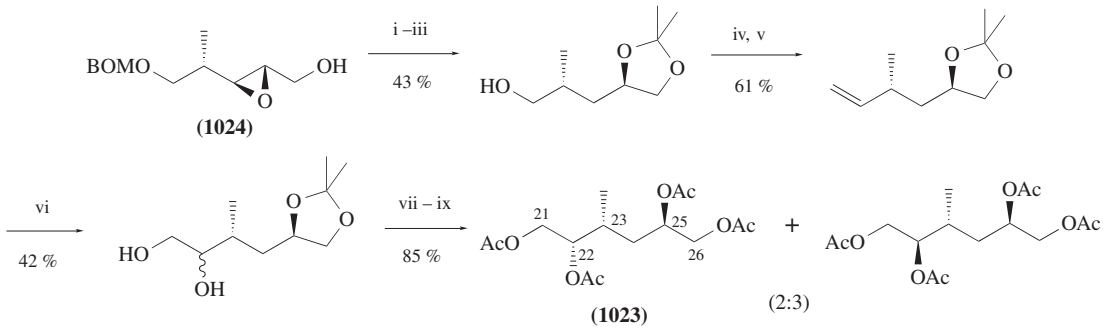


i, (4 steps); [163] ii, CuI (12 eq), MeLi (24 eq), Et_2O, –40°C, 4–9 h, then –23 °C, 30 min; iii, 1M HCl, THF, room temperature, 7–25 h or AcOH/H_2O (4:1), 40 °C, 4 h; iv, Ac_2O, pyridine, room temperature, 11–20 h; v, HPLC separation

Scheme 33

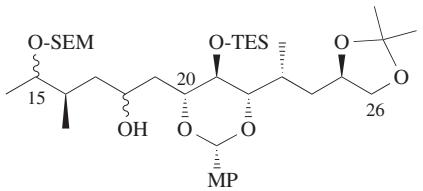
thus determining the 13*R*,14*R*,15*R*-configurations for (**994**). From these results the structure of amphidinolide J was firmly established as (**994**) including the absolute stereochemistry of the six chiral centers.

In connection with our studies on cytotoxic macrolides from the dinoflagellate *Amphidinium* sp. (strain Y-5), we also continued examining the extract of the strain Y-25 *Amphidinium* sp., which was isolated from the Okinawan flatworm *Amphiscolops breviviridis* to result in the isolation of a new 27-membered macrolide, amphidinolide L (**1002**).[845] Amphidinolide L (**1002**), $C_{32}H_{50}0_8$, was isolated as a colorless oil in 0.0004% yield from ca. 1800 g (wet weight) of the harvested cells obtained from 17501 of culture of this alga. Detailed analysis of the 2D NMR data (^{1}H-^{1}H COSY, HOHAHA, HMQC, and HMBC) led to the planar structure of amphidinolide L (**1002**) as constructed from a 27-membered lactone ring with an epoxide and tetrahydropyran moiety, which corresponded to the 20-dihydro-21-dehydro derivative of amphidinolide G (**988**). The relative stereochemistry of the tetrahydropyran moiety (C-21, C-22, C-23, and C-25 positions) was elucidated on the basis of NOE and coupling constant data, and the absolute configurations of the C-23 and C-25 positions were established by synthesis of the tetraacetate (**1023**), corresponding to the C-21–C-26 fragment of (**1002**), starting from the optically active epoxy alcohol (**1024**)[846] (Scheme 34). The synthetic tetraacetate (**1023**) showed completely identical spectral data including the signs of the optical rotations (synthetic, $[\alpha]_D$ +64° (*c* 0.2, $CHCl_3$); natural, $[\alpha]_D$ +72±8° (*c* 0.01, $CHCl_3$)), with those of compound (**1023**), which was obtained from (**1002**) by treatment with $NaIO_4$ followed by $NaBH_4$ reduction and acetylation, indicating the 21*R*,22*S*,23*R*,25*S*-configurations. Investigations are continuing into the synthesis of the diastereomers of the C-15–C-26 fragment of (**102**) to determine the stereochemistry of that moiety; preparation of a diastereomer (**1025**) possessing 16*R*,20*R*-configuration from (−)-methyl 3-hydroxy-2(*R*)-methylpropionate (**1010**) (Scheme 31) has been achieved in an efficient manner with good yields.[847]



i, DIBAH, benzene, room temperature, 1 h; ii, $(MeO)_2CMe_2$, PPTS, CH_2Cl_2, room temperature, 5 h; iii, H_2, $Pd(OH)_2$, EtOH, room temperature, 18 h; iv, DMSO, $(COCl)_2$, CH_2Cl_2, -78 °C, 30 min, then Et_3N, –20 °C, 30 min; v, Ph_3PCH_3Br, Bu^nLi, THF, room temperature, 2 h; vi, OsO_4, pyridine, THF, room temperature, 4 h; vii, 1N HCl, THF, room temperature, 5 h; viii, Ac_2O, pyridine, room temperature, 18 h; ix, HPLC separation

Scheme 34



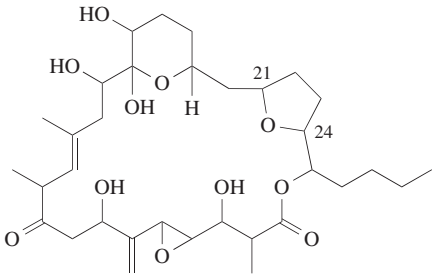
(1025)

The cytotoxic fraction mainly containing amphidinolide J (**994**) was further examined carefully by separation using reversed-phase HPLC to give four new compounds, amphidinolides K (**995**), M (**996**), N (**997**), and amphidinin A (**1001**). Amphidinolide K (**995**),[848] $C_{27}H_{40}O_5$, was isolated in 0.0002% yield (wet weight), and its gross structure was elucidated by applying several types of 2D

NMR techniques using a 600 MHz spectrometer to deduce the planar structure as (**995**) containing a 19-membered macrocyclic lactone along with a diene, an epoxide, and a tetrahydrofuran moiety. We proposed the relative stereochemistry of the epoxide-tetrahydrofuran portion (C-9, C-10, C-11, C-12, and C-15) on the basis of the NOESY and coupling constant data, and the diene moiety was inferred to have *S-trans* conformation from the NOESY correlations (H-5 and one of H_2-8; H_3-25 and one of H_2-26).

Amphidinolide M (**996**),[849] $C_{43}H_{66}O_9$, was first isolated in 1986 from the dinoflagellate of this species (strain number Y-5) in ca. 0.0005% yield (wet weight). Unfortunately, the sample of (**996**) decomposed extensively during storage as a $CDCl_3$ solution, and the structural studies were interrupted. The quantity of these macrolides contained in the extracts of the cultured cells varied a little during the course of time and amphidinolide M (**996**) was not isolated again for several years. When amphidinolide M (**996**) was reisolated from the same strain of this cultured alga by careful HPLC examination, spectral studies of (**996**) and its tetraacetate were extensively carried out to suggest that compound (**996**) was a 29-membered macrolide with two dienes, two tetrahydrofuran (THF) rings, an exomethylene, and an epoxide. The stereochemistry of (**996**) remained undetermined; the NOESY data of (**996**), however, may have implied that the angular hydrogens of two THF portions were both *trans* since NOESY cross-peaks were significantly observed for H-15/H-20 and H-22/H-27 while no correlations between angular protons (H-17/H-20 and H-22/H-25) were visible.

Amphidinolide N (**997**)[850] was isolated from a relatively polar fraction by reversed-phase HPLC (Develosil ODS-5, 60% CH_3CN) separation. Compound (**997**) was extremely cytotoxic against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* (*vide infra*); the cytotoxicity of (**997**) was the most potent of all amphidinolides that have ever been isolated. The structure of this macrolide, $C_{33}H_{54}O_{12}$, was interpreted by the extensive analysis of its spectroscopic data and was proposed to be (**997**), which was composed of a 26-membered macrolide containing a tetrahydropyran (THP) moiety with a hemiketal group, an epoxide, and an exomethylene group. The NOESY cross-peak observed between H-14 and H-19 might suggest that C-14 and H-19 were both axially oriented on the THP ring. The hydroxyl group on C-16 was deduced to be axial from the coupling constants ($J_{16,17a} = J_{16,17b} = 2.5$ Hz). After the isolation and gross structure of amphidinolide N (**997**) was published, the isolation and structure of caribenolide I (**1026**),[851] a related compound to (**997**), was described by Shimizu and co-workers; they isolated compound (**1026**) from a cultured free-swimming Caribbean dinoflagellate *Amphidinium* sp. Caribenolide I (**1026**) was reported to show strong cytotoxicity (IC_{50} 0.001 μg ml^{-1} or 1.6 nM) against both human colon tumor cell line (HCT 116 and its drug-resistant cell line, HCT 116/VM 46). This cytotoxicity was about 100 times higher than that of amphidinolide B (**983**) (IC_{50}, HCT 116, 0.122 μg ml^{-1}). Compound (**1026**) was also described to exhibit *in vivo* activity against murine tumor P388 (T/C: 150 at a dose of 0.03 mg kg^{-1}).

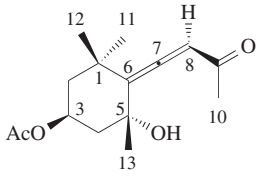


(1026)

Amphidinin A (**1001**), $C_{22}H_{38}O_4$,[852] was isolated from the macrolide-containing fraction by reversed-phase HPLC (Develosil ODS-5; 10×250 mm; 59% MeCN; flow rate: 2.5 ml min^{-1}; detection: RI and UV at 220 nm). Under this separation condition, compound (**1001**) had a very close retention time (t_R 27.6 min) to those of amphidinolides A (**981**) (t_R 29.1 min) and E (**986**) (t_R 26.0 min). Being different from all other cytotoxic metabolites isolated from this microalga, amphidinin A (**1001**) did not have the macrolide structure; the IR spectrum of (**1001**) showed no characteristic band due to a carbonyl group. Extensive NMR studies revealed that compound (**1001**) possessed a linear backbone skeleton with one THF moiety. Three hydroxyl groups are located on one end of the molecule, while there is a 2-methyl-1,4-pentadiene unit on the other end, constructing a hydrophilic and a hydrophobic moiety, respectively, in a linear molecule. The relative stereochemistry of

the THF portion was suggested by the NOESY data. As a result, the gross structure of amphidinin A was deduced as (**1001**), but the relative and absolute configurations of the chiral centers of (**1001**) remained undefined. Although amphidinin A (**1001**) is a nonmacrolide, this compound has several structural relationships to previously isolated amphidinolides, implying that biogenesis of amphidinin A (**1001**) may be closely related to amphidinolides.

During examinations of the cytotoxic fraction of the extract of the Y-5 strain of this microalga, an allenic compound (**1027**) was also isolated;[853] this compound was identified including the CD spectral data as apo-9′-fucoxanthinone,[854] which was previously reported as a permanganate oxidation product of fucoxanthin. The deacetyl derivative of (**1027**) was known as a grasshopper ketone isolated from ant-repellent secretions of the large flightless grasshopper *Romalea microptera*[855] and also isolated from *Edgeworthia chrysantha*.[856]



(1027)

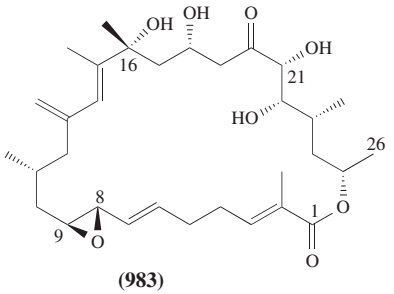
During studies on the Y-5 strain of *Amphidinium* sp., the cytotoxic fractions which were less polar than amphidinolide J (**994**) were examined. The ^{1}H NMR spectra of these crude fractions exhibited significantly exomethylene signals. Previously isolated amphidinolides all contain exomethylene groups, which were detected as sharp singlets around 5 ppm in the ^{1}H NMR spectra of crude fractions in the latter stage of the isolation process. Thus, these fractions were further purified by reversed-phase HPLC to result in the isolation of three novel macrolides, amphidinolides O (**998**), P (**999**), and Q (**1000**). Amphidinolides O (**998**) ($C_{21}H_{28}O_6$) and P (**999**) ($C_{22}H_{30}O_5$)[857] were both novel 15-membered macrolides, and these two compounds are structurally related to each other. They both contain a THP moiety with a hemiketal group, an epoxide, and at least two exomethylene groups. The structural difference was found at the C-11 position; the C-11 ketone group of (**998**) was replaced by an exomethylene group for (**999**), which was indicated from the following observations: (i) two IR absorption bands due to carbonyl groups were observed for (**998**), but one (ν_{max} 1730 cm^{-1}) for (**999**); (ii) compound (**998**) showed a UV absorption maximum at 231 nm due to an enone moiety, while the UV absorption of (**999**) underwent a blue shift (λ_{max} 225 nm), which was assignable to a diene chromophore; (iii) the ^{13}C NMR of (**999**) showed no signal due to a conjugated ketone, instead of which NMR signals for another exomethylene group were observed (δ_C 118.1 (C-22) and 142.3 (C-11); δ_H 4.98 (1H, br s) and 4.85 (1H, br s) for H_2-22).

Amphidinolides O (**998**) and P (**999**) possess seven chiral centers; the relative configurations of five chiral centers contained in the THP and epoxide ring portion were both elucidated on the basis of the NMR data as 3*S**, 4*R**, 7*S**, 8*S**, and 9*S** for (**998**) and 3*S**, 4*R**, 7*S**, 8*R**, and 9*S** for (**999**). The relative stereochemistries of the remaining two chiral centers (C-14 and C-15) were investigated by the combination of the ^{1}H NMR data and molecular mechanics calculations. Four diastereomers were considered for each compound with (14*R**,15*R**)-, (14*R**,15*S**)-, (14*S**,15*R**)-, and (14*S**,15*S**)-configurations, and the Monte Carlo lowest-energy conformations were calculated with the MM2 force field. The NOESY spectrum of amphidinolide O (**998**) in C_6D_6 solution clearly revealed cross-peaks due to H-8/H-12 and H-10b/H-12 with no correlations for H-8/H-13 or H-10b/H-13 observed. On the other hand, the NOESY spectra of amphidinolide P (**999**) in C_6D_6 showed substantial correlations for H-8/H-13, H-10b/H-13, and H-12/H-22a, but no cross-peaks due to H-8/H-12, H-10b/H-12, or H-13/H-22a were visible. These observations implied that, in the C_6D_6 solution states, the C-11–C-13 enone moiety of (**998**) is abundantly *S-cis* while the *S-trans* conformation is predominant for the C-11–C-13 diene moiety of (**999**). This result was consistent with the calculation data of 14*R**-diastereomers for both (**998**) and (**999**). The relative stereochemistry of the remaining chiral center at C-15 was analyzed on the basis of comparison of the proton–proton coupling constant ($J_{14,15}$) between the observed and the calculated values. The observed $J_{14,15}$-values were 7.4 Hz and 9.3 Hz for (**998**) and (**999**), respectively. The calculated average values of $J_{14,15}$ for the 15*R**-diastereomers (9.2 Hz for (**998**) and 9.5 Hz for (**999**)) corresponded better than those of the 15*S**-diastereomers (2.5 Hz for (**998**) and 3.7 Hz for (**999**)). Thus, the relative configurations of amphidinolides O and P were deduced as 3*S**, 4*R**, 7*S**, 8*S**, 9*S**, 14*R**, and 15*R** for (**998**) and 3*S**, 4*R**, 7*S**, 8*R**, 9*S**, 14*R**, and 15*R** for (**999**). The structural difference

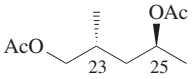
between (**998**) and (**999**) is found only at the C-11 position, viz., the ketone group for (**998**) and the exomethylene group for (**999**). Natural product analogues with this type of structural difference (ketone/exomethylene) are believed to be quite rare. Compounds (**998**) and (**999**) are likely to be biogenetically related to each other; one may be a precursor of the other, but it is unknown which one preceded the other.

Amphidinolide Q (**1000**) ($C_{21}H_{34}O_4$)[858] was revealed to possess one ketone, one exomethylene, and four methyl groups by spectral data. The ^{13}C chemical shift of the C-17 methyl (δ_C 16.6) argued that the Δ^2-alkene was *E*, and this double bond was suggested to be conjugated with the C-1 ester carbonyl from the ^{13}C chemical shifts (C-2: δ_C 117.4; C-3: δ_C 155.4), which were also consistent with the UV absorption data of (**1000**) (MeOH, λ_{max} 222 nm, ε 10 300). Since the molecule of (**1000**) was inferred to contain one ring from the unsaturation degrees, the C-1 carbonyl had to be linked to the C-11 oxymethine to form a 12-membered lactone ring, which was coincident with the low-field resonance of H-11 (δ_H 5.28). The gross structure of amphidinolide Q was thus elucidated as (**1000**) having a novel backbone skeleton with a 12-membered macrocyclic lactone ring. Among the NOESY correlations observed for (**1000**), cross-peaks for H-2/H-8a, H-7/H-9, H-8a/H-10a, and H-9/H-11 were noteworthy, which may suggest that the H-7, H-9, and H-11 are oriented to the same side of the macrocycle plane, whereas the H-2, H-8a, and H-10a are directed otherwise. Further convincing evidence, however, has not been provided for the stereochemical assignment of the molecule of (**1000**).

In 1987 the authors first reported the isolation and basic structure of amphidinolide B (**983**) from the Y-5 strain of *Amphidinium* sp.,[833] and the basic structure was later revised partially.[835] In 1994 Shimizu and co-workers isolated three macrolides belonging to the amphidinolide B group (amphidinolides B_1 (**983**), B_2 (**1028**), and B_3 (**1029**)) from a free-swimming dinoflagellate *Amphidinium* sp. and reported their relative stereochemistry on the basis of the X-ray crystal structure of amphidinolide B_1.[859] The identity of amphidinolides B and B_1 was unambiguously established by direct comparison of HPLC and ^1H NMR data using each authentic sample.[860] The signs of the optical rotations of these two samples were the same. The absolute stereochemistry of amphidinolide B (**983**) was studied based on the synthesis of a degradation product (**1030**) and chiral HPLC analysis.



(983)
(1028) C-18 epimer
(1029) C-22 epimer

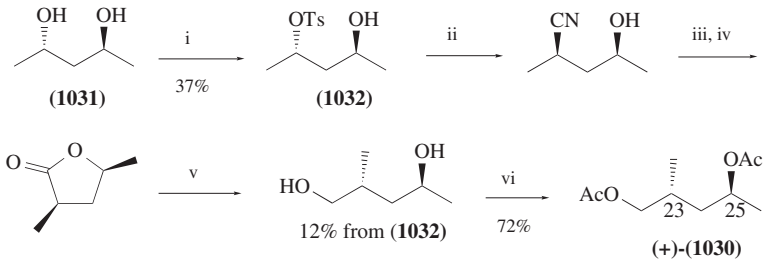


(+)-(**1030**)

23*R*, 25*S*

In advance of the degradation experiment both enantiomers of the C-22–C-26 fragment, (+)-(**1030**) and (−)-(**1030**) were prepared, as shown in Scheme 35, from (2*S*,4*S*)-(+)-pentanediol (**1031**) and (2*R*,4*R*)-(−)-pentanediol, respectively, both of which were available commercially. The chiral HPLC analysis (CHIRALCEL OD, Daicel Chemical Ind. Ltd.; 4.6 × 250 mm; flow rate: 1.0 ml min^{-1}; eluent: hexane/2-propanol (500:1); UV detection at 215 nm) of the enantiomers (+)- and (−)-(**1030**) showed that they were separable [(+)-(**1030**), t_R 23.2 min; (−)-(**1030**), t_R 22.3 min]. A MeOH adduct of amphidinolide B (**983**), which was obtained as an artifact of isolation and has a structure with a methoxyl and a hydroxyl group at the C-8 and C-9 positions,[835] was treated with $NaIO_4$ followed by $NaBH_4$ reduction and acetylation (Ac_2O/pyridine) to give the C-22–C-26 fragment (**1030**) after separation by normal-phase HPLC. The fragment (**1030**) thus obtained was subjected to chiral HPLC analysis as above and proved to be identical with (+)-(**1030**) (t_R 23.2 min), thus revealing that the C-22–C-26 fragment (**1030**) has (23*R*, 25*S*)-configurations. Since the relative stereochemistry of amphidinolide B_1 identical with (**983**) is known,[859] the absolute configurations of amphidinolide B (**983**) were concluded as 8*S*, 9*S*, 11*R*, 16*R*, 18*S*, 21*R*, 22*S*, 23*R*, and 25*S*, which was in agreement with results on the absolute configurations of amphidinolide L (**1002**).[845]

Shimizu and co-workers reported that amphidinolides B_2 (**1028**) and B_3 (**1029**), which they isolated concurrently with amphidinolide B_1 (**983**), were C-18 and C-22 epimers of (**983**), respectively.[859] The ^1H NMR spectra of amphidinolide B_2 (**1028**) and amphidinolide D (**985**) resembled each other very

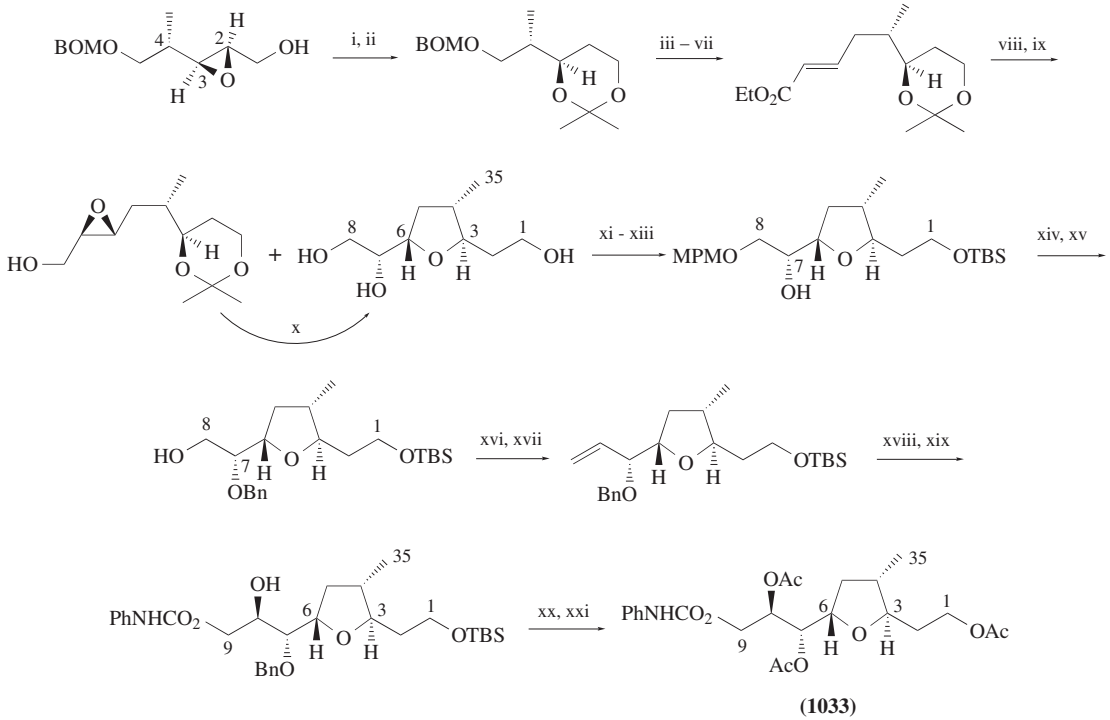


i, TsCl, pyridine; ii, NaCN, DMSO; iii, NaOH, H_2O_2, EtOH; iv, 2N HCl; v, LAH, Et_2O; vi, Ac_2O, pyridine

Scheme 35

well, indicating that these two compounds were identical. The authors had assigned the structure of amphidinolide D (**985**) to the C-21 epimer of (**983**),[835] and the conclusion of this different structural assignment ((**985**) or (**1027**)) was not provided.

The stereochemistry of amphidinolide C (**984**) has also been studied on the basis of NOESY experiments on (**984**) to propose the relative stereochemistry around a tetrahydrofuran ring moiety (C-1–C-9). A diastereomer of the C-1–C-9 fragment (**1033**) proposed from the NOESY data was synthesized as an optically active form, as shown in Scheme 36, to provide an authentic sample for use in studies on the degradation of amphidinolide C (**984**).[861]



(1033)

i, Red-Al (96%); ii, DMP, PPTS (99%); iii, H_2, Raney Ni (90%); iv, TsCl, Et_3N, DMAP (93%); v, NaCN, DMSO; vi, DIBAH; vii, $EtO_2CCH{=}PPh_3$ (3 steps, 79%); viii, DIBAH (86%); ix, (–)-DET, TBHP, $Ti(Pr^iO)_4$ (**7**, 62%; **8**, 16%); x, 1N H_2SO_4 (90%); xi, *p*-anisaldehyde dimethylacetal, TsOH (62%); xii, TBSCl, imidazole (86%); xiii, DIBAH (61%); xiv, BnBr, NaH (81%); xv, DDQ, phosphate buffer (81%); xvi, $(COCl)_2$, DMSO, Et_3N; xvii, Ph_3PCH_3Br, Bu^nLi (2 steps, 51%); xviii, AD-mix-α (56%); xix, PhNCO, DMAP, pyridine, then silica gel column (hexane/EtOAc, 5:2) (58%); xx, H_2, $Pd(OH)_2/C$; xxi, Ac_2O, pyridine (2 steps, 68%)

Scheme 36

Macrolide antibiotics from terrestrial microorganisms generally possess even-numbered macrocyclic lactones, which are reasonably derived from the polyketide biosynthesis. However, many amphidinolides comprise unusual odd-numbered macrocyclic lactone rings (amphidinolides C (**984**), 25-membered; E (**986**), 19-membered; F (**987**), 25-membered; G (**988**), 27-membered; J (**994**), 15-membered; K (**995**), 19-membered; L (**1002**), 27-membered; M (**996**), 29-membered; O (**998**), 15-membered; and P (**999**), 15-membered). The amphidinolides have some other unique structural features: (i) they have a variety of novel backbone-skeletons, isolated from one genus of microalga: (ii) all amphidinolides contain one or more exomethylene units; and (iii) vicinally located one-carbon branches (viz., methyl or exomethylene) are present in amphidinolides B (**983**), C (**984**), D (**985**), F (**987**), G (**988**), H (**989**), J (**994**), K (**995**), L (**1002**), M (**996**), O (**998**), P (**999**), and Q (**1000**), and amphidinin A (**1001**). In particular, the generation of an odd-numbered macrocyclic lactone ring, as well as structural feature (iii), was unable to be accounted for by the classical polyketide biosynthesis. The investigation of the biosynthesis of amphidinolides based on stable isotope incorporation experiments, although the sample size of the macrolides produced by the alga was not very high, requiring a large scale of culturing and a considerable number of ^{13}C-labeled precursors. The experimental results as well as the hypothesis on the biosynthesis of amphidinolide J (**994**), the most abundant macrolide in *Amphidinium* sp. (strain Y-5), are described below.[862]

The dinoflagellate *Amphidinium* sp. (strain Y-5) was cultured in 3 L glass bottles containing nutrient-enriched seawater medium, and feeding experiments were carried out with [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$] sodium acetate and [methyl-^{13}C]-L-methionine. A summary of the conditions of the feeding experiments is shown in Table 23. The ^{13}C-labeled precursors were fed to the alga (610 μM for labeled sodium acetate and 93 μM for labeled methionine) in one portion 10–12 days after inoculation, and 2 days later the culture was harvested. The extract of the harvested cells was purified by improved procedures to afford ^{13}C-labeled amphidinolide J (**994**) (0.5–1 mg from 80–100 L of culture). The assignments of the ^{13}C NMR signals of (**994**) in C_6D_6 solution were fully established by HMQC and HMBC spectra and are presented in Table 24.

Table 23 Feeding experiments of ^{13}C-labeled precursors to *Amphidinium* sp. (Y-5).

				The day after inoculation	
Run	*^{13}C-Labeled precursors*	*Culture* (L)	*Concentration* (μM)	*Addition* (day)	*Harvest* (day)
1	1-^{13}C-NaOAc	200	610	10	14
2	1-^{13}C-NaOAc	100	610	10	12
3	2-^{13}C-NaOAc	100	610	12	14
4	1,2-$^{13}C_2$-NaOAc	100	610	10	12
5	methyl-^{13}C-(L)-methionine	80	93	10	12

The ^{13}C NMR spectrum of amphidinolide J (**984**) labeled from sodium [1-^{13}C] acetate showed significant enrichment of nine carbons (C-1, C-4, C-6, C-8, C-10, C-13, C-15, C-17, and C-19), while 15 carbons were enriched by sodium [2-^{13}C] acetate (C-2, C-3, C-5, C-7, C-9, C-11, C-12, C-14, C-16, C-18, C-20, C-21, C-22, C-23, and C-24). The ratios of the signal intensities over those of nonlabeled (**994**) are given in Table 24. Thus, all 24 carbons contained in amphidinolide J (**994**) were revealed to be derived from acetates. The ^{13}C NMR of (**994**) obtained from the feeding experiment of [methyl-^{13}C]-L-methionine did not show appreciable enrichment of any carbon. The ^{13}C-^{13}C coupling constants ($^1J_{CC}$) of (**994**) labeled with [1,2-$^{13}C_2$]-acetate (Table 24) indicated that the C_2 units for C-1/C-2, C-4/C-5, C-6/C-7, C-8/C-9, C-10/C-11, C-13/C-14, C-15/C-16, C-17/C-18, and C-19/C-20 originate from the same acetates. Interestingly, when the culture was harvested 4 days after feeding of sodium [1-^{13}C] acetate to the alga (run 1 of Table 23), the ^{13}C NMR of isolated (**994**) showed that all carbon atoms of (**994**) were enriched and almost all signals were observed with double satellite signals due to vicinal ^{13}C-^{13}C couplings. This phenomenon was considered to be observed probably because C-1 of acetate was cleaved via decarboxylation during passage through the TCA cycle and the released $^{13}CO_2$ was reincorporated during photosynthesis to give randomly labeled acetates, which led to all-carbon enriched (**994**). The labeling patterns of amphidinolide J (**994**) shown by the feeding experiments were quite unusual and are presented in Figure 18.

Table 24 Isotope incorporation results from the ^{13}C NMR data of amphidinolide J (**994**).[a]

Position	δ_C	*Intensity ratio (labeled/unlabeled)*[b]		*Assignment* "c" or "m"[c]	J_{CC} (Hz)
		[1-^{13}C]-*acetate*	[2-^{13}C]-*acetate*		[1,2-$^{13}C_2$]-*acetate*
1	171.6	1.41	1	c	57.8
2	39.9	1.01	1.72	m	57.8
3	34.6	0.88	1.59	m	
4	151.9	1.34	0.97	c	42.5
5	36.1	1.05	2.02	m	41.4
6	29.7	1.68	1.36	c	43.6
7	130.8	0.87	1.88	m	43.6
8	136.5	2.10	1.10	c	49.0
9	78.8	0.95	1.66	m	48.0
10	45.7	1.51	0.99	c	43.6
11	133.5	1.03	2.09	m	43.6
12	132.6	0.87	1.51	m	
13	72.6	1.46	1.05	c	42.5
14	79.9	1.15	2.33	m	41.4
15	39.5	1.52	1.16	c	42.5
16	133.6	0.76	1.61	m	43.6
17	131.5	1.53	0.95	c	42.5
18	35.3	0.94	2.50	m	42.5
19	23.4	1.58	1.01	c	34.9
20	14.2	1	1.98	m	34.9
21	22.2	0.96	1.81	m	
22	108.7	1.12	1.99	m	
23	19.0	1.03	1.78	m	
24	17.5	1.14	2.21	m	

[a] The ^{13}C NMR spectra were recorded in C_6D_6 solution on a Bruker ARX500 spectrometer at 125 MHz with sweep width of 35 700 Hz using Bruker's pulse program "zgpg30." Numbers of scans were ca. 13 000 and 25 728, for the samples from feedings of mono- and double-^{13}C labeled precursors, respectively. [b]Intensity of each peak in the labeled (**994**) divided by that of the corresponding signal in the unlabeled (**994**), normalized to give a ratio of 1 for an unenriched peak (C-20 for [1-^{13}C]-acetate labeling and C-1 for [2-^{13}C]-acetate labeling). [c]"c" denotes "carbon derived from C-1 of acetate," while "m" indicates carbon derived from C-2 of acetate."

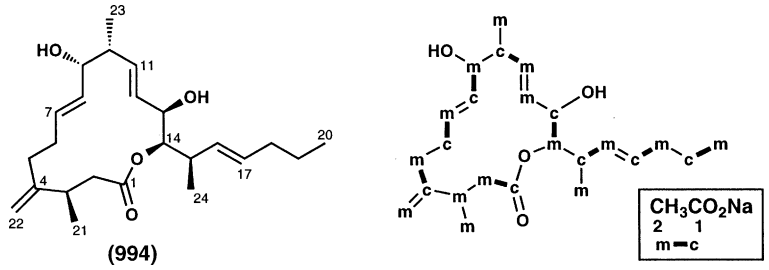
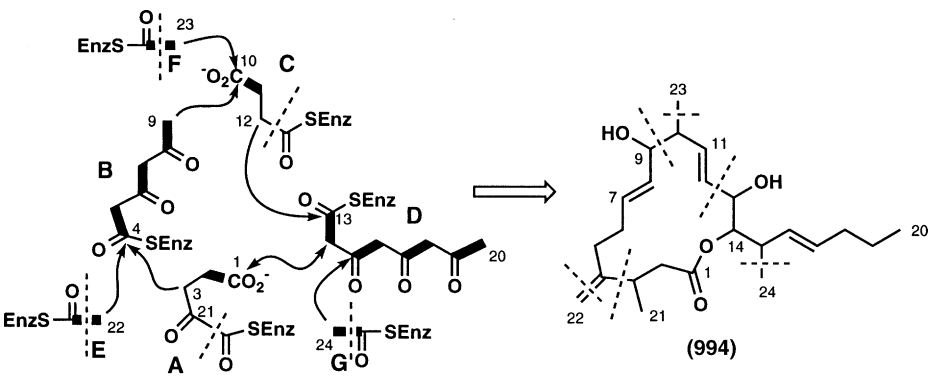


Figure 18 Labeling patterns of amphidinolide J (**994**).

Significantly, the C-3 and C-12 of (**994**) were derived from the methyl carbons of acetates, the carboxyl carbons of which were lost. Thus, the carbons constituting the 15-membered lactone ring were not constructed from the consecutive polyketide chain. This finding seems to justify the fact that the lactone ring size of (**994**) does not have to be even. The irregular labeling pattern of (**994**) could be interpreted as one possibility by assuming that the backbone carbons of (**994**) were biosynthetically derived from the precursors depicted in Figure 19. Units B (C-4 to C-9) and D (C-13 to C-20) are likely to be classical polyketides derived as a result of the condensation of three and four acetate units, respectively. Unit A (C-1/C-2/C-3/C-21) contains the "c-m-m-m" moiety and many come from a dicarboxylic acid like α-ketoglutarate after passage of acetate through the TCA cycle, which has been observed in the biosynthesis of brevetoxin B and okadaic acid (see Sections 8.07.8.3.1 and 8.07.8.5.2). Unit C (C-10/C-11/C-12) labeled "c-m-m" may be derived from succinate, corresponding to the six units in brevetoxin B. Units E, F, and G (C-22, C-23, and C-24) are one-carbon branches (an exomethylene and two secondary methyls), and they were demonstrated

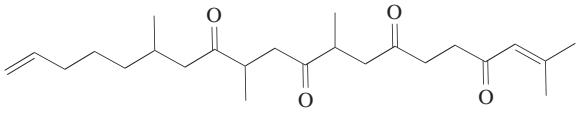
to be derived from the C-2 of acetates and attached to carbons in a linear chain derived from the C-1 of acetates (C-4, C-10, and C-15, respectively). One-carbon branching of this type is unusual in polyketide biosynthesis and has been previously reported only in few cases. Another one-carbon branch of C-21 also came from the C-2 of acetate. However, the condensation of this carbon to the linear chain occurred at the carbon (C-3) derived from the C-2 of acetate; thus, the participation of a dicarboxylic acid precursor was proposed for this moiety. How the vicinal locations of one-carbon branches are brought about in amphidinolides is an interesting question, and the results argued that two vicinal one-carbon branches (C-21 and C-22) of (**994**) were both derived from the C-2 of acetate but attached to the linear chain through different processes. It should be noted that the oxymethines at C-9 and C-14 of (**994**) are derived from the C-2 of acetate, and the origins of the oxygen atoms are unknown.



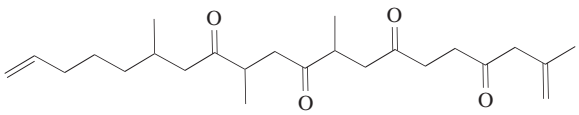
A: α-ketoglutarate; **B**, **D**: classical polyketide;
C: succinate; **E**, **F**, **G**: C-2 of acetate

Figure 19 Possible biosynthetic building blocks of amphidinolide J (**994**).

In connection with this work, the unusual 1,4-polyketides, amphidinoketides I (**1034**) and II (**1035**) were isolated from *Amphidinium* sp. by Shimizu's group[863] and a possible biosynthetic pathway involving the condensation of succinates was proposed. The 1,4-polyketide moieties of (**1034**) and (**1035**) may correspond to the C-15–C-18 portion of amphidinolides C (**984**) and F (**987**).



(**1034**)



(**1035**)

Wright and co-workers[329] proposed the biosynthetic path of amphidinolide J (**994**) through a carbon-deletion step involving oxidation of a methyl-derived carbon to yield an α diketide followed by a Favorski-type rearrangement, peroxide attack, and collapse of the cyclopropanone, which may account for the interrupted pattern of acetate units in the chain instead of the participation of dicarboxylic acid precursors (see Section 8.07.8.3.1).

The cytotoxicity data of amphidinolides against murine lymphoma L1210 and human epidermoid carcinoma KB cells *in vitro* are summarized in Table 25 together with their isolation yields from the four strains of the dinoflagellates *Amphidinium* sp. The level of the cytotoxic activity of

amphidinolides B_1 (**983**), B_2 (**1028**), and B_3 (**1029**) against a different cell line (human colon tumor cell line HCT 116 (IC_{50} 0.122, 7.5, and 0.206 μg ml^{-1}, respectively), reported by Shimizu and coworkers,[859] were significantly lower than those of amphidinolides B (**983**) and D (**985**).

Table 25 Isolation yields and cytotoxicity data of amphidinolides.

Compounds		*Lactone ring size*	*Isolation yields* ($\times 10^{-4}$%)[a] *Strain number* *Y-5*[b]	*Y-5′*	*Y-25*	*Y-26*	*Cytotoxicity* (IC_{50}, μg ml^{-1}) *L1210*	*KB*
Amphidinolide A	(**982**)	(20)	20	4			2.0	5.7
Amphidinolide B	(**983**)	(26)	10			0.8	0.00014	0.0042
Amphidinolide C	(**984**)	(25)	15			0.3	0.0058	0.0046
Amphidinolide D	(**985**)	(26)	4				0.019	0.08
Amphidinolide E	(**986**)	(19)	4	3			2.0	10
Amphidinolide F	(**987**)	(25)				0.1	1.5	3.2
Amphidinolide G	(**988**)	(27)			20		0.0054	0.0059
Amphidinolide H	(**989**)	(26)			17		0.00048	0.00052
Amphidinolide J	(**994**)	(15)	60				2.7	3.9
Amphidinolide K	(**995**)	(19)	0.3				1.65	2.9
Amphidinolide L	(**1002**)	(27)			2		0.092	0.1
Amphidinolide M	(**996**)	(29)	4				1.1	0.44
Amphidinolide N	(**997**)	(26)	9				0.00005	0.00006
Amphidinolide O	(**998**)	(15)	1				1.7	3.6
Amphidinolide P	(**999**)	(15)	2				1.6	5.8
Amphidinolide Q	(**1000**)	(12)	0.5				6.4	> 10
Amphidinin A	(**1001**)		0.6				3.6	3.0

[a] Based on the wet weight of the harvested cells. [b] Isolation yields vary during the course of time.

8.07.10 REFERENCES

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