Bioactive Sponge Peptides

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Contents

Nobuhiro Fusetani was born in Tsu-shi, western Japan, and received his B.Agric. in 1966 from Faculty of Agriculture, the University of Tokyo. After he completed his M.S. degree with the late Professor Yoshiro Hashimoto at the University of Tokyo, he spent two years with Professor Paul J. Scheuer at the University of Hawaii. He then took up an assistant professorship in 1971 at Faculty of Agriculture, University of Tokyo. In 1973 he was awarded his Ph.D. under the guidance of Professor Hashimoto, from the University of Tokyo. He was appointed to Associate Professor in 1977 and has been a full Professor since 1990. He has been involved in marine toxin research with Professors Y. Hashimoto and P. J. Scheuer. Since the early 1980s he has been leading a research group investigating bioactive metabolites from Japanese marine invertebrates. Currently this group's focus is on the discovery of useful leads from marine sponges and on chemical signals in nudibranch molluscs and crustaceans. In October 1991 he was appointed to Project Leader of the Fusetani Biofouling Project, a 5-year project of the Exploratory Research for Advanced Technology (ERATO), Research Development Corporation of Japan. He is leading a research group of 20 persons working on mechanisms of larval settlement and metamorphosis of marine organisms.

Shigeki Matsunaga was born in Nakano, Tokyo, in 1957. He received his B.A. degree and his Ph.D. degree from The University of Tokyo. After postdoctoral work at The University of Tokyo (with Nobuhiro Fusetani), University of Hawaii (with Richard E. Moore), and National Cancer Center Research Institute (with Hirota Fujiki), he moved to a faculty position at The University of Tokyo in 1991. Research interests include discovery of marine natural products with interesting biological activities and mode of actions of bioactive marine natural products.

I. Introduction

Sponges appeared on the earth in the Cambrian Age (more than 6 million years ago) and are widely found from pole to pole and from intertidal zones to water thousands of meters deep. As biomass, they are enormous and naturally have been a target for extensive studies to isolate new substances since Bergmann's pioneering work on sterols and novel nucleosides in the 1940s.[1] These research activities have resulted in isolation of a variety of new compounds, among which are included structurally novel and highly biologically active metabolites.[2]

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Table I. Classification of Demospongiae

subclasss	order	representative genus
Homoscleromorpha	Homosclerophorida	*Plakortis*
Tetractinomorpha	Choristida	*Stelletta, Geodia, Jaspis*
	Spirophorida	*Cinachyra*
	Lithistida	*Discodermia, Theonella*
	Hadromerida	*Cliona, Tethya, Aaptos, Chondrilla, Latrunculia*
	Axinellida	*Axinella, Phakellia, Acanthella, Agelas*
Ceractinomorpha	Halichondria	*Halichondria, Hymeniacidon*
	Poecilosclerida	*Mycale, Tedania*
	Nepheliospongida	*Petrosia, Xestospongia*
	Haplosclerida	*Haliclona, Adocia*
	Dictyoceratida	*Spongia, Cacospongia, Ircinia, Dysidea*
	Dendroceratida	*Aplysilla*
	Verongida	*Aplysina, Psammaplysilla, Ianthella*

Why do sponges often contain unusual compounds? Most marine natural products chemists would ascribe this to the occurrence of symbiotic microbes. Sponges are actually simple cell aggregates, which are usually referred to as "the most undeveloped multicellular animals". Therefore, sponges provide lodging for many organisms; brittle stars, bivalves, gastropods, crustaceans, and annelid worms are common guests. In addition to these macroorganisms, bacteria, blue-green algae, and dinoflagellates are observed in many species.[3] In an extreme case, bacteria occupy more than 40% of the tissue volume of a sponge.[3] It is therefore reasonable to believe that some sponge metabolites are produced by symbiotic microorganisms. In fact, certain classes of compounds are structurally identical with or similar to those known from terrestrial microorganisms; representative examples are dysidin from *Dysidea herbacea*,[4] malyngolide A from the blue-green alga, *Lyngbya majuscula*,[5] swinholides and bistheonellides from *Theonella swinhoei*,[6] scytophycins from the blue-green algae *Scytonema* spp.,[7] aurantosides from *Theonella swinhoei*,[8] lipomycin from *Streptomyces aureofaciens*,[9] cylindramide from *Halichondria cylindrata*,[10] and ikarugamycin from *Streptomyces phaeochromogenes*.[11]

Peptides are also suspected to be of microbial origin from the presence of both D amino acids and unusual amino acids. In 1985 we isolated the first bioactive peptide, discodermin A[12] from the marine sponge *Discodermia kiiensis*, which contains the rare *tert*-leucine and cysteic acid, in addition to several D amino acids. Heretofore, *tert*-leucine was only known as a constituent amino acid of peptidic antibiotics isolated from actinomycetes.[13] Since our isolation of discodermins almost 50 peptides have been reported from marine sponges, some of which not only show interesting biological activities but also contain new amino acids.

Several reasons can be listed for progress in the chemistry of sponge peptides: (1) Development of reversed-phase HPLC enabled the isolation of peptides from a mixture of related metabolites. (2) Advances in spectroscopy, especially 2D NMR and FAB mass spectroscopy were indispensable for the structural study of peptides from marine sponges, because sequence analysis of unusual peptides cannot be accomplished by Edman degradation due to the presence of blocked N-termini and β- or γ-amino acid residues. (3) Progress in chiral chromatography allowed the assignment of absolute configuration of amino acids with small amounts of material. (4) Last but not least, marine natural product chemists encountered sponges of the order Lithistida, which includes sponges rich in bioactive peptides.

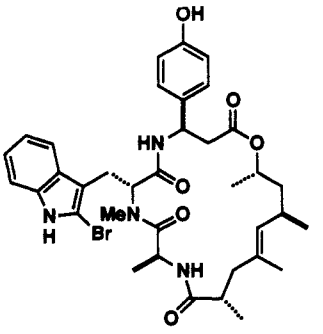
Ireland and collaborators[14] reviewed marine peptides which appeared in the literatures before 1987. However, numerous interesting peptides have been isolated from marine sponges since that review. In this account, we describe isolation, structure elucidation, and biological activities of peptides from marine sponges, according to their taxonomical classification as shown in Table I. We also discuss their "real producers" from the viewpoint of their structural similarity to peptides from terrestrial microbes.

II. Choristida

Chemistry of the sponges of the order Choristida includes isomalabaricane triterpenes from *Jaspis stellifera*,[15] amino acid-derived metabolites from an undescribed Jaspidae sponge,[16] stellettamide A from a *Stelletta* sp.,[17] and a steroid glycoside, pachastrelloside A from a *Pachastrella* sp.[18] No general trend is observed in the type of the metabolites from this order.

A. Jaspamide (Jasplakinolide) from *Jaspis* sp.

Jaspamide was the first bioactive peptide from sponges of the order Choristida;[19] isolation of geodiamolides followed shortly.[20] They are four-residue cyclic depsipeptides sharing similar structural features: presence of an 11-carbon hydroxy acid and a halogenated aromatic amino acid.



jaspamide
(jasplakinolide)

Jaspamide was isolated from a sponge of the genus *Jaspis* from Fiji and Palau.[19] Structures of component amino acids were elucidated by interpretation of the NMR data, while the amino acid sequence was determined by analysis of HRFAB mass spectra of a

saponified product. The structure, including relative stereochemistry, was firmly established by X-ray crystallography of the acetate. Absolute stereochemistry was secured from the configuration of the Ala residue (L form), which was assigned by chiral HPLC analysis of the acid hydrolyzate.

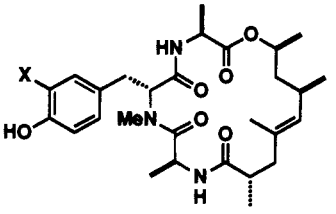
The same compound was also reported under the name of jasplakinolide, which was isolated from a Fijian *Jaspis* sponge.[21] Structural study of jasplakinolide was done by interpretation of NMR data (COSY, long-range COSY, and long-range C,H-COSY), isolation of hydrolysis products, and analysis of CIMS fragment ion peaks, leading to the same gross structure as jaspamide. Conformational analysis made from NMR data, molecular mechanics, and molecular dynamics calculations concluded that jasplakinolide adopts two major conformations.[22] Lithium complexation study on jasplakinolide was also reported.[23]

Japspamide (jasplakinolide) exhibited insecticidal activity against *Heliothis verescens* with an LC_{50} of 4 ppm. It was also toxic to the nematode *Nippostrongylus braziliensis* ($LD_{50} < 1$ μg/mL). Jaspamide (jasplakinolide) showed *in vivo* topical activity against a vaginal *Candida* infection in mice and was cytotoxic against a larynx epithelial carcinoma cell line (IC_{50} 0.32 μg/mL) and a human embryonic lung cell line (IC_{100} 0.01 μg/mL).

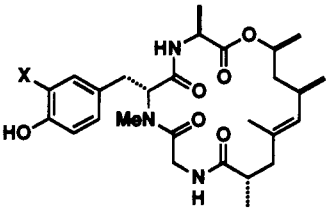
Total synthesis of jaspamide (jasplakinolide) has been accomplished.[24]

B. Geodiamolides A and B from *Geodia* sp. and Geodiamolides C–F from *Pseudoaxinyssa* sp. (Axinellida)

Geodiamolides A and B were first isolated from a Caribbean sponge *Geodia* sp.[20] The structures of



geodiamolide A X=I
geodiamolide B X=Br
geodiamolide C X=Cl



geodiamolide D X=I
geodiamolide E X=Br
geodiamolide F X=Cl

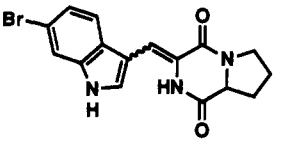
constituents in geodiamolides A and B were determined by interpretation of NMR data, and their total structures were determined by X-ray crystallography. Absolute configurations of geodiamolides were based on the anomalous scattering of the halogen atoms in the tyrosine units. Geodiamolide A differed from B in the halogen atom in the tyrosine residues.

Recently, geodiamolides C–F were isolated, together with geodiamolides A and B, from a Papua New Guinean sponge, *Pseudoaxinyssa* sp., belonging to the order Axinellida, family Axinellidae, which is taxonomically distant from *Geodia*.[25] Structure elucidation of geodiamolides C–F was carried out by analysis of the NMR and mass spectral data as well as comparison of NMR data with those of geodiamolides A and B. Geodiamolide C was a chlorine-containing analog of geodiamolide A, while geodiamolides D, E, and F had a glycine residue in place of the alanine residue in geodiamolides A, B, and C, respectively. Isolation of the same compounds from taxonomically remote species suggests an involvement of symbionts in the production of geodiamolides.

Total synthesis of geodiamolides A and B has been reported.[26]

C. Barettin from *Geodia baretti*

From the extract of *Geodia baretti* collected from the Swedish coast, an unrelated peptide, barettin, was reported.[27] Barettin strongly affected an isolated guinea pig ileum. A diketopiperazine structure composed of proline and 6-bromodehydrotryptophan residues was proposed on the basis of spectral data. However, total synthesis of the diketopiperazine disclosed that the proposed structure was incorrect, suggesting that barettin might have a dimeric cyclic tetrapeptide structure.[28]



barettin

III. Lithistida

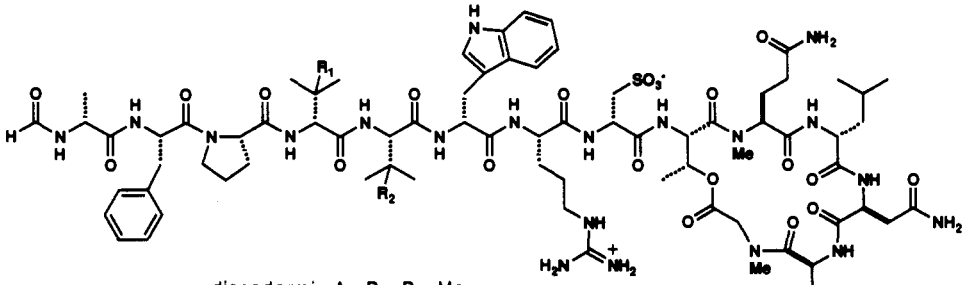
Sponges of two genera *Discodermia* and *Theonella* have proved to be a rich source of bioactive metabolites. Most of the secondary metabolites reported from the sponges of this order are nitrogenous, viz. isocyano or amino sesquiterpenes, indole derivatives, tetramic acids, and peptides.[2] The exceptions are polyketides, swinholides, bistheonellides, and discodermolide.[2]

It had been proposed that soft-bodied sponges have a higher probability of containing bioactive compounds than those with hard bodies, because they need chemical defense against predators. However, *Discodermia kiiensis* and calyculin-containing *D. calyx*, which not only have hard bodies, but also epiphytes, contain large amount of bioactive metabolites. This is the case for other sponges of the order Lithistida.

A. Discodermins from *Discodermia kiiensis*

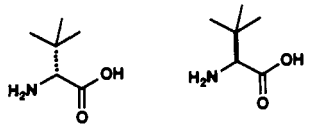
Discodermins were the first bioactive peptides isolated from marine sponges,[12] although the structures of peracetylated celenamides had been reported a few years earlier without description of their biological activities. We isolated discodermins, A–D (Chart I) from *D. kiiensis* as antimicrobial constituents. They are tetradecapeptides with the N-terminus blocked by a formyl group and the C-terminus lactonized with the ninth (Thr) residue from the N-terminus. Structural study was mainly performed on the major metabolite, discodermin A. Component amino acids were isolated

Chart I



discodermin A R_1=R_2=Me
discodermin B R_1=H R_2=Me
discodermin C R_1=Me R_2=H
discodermin D R_1=R_2=H

either by ion exchange chromatography or high-voltage paper electrophoresis, and their chiralities were determined by CD spectroscopy. An initial assignment of D-Pro was later revised to L-Pro on the basis of chiral GC analysis. Sequence analysis up to the sixth residue was accomplished by the Edman degradation of the deformylated discodermin A. For further sequence analysis, discodermin A was treated with BNPS-skatol [2-[2′-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine], which selectively cleaved the carboxyl side of the peptide bond of Trp residue, yielding an octapeptide containing the C-terminus. Although Edman degradation of this octapeptide gave a sequence of only three residues, a corresponding octapeptide fragment obtained from the base-treated discodermin A provided a sequence of six residues by Edman degradation. C-Terminal analysis with hydrazinolysis as well as Hofmann-type degradation with bis(trifluoroacetoxy)-iodobenzene, which confirmed the presence of an α-linked Asn residue, allowed assignment of a total structure. A differentiation between D-*t*-Leu and L-*t*-Leu was accomplished by cleaving deformyldiscodermin A up to the fourth residue from the N-terminus by Edman degradation and subjecting the remaining decapeptide to acid hydrolysis followed by chiral GC/MS analysis.



D-*t*-Leu L-*t*-Leu

The structures of discodermins B–D differed in the fourth and fifth residues, which were Val-*t*-Leu in discodermin B, *t*-Leu-Val in discodermin C, and Val-Val in discodermin D. Amino acid sequences of discodermins B–D from the N-terminus to the fifth residue were determined by a combination of Edman degradation and analysis of fragment ions in the EI mass spectra, whereas those from the sixth residue to the C-terminus were disclosed by obtaining the same octapeptide as from discodermin A upon treatment with BNPS-skatol.

Peptides with a cysteic acid residue are rare among natural peptides,[29] though cysteine residues are usually transformed to cysteic acid residues to facilitate sequence analysis. The most unusual component in the discodermins is the *t*-Leu residue, which had only been reported as a constituent of actinomycete peptides,

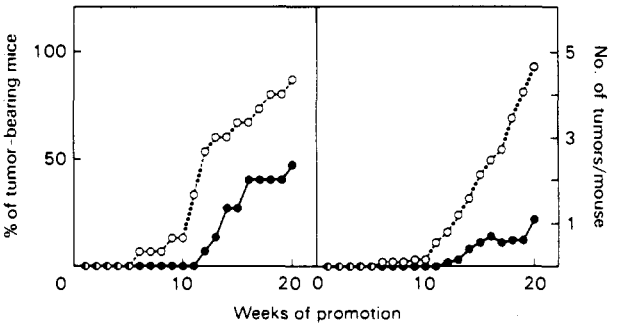


Figure 1. Inhibition of tumor promotion by discodermin A. The groups treated with 7,12-dimethylbenz[*a*]anthracene (DMBA) and okadaic acid (○) and DMBA and okadaic acid plus discodermin A (●).

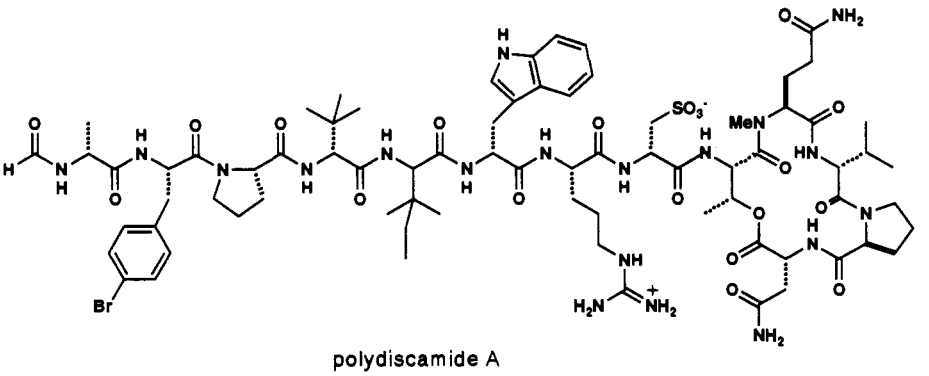
bottromycins.[13] These facts together with the presence of many D amino acids may suggest the microbial origin of discodermins.

Discodermin A inhibited *Bacillus subtilis* and *Proteus mirabilis*, with minimum inhibitory concentrations of 3 and 1.6 μg/mL, respectively. Discodermins also inhibited the development of starfish embryos at concentrations of 2–20 μg/mL. Later, discodermins were found to be potent inhibitors against phospholipase A_2 (IC_{50} 3.5–7.0 × 10^{-7} M).[30] As expected, discodermin A exhibited antiinflammatory activity in the mouse ear pretreated with okadaic acid. Discodermin A also inhibited tumor promotion by okadaic acid. Treatment with 500 μg of discodermin A before application of okadaic acid (1 μg) reduced the percentages of tumor-bearing mice from 86.7% to 46.7% and the average number of tumors per mouse from 4.7 to 1.1 (Figure 1).[31]

B. Polydiscamide A from a Deep-Sea Species of *Discodermia*

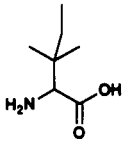
Polydiscamide A (Chart II) was isolated from a Caribbean sponge *Discodermia* sp. which was collected at a depth of 274 m off the coast of St. Lucia, Lesser Antilles, by using a submersible.[32] Polydiscamide A possesses common structural features with discodermins: the presence of a formylated N-terminus; *t*-Leu, Cys(O_3H), Arg, and Trp residues; and the C-terminus being lactonized with a Thr residue. Stereochemistry of the component amino acids in polydiscamide A was determined by HPLC analysis of the acid hydrolyzate after derivatization with Marfey's reagent. The presence of a novel amino acid, 3-methylisoleucine, was inferred from the NMR data, which

Chart II



polydiscamide A

was supported by isolating the amino acid from the acid hydrolyzate. Sequence analysis was carried out by interpretation of the NMR data.



3-methylisoleucine

Polydiscamide A is cytotoxic against the cultured human lung cancer A549 cell line with an IC_{50} of 0.7 μg/mL and inhibits *B. subtilis* with an MIC of 3 μg/mL. It is an interesting question whether polydiscamide A, with a smaller lactone ring, inhibits phospholipase A_2 or not.

C. Discokiolides from *Discodermia kiiensis*

Discokiolides are unrelated peptides from *D. kiiensis*.[33] They are cyclic depsipeptides containing unusual β-hydroxy acids named discokiic acids as well as β-methoxyphenylalanine residues (Chart III). Actually, discokiolides could not be isolated as their natural free carboxylic acid forms; they were separated by reversed-phase HPLC after conversion to the methyl esters. Structural study was performed on the major constituent, discokiolide B methyl ester, by means of NMR and mass spectroscopy. Component amino acids were deduced by the COSY, HOHAHA, and HMQC spectra, whereas the amino acid sequence was determined on the basis of HMBC and ROESY data. The gross structure was supported by accurate mass measurements for the fragment ions in the liquid secondary ion mass spectrum. Stereochemistry of the component amino acids and hydroxy acid remain to be elucidated. The structures of three related metabolites, discokiolides A, C, and D, were determined in the same way.

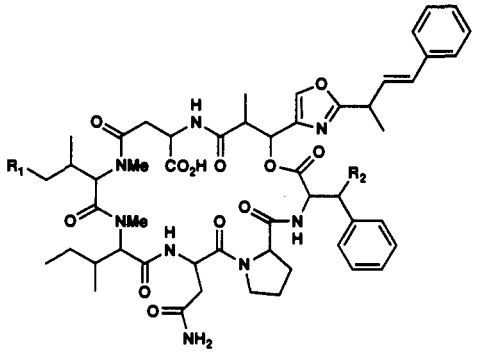
As a mixture of carboxylic acids, discokiolides exhibited marginal cytotoxic activity: IC_{50} (μg/mL) P388, 2.6; P388/ADM, 7.2; B16-BL6, 1.6; Lewis, 1.2; Lu-99, 0.7; HT-29, 1.2; CCD-19Lu, 0.5. However their methyl esters were no longer active.

D. Calyculins from *Discodermia calyx*

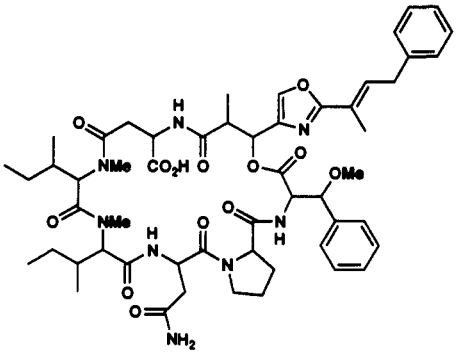
Calyculins can be categorized as peptides containing an octamethyl polyhydroxylated C_{28} fatty acid linked to two γ-amino acids. We came across the sponge *Discodermia calyx* during our screening program for cell division inhibitors of fertilized starfish eggs.[34] The extract of *D. calyx* also exhibited potent antifungal activity. Isolation of calyculins was unexceptional, because they were the major components of the lipophilic extract and in spite of their ionic nature they behaved like nonionic organic compounds.

The structure of calyculin A including relative stereochemistry was established by X-ray crystallography, whereas the structures of calyculins B-F were elucidated by interpretation of NMR spectral data.[35] During isolation, calyculins A, B, E, and F as well as calyculins C, D, G, and H interconvert due to photochemical isomerization of the terminal tetraene moiety. Since calyculins A and C are the predominant components in the fresh extract, other calyculins may be

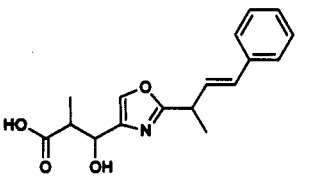
Chart III



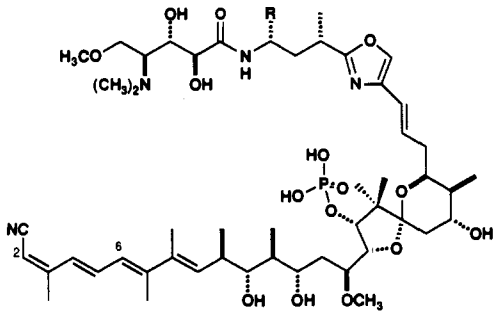
discokiolide A R_1=H, R_2=OMe
discokiolide B R_1=Me, R_2=OMe
discokiolide C R_1=Me, R_2=H



discokiolide D

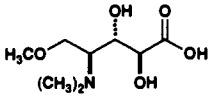


discokiic acid



calyculin A R=H
calyculin B R=H (2*E*)
calyculin C R=CH_3
calyculin D R=CH_3 (2*E*)
calyculin E R=H (6*Z*)
calyculin F R=H (2*E*, 6*Z*)
calyculin G R=CH_3 (6*Z*)
calyculin H R=CH_3 (2*E*, 6*Z*)

artifacts. Absolute stereochemistry of the calyculins was determined on the basis of the CD spectrum of an amino acid fragment obtained by acid hydrolysis,[36] which was later confirmed by a synthesis of the fragment[37] and total synthesis of an enantiomer of calyculin A.[38]



a hydrolytic fragment of calyculin A

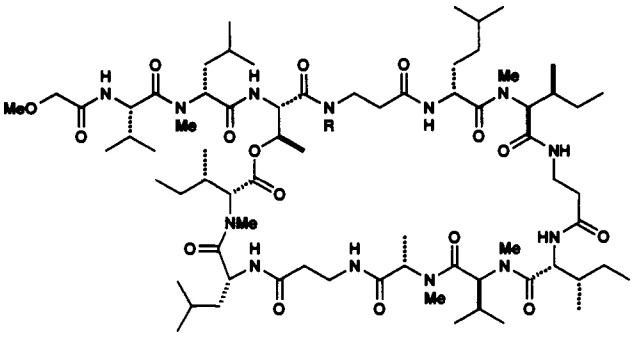
Calyculin A had *in vivo* antitumor activity as well as cytotoxic activity and antifungal activity. Later, calyculin A turned out to be a potent tumor promoter as is the case for okadaic acid.[39] This activity and contraction of smooth muscles were found to be attributable to inhibition of protein phosphatases 1 and 2A (Table II).[40]

Table II. Inhibitory Activity of Calyculins against Protein Phosphatases 1 and 2A

	IC_{50} nM	
	phosphatase 1	phosphatase 2A
calyculin A	1.4	2.6
calyculin B	1.0	3.6
calyculin C	0.6	2.8
calyculin D	4.0	4.8
calyculin E	1.4	5.2
calyculin F	1.4	4.8
calyculin G	6.4	8.5
calyculin H	7.5	14.0

E. Theonellapeptolides from *Theonella* sp.

Theonellapeptolides are tridecapeptides rich in *N*-methyl and D amino acids. They were isolated from an Okinawan *Theonella* sp. that contained swinholides.[41] Due to the presence of β-alanines and a blocked N-terminus, theonellapeptolides resisted sequence analysis by Edman degradation. The structure of theonellapeptolide Id, the major constituent, was determined by extensive analysis of fragment peptides which were obtained by treating the parent peptide with 30% TFA, 110 °C, 40 min.[42] Fragment peptides were characterized by ^1H NMR, FABMS, N-terminal analysis after dansylation, and amino acid analysis developed in this study. It should be noted that component amino acids were successfully separated by ODS HPLC with a water eluent by detecting UV absorption at 205 nm. This methodology is effective in separating amino acids including *N*-methyl or *N,N*-dimethyl amino acids with hydrophobic side chains. Theonellamine B, an inhibitor of Na,K-ATPase, was identical with theonellapeptolide Id.[43] Theonellamine B was isolated from probably the same Okinawan *Theonella* sp. The structure elucidation of theonellamine B was mainly carried out by interpretation of NOESY and COLOC data. Structure elucidation of theonellapeptolide Ie, a related peptide, was also reported.[44]



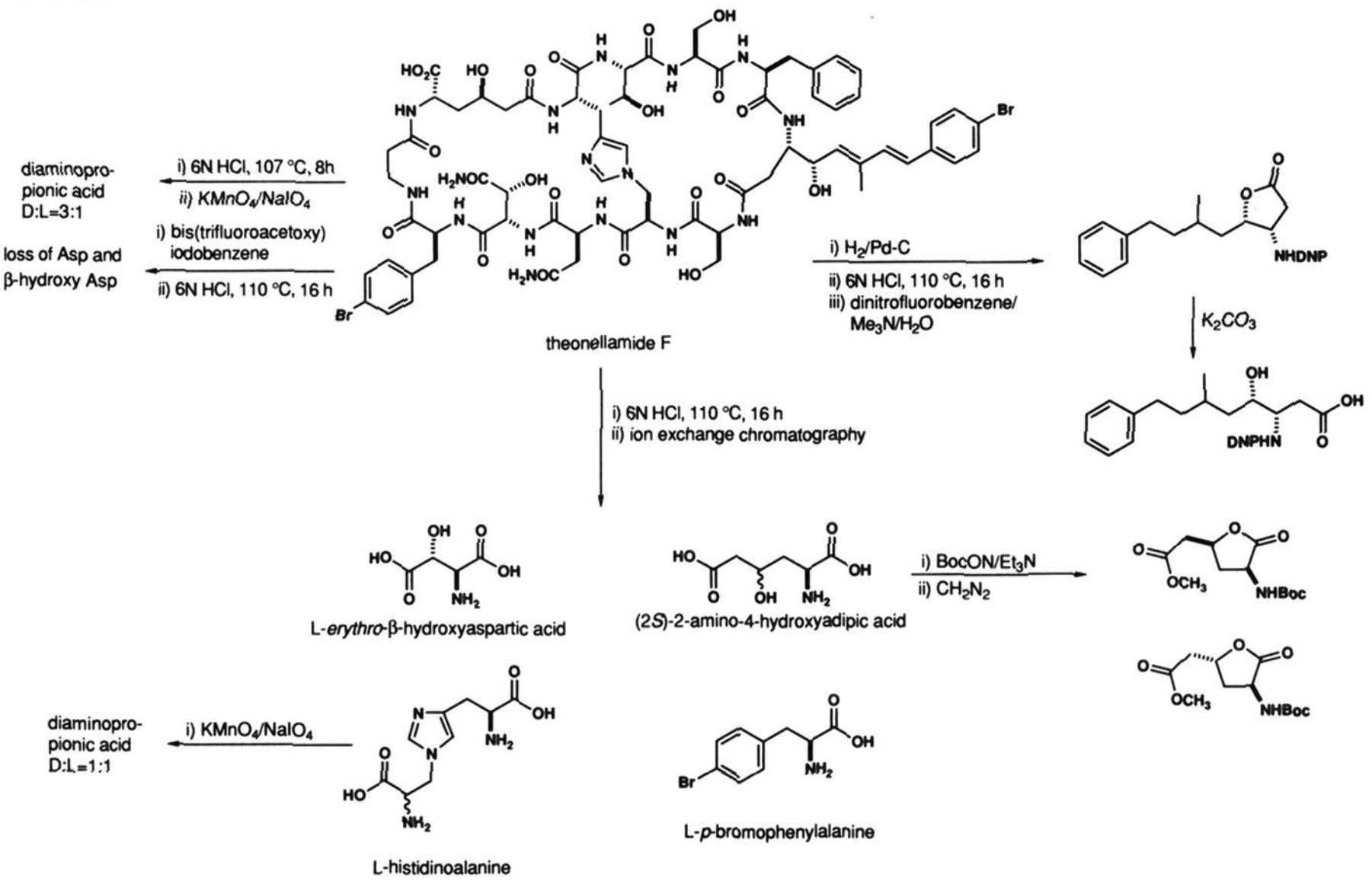
theonellapeptolide Id R=H
theonellapeptolide Ie R=CH_3

F. Theonellamide F from *Theonella* sp.

In our studies on inhibitors of development of echinoderm embryos from a *Theonella* sponge collected off Hachijo-jima Island, we isolated bistheonellides A and B from the less polar fraction of the EtOH extract.[45] The polar fraction was also active and bioassay-guided isolation afforded theonellamides. Although swinholides and bistheonellides are closely related to each other, theonellamides turned out to be quite different from theonellapeptolides. We have elucidated the structure of the major metabolite, theonellamide F[46] (Scheme I). Theonellamide F, an antifungal and cytotoxic cyclic dodecapeptide had a characteristic histidinoalanine bridge and Aboa residue (*vide infra*). Half of the constituent amino acids in theonellamide F were unusual. (2*S*,3*R*)-3-Hydroxyasparagine had been reported as a constituent of proteins and microbial peptides, however, (2*S*,4*R*)-2-amino-4-hydroxyadipic acid, τ-L-histidino-D-alanine, L-*p*-bromophenylalanine, and (3*S*,4*S*,5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-(*p*-bromophenyl)-5,7-octadienoic acid (Aboa) were new amino acids from natural sources.

The structures of unusual amino acids were determined by NMR and CD spectroscopy after isolation by ion-exchange chromatography. Since the Aboa residue decomposed during acid hydrolysis, this amino acid was isolated as a saturated debromo derivative from the hydrolyzate of hydrogenated theonellamide F. It should be noted that C4 of 2-amino-4-hydroxyadipic acid and the methine carbon in the alanine portion of histidinoalanine were epimerized during standard acid hydrolysis. The stereochemistries of these two chiral carbons were determined by hydrolysis under milder acidic conditions. Throughout the structural study, chiral GC/MS analysis on a Chirasil Val column was quite helpful for the determination of stereochemistry of the amino acids.

Scheme I



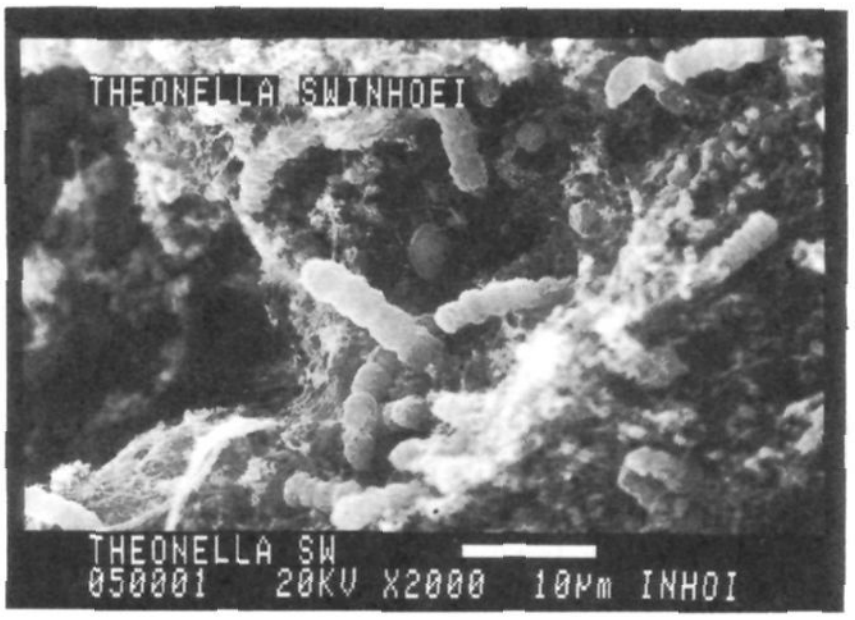
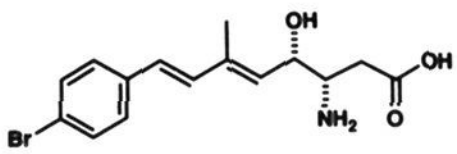


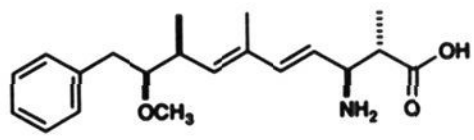
Figure 2. Scanning electron micrograph of *Theonella swinhoei* which contained swinholides and theonellapeptolides.

The amino acid sequence was determined from the NMR spectral data, since theonellamide F resisted partial hydrolysis with acids or enzymes. Although the ^{1}H NMR signals were broad at room temperature, theonellamide F gave sharper signals at 60 °C, which enabled us to measure proton-detected 2D NMR spectroscopies. The NOESY and/or ROESY data in H_2O/D_2O (9:1) or in DMSO-d_6 gave sequential information from correlations between amide protons and α-protons of the adjacent residues via amide bonds.

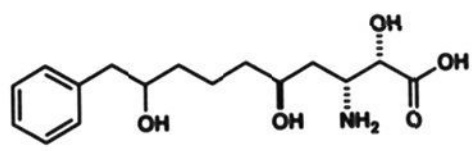
Structural features of theonellamide F, especially the presence of a histidinoalanine bridge, were unprecedented among peptides from natural sources. Another unusual amino acid, Aboa, was interesting in view of biogenetic considerations, since closely related amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) is found in microcystins,[47] hepatotoxic cyclic peptides, and (2*S*,3*R*,5*S*)-3-amino-2,5,9-trihydroxy-10-phenyldecanoic acid (Ahad) is contained in scytonemin A,[48] a peptide with calcium antagonistic activity, both of which were reported from blue-green algae. Also a biogenetically related δ-hy-



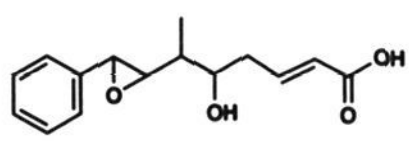
(3*S*,4*S*,5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-(*p*-bromophenyl)-5,7-octadecanoic acid (Aboa)



Adda



Ahad

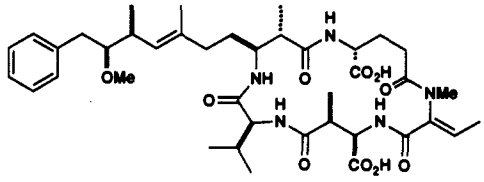


δ hydroxy acid component in cryptophycin

droxy acid was found in cryptophycin, a cytotoxic peptide from a blue-green alga.[49] To date, β-amino acids of these classes have never been encountered in peptides from bacteria, fungi, or plants. Therefore, it is likely that a symbiotic blue-green alga (algae) play(s) important parts in the production of theonellamide F. Bistheonellides A and B are also related to scytophycins, metabolites of blue-green algae of the genus *Scytonema*. Actually, numbers of blue-green alga(e) were observed in the tissue of a *Theonella* sponge which contains swinholides and thonellapeptolides (Figure 2).

G. Motuporin from *Theonella swinhoei*

Motuporin has been isolated from the Papua New Guinean *Theonella swinhoei*.[50] Motuporin is a cyclic



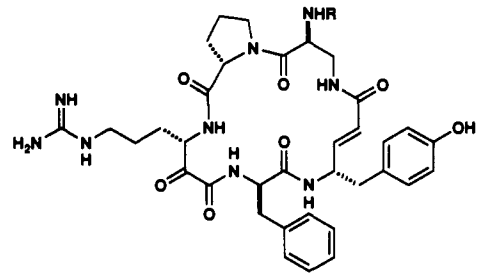
motuporin

pentapeptide with potent inhibitory activity against protein phosphatase 1 (IC_{50} < 1 nM) and cytotoxic activity [IC_{50} (μg/mL) P388, 6; A549, 2.4; HEY, 2.8; LoVo 2.3; MCF7, 12.4; U373MG, 2.4]. Motuporin may provide direct evidence for the participation of symbiotic blue-green algae in the biosynthesis of cyclic peptides from sponges; motuporin is an analog of nodularin, a hepatotoxic cyclic pentapeptide from the brackish water blue-green alga *Nodularia spumigena*.[51] Both peptides can be considered as members of the microcystin class of cyclic peptides, which have the characteristic Adda residue in common. The Arg residue in nodularin was replaced by a Val residue in motuporin.

The gross structure of motuporin was assigned by interpretation of NMR data, while absolute stereochemistry of amino acid residues except for the Adda residue were determined by HPLC analysis of their Marfey's derivatives. Relative stereochemistry of Adda moiety in motuporin was believed to be identical with that in nodularin and microcystins as judged from the NMR data. Finally, absolute stereochemistry of the Adda residue in motuporin was determined to be identical with that in nodularin by the fact that the ozonolysis product of motuporin liberated a second equivalent of D-*erythro*-β-methyl-Asp upon acid hydrolysis.

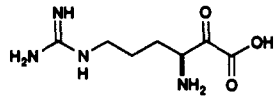
H. Cyclotheonamides from *Theonella swinhoei*

This particular sponge *Theonella swinhoei*, having bright yellow inner body, collected off Hachijo-jima Island has been extremely productive; a number of unusual metabolites with strong bioactivities have been isolated. The first example consists of cyclotheonamides.[52] Cyclotheonamides are cyclic pentapeptides possessing potent inhibitory activity against thrombin, trypsin, and plasmin. They contain two unusual amino

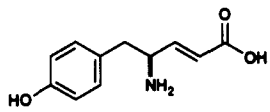


cyclotheonamide A R=CHO
cyclotheonemide B R=Ac

acid residues: a vinylogous derivative of Tyr and α-keto homolog of Arg.



α-keto-homolog of Arg



vinylogous Tyr

Amino acid analysis of the acid hydrolyzate of cyclotheonamide A revealed the presence of Phe, Pro, and 2,3-diaminopropionic acid (Dpr), while the two unusual amino acids were deduced by interpretation of COSY and HMBC spectra in D_2O. The sequence of the amino acids in cyclotheonamide A was assigned on the basis of NOESY data in DMSO-d_6. Cyclotheonamide A was decomposed upon storage in DMSO-d_6, presumably due to a reaction between the guanidine and the α-keto amide groups by analogy with a reaction between the guanidine group in Arg and phenylglyoxal. The stereochemistry of the Pro and Phe residues was determined by chiral GC analysis of a standard acid hydrolyzate, whereas the stereochemistry of the Dpr residue was determined after acid hydrolysis under milder conditions, otherwise the methine carbon in Dpr was easily epimerized. The stereochemistry of the methine carbon in the vinylogous Tyr residue, originally assigned as *R* by the chiral GC analysis of a $KMnO_4$/$NaIO_4$ oxidation product of cyclotheonamide A, was revised to *S* by the total synthesis of cyclotheonamide B, which also disclosed the stereochemistry of the methine carbon in the keto homologue of the Arg residue to be *S*.[53] Due to an equilibrium between the ketone, hydrated ketone, and hemiacetal forms in aqueous alcoholic solvents, cyclotheonamide A not only gave three spots by TLC, but also a very broad peak or three peaks in ODS-HPLC with alcoholic mobile phases. The FAB mass spectrum also exhibited molecular ion species corresponding to the three forms.

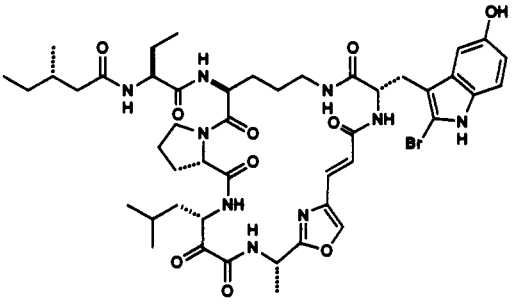
The structure of cyclotheonamide B was determined on the basis of spectral data. The difference between cyclotheonamide A and cyclotheonamide B lay only in the substituent on the amino group of the Dpr residue: a formyl group in cyclotheonamide A was replaced by an acetyl group in cyclotheonamide B. The NMR experiments on cyclotheonamide B were done in H_2O/

D_2O (9:1), because the amide protons were crucial to sequence analysis by NMR spectroscopy. Cyclotheonamide B was also expected to be decomposed in DMSO-d_6. Amide protons of cyclotheonamide B gave sharp signals in H_2O/D_2O (9:1), which enabled sequence analysis by the HMBC spectrum, confirming the sequential information obtained from the NOESY and ROESY spectra.

Modifications found in the two unusual amino acids in cyclotheonamides have rarely been encountered in peptides of natural origin. A vinylogous analog of Ala was found in glydobactins, metabolites of a Gram-negative bacterium *Polyangium brachysporum* sp. nov.,[54] while α-keto homologs of amino acids were later found in poststatins[55] and eurystatins,[56] metabolites of *Streptomyces* as well as in metabolites of the same sponge described below.

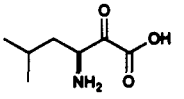
I. Orbiculamide A from *Theonella swinhoei* and Keramamides B–D and F from *Theonella* sp.

The second example is another cyclic peptide, orbiculamide A, which was obtained as a cytotoxic constituent.[57] Incidentally, this sponge contained other

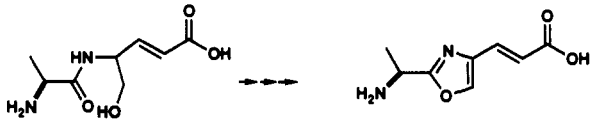


orbiculamide A

potent cytotoxins, theopederins[58] and onnamides.[59] Orbiculamide A contained three unusual amino acids, 2-bromo-5-hydroxytryptophan, theonalanine, and theoleucine. Standard amino acid residues in orbiculamide



theoleucine

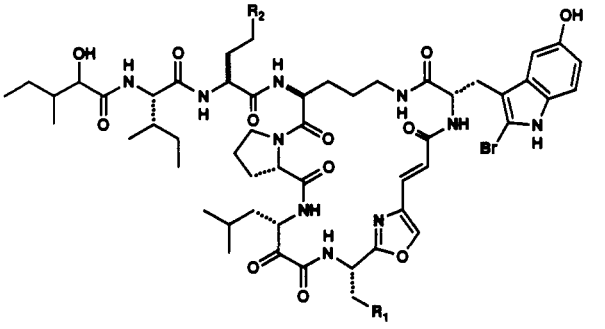


theonalanine

A were assigned by amino acid analysis and HPLC analysis of the acid hydrolyzate after derivatization with Marfey's reagent. The structures of the unusual amino acids as well as β-methylvaleric acid unit were elucidated by 2D NMR spectra in CD_3OH and in DMSO-d_6. The theonalanine residue liberated 1 mol of Ala upon acid hydrolysis. The sequence of the components was determined by interpretation of the NOESY and ROESY data, which was confirmed by the HMBC spectrum.

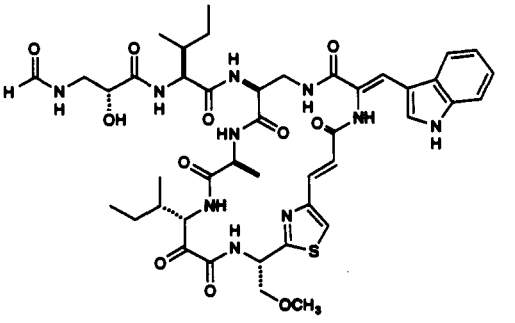
As in the case of cyclotheonamides, orbiculamide A contained an α-keto homolog of an amino acid, this time a homolog of Leu. It should be noted that the other unusual amino acid, theonalanine may be formed by cyclization of a dipeptide, alanyl–vinylogous-serine.

Closely related peptides, keramamides B–D, were isolated from an Okinawan *Theonella* sp.[60] Their



keramamide B $R_1=R_2=CH_3$
keramamide C $R_1=CH_3$, $R_2=H$
keramamide D $R_1=R_2=H$

structures were determined by interpretation of the 2D NMR data and FABMS/MS data. Another related peptide, keramamide F, was also obtained from the same sponge.[61] As in the cases of other cyclic peptides



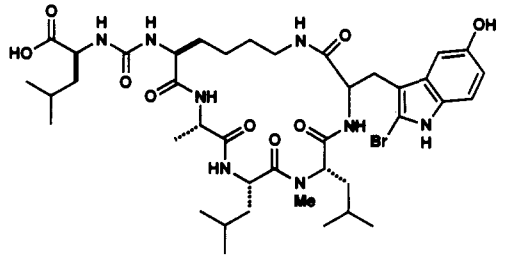
keramamide F

from *Theonella* sponges, keramamide F contained an α-keto homolog of Ile and "(*O*-methylseryl)thiazole", the latter of which may be a cyclization product of a dipeptide, *O*-methylseryl–vinylogous-cysteine. The gross structure was determined by interpretation of the NMR data and the stereochemistry of standard amino acid residues was assigned by GC or HPLC analysis on chiral columns. The absolute configuration of unusual residues was correlated with known compounds by oxidative degradation.

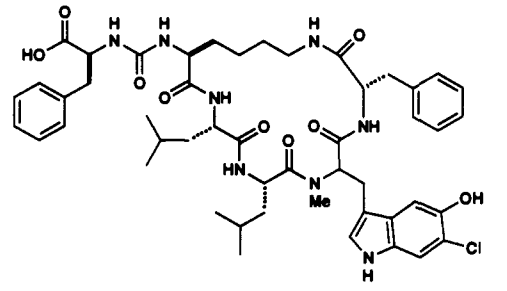
Orbiculamide A and keramamide F were moderately cytotoxic, whereas keramamides B–D inhibited the generation of superoxide anion in human neutrophils treated with formyl-Met-Leu-Phe at concentrations of 5×10^{-8} M.

J. Konbamide and Keramamide A from *Theonella* sp.

Konbamide[62] and keramamide A,[63] metabolites of an Okinawan *Theonella* sponge, possess a common



konbamide



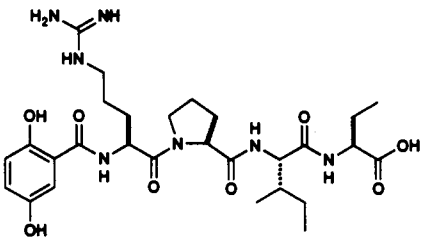
keramamide A

pentapeptide feature; the side-chain amino group in the N-terminal Lys residue forms an amide bond with the C-terminal carboxyl group and the α-amino group of the Lys residue forms a urea bond with an amino acid. Structure elucidation of these peptides was performed by the same procedure, viz., (1) interpretation of the 2D NMR data to obtain the gross structure including component amino acids, (2) amino acid analysis by chiral GC and chiral HPLC, and (3) interpretation of the FABMS/MS data to confirm the sequence determined by NMR experiments. Konbamide and keramamide A were moderate inhibitors of calmodulin-activated phosphodiesterase and Ca^{2+}-ATPase, respectively.

A cyclic peptide with the identical peptide backbone was isolated as a bitter principle from oysters harvested in Korea.[64] An involvement of symbiotic microorganisms in the biosynthesis of the konbamide/keramamide A family of peptides was again evident, since oysters filter-feed microalgae.

K. Nazumamide A from *Theonella swinhoei*

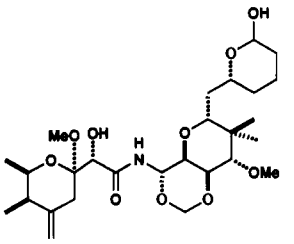
Nazumamide A was isolated from the same *T. swinhoei* that contained cyclotheonamides.[65] Nazumamide A was a linear tetrapeptide with a 2,5-dihydroxybenzoylated N-terminus and a free C-terminus. The structure of nazumamide A was elucidated on the basis of 2D NMR data, viz. COSY, HOHAHA, HMQC, and HMBC, in CD_3OH. Although nazumamide A was reported to inhibit thrombin with an IC_{50} of 2.8 μg/mL, a synthetic material,[66] which had the same spectral data as the natural product, exhibited only weak inhibitory activity at a concentration of 300 μg/mL. The potency reported for the natural product was mostly due to a contamination of ca. 0.1% of cyclotheonamide A. Under the usual analytical procedures, a contamination of the order of 0.1% can easily be missed.



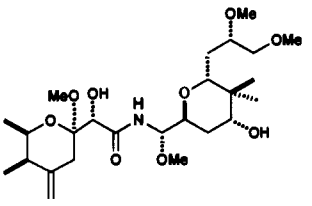
nazumamide A

L. Onnamides from *Theonella* sp. and *Theonella swinhoei*

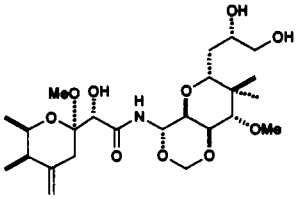
Onnamide A was first isolated from an Okinawan sponge of the genus *Theonella* as an antiviral principle.[67] The structure of onnamide A, including relative stereochemistry of the substituents on two oxane rings, was deduced by interpretation of the NMR spectral data. The chirality of the Arg residue was shown to be *S* on the basis of TLC analysis of the hydrolysis product of a pyrimidine derivative after treatment with Marfey's reagent. Later, we isolated seven related metabolites of onnamide A, viz. 13-des-*O*-methylonnamide A, dihydroonnamide A, onnamide B, 17-oxo-onnamide B, onnamide C, onnamide D, and onnamide E, from the Hachijo-jima Island sponge *Theonella swinhoei*, and pseudoonnamide A from a morphologically different sponge of the genus *Theonella*[59] (Chart IV). Their structures were assigned on the basis of spectral data. Interestingly, the same extract contained truncated derivatives, which we named theopederins by analogy with the name pederin, a toxic principle of the brister beetle *Paederus fuscipes*.[68] Theopederins exhibit stronger cytotoxic activity than the onnamides, as observed in related metabolites, mycalamides isolated from a New Zealand sponge, *Mycale* sp.[69]



theopederin A



pederin

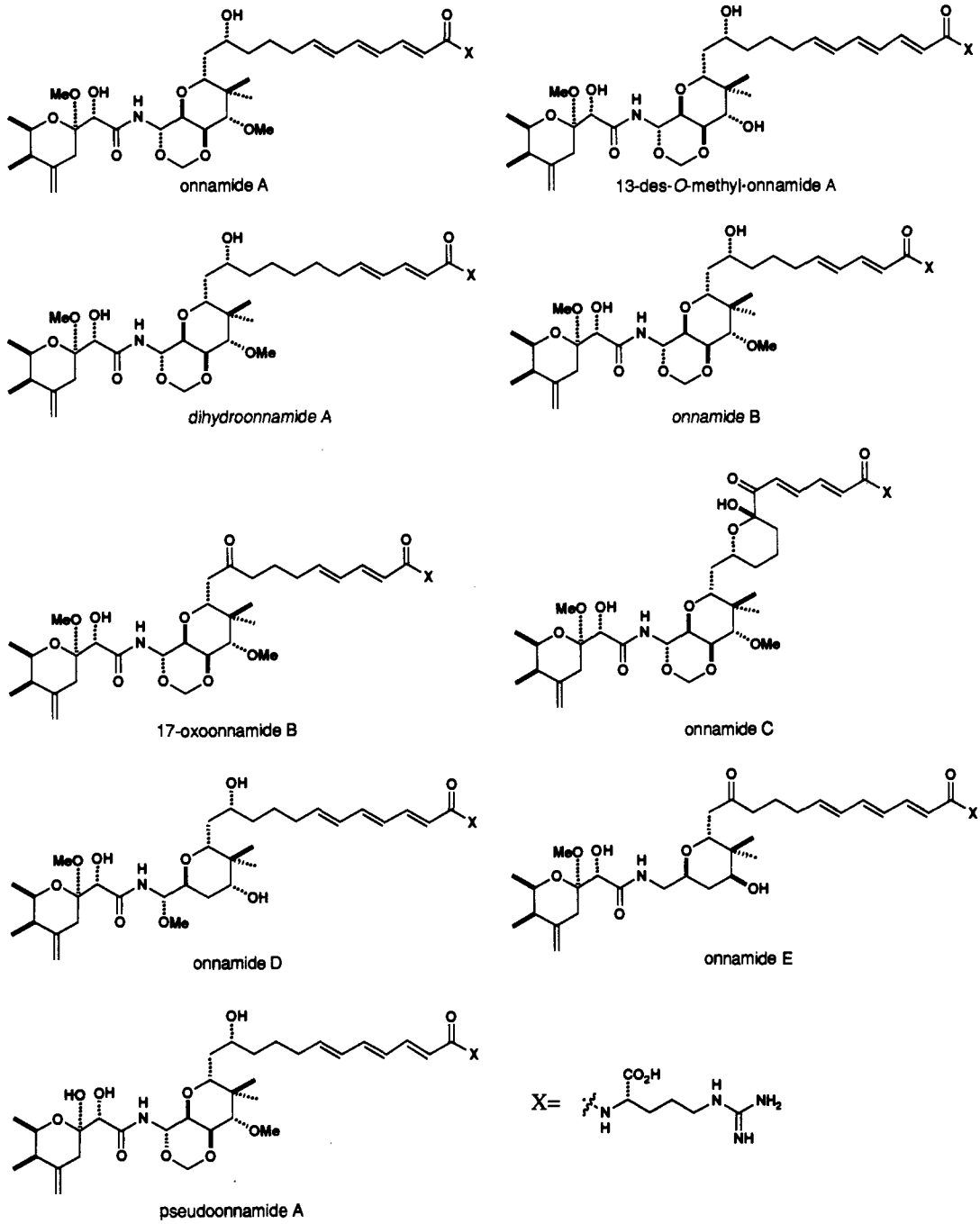


mycalamide A

The occurrence of closely related compounds in taxonomically remote animals, sponges and a terrestrial beetle, may indicate the connection of a common producer, probably symbiotic microorganisms.

Total synthesis of onnamide A was accomplished, defining the stereochemistry, including absolute configuration.[70]

Chart IV



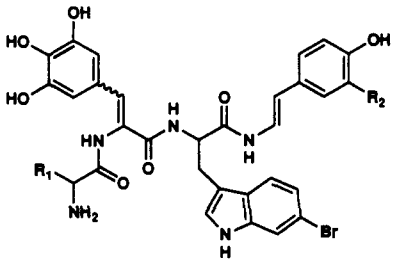
IV. Hadromerida

The order Hadromerida includes *Cryptotethia crypta*, from which spongouridine and spongothymidine were isolated.[1] Cyclic peroxides have frequently been found in sponges belonging to this order.[2] From the red sea *Latrunculia magnifica* latrunculins, thiazolone-containing macrolides, were isolated.[71] Polycyclic heteroaromatic alkaloids were isolated from *Aaptos aaptos* and *Latrunculia brevis*.[2]

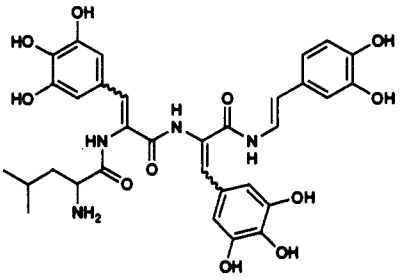
A. Celenamides from *Clíona celata*

Celenamides A–D were isolated from the East Pacific sponge *Cliona celata*.[72] Due to the instability of the molecule, isolation was performed after converting to the peracetates. Acetylation of a crude extract with deuteriated acetic anhydride followed by isolation of the deuterioacetylated celenamides disclosed that the natural celenamides have no acetyl groups. Structure elucidation was based on the NMR data, analysis of ozonolysis products, partial hydrolysis with dilute acid, and interpretation of fragment ions in the EI mass spectra. Celenamide A was a tetrapeptide with two dehydroamino acid residues. In celenamide B, the N-terminal Leu residue in celenamide A was replaced by a Val residue. One of the phenolic hydroxyl groups in celenamide A was replaced by a hydrogen in celenamide C, whereas celenamide D had a second dihydroxydehydrotyrosine residue instead of a bromotryptophan residue in celenamide A.

Celenamides are closely related to integerrin, a peptide alkaloid from the Rhamnaceae plant *Ceanothus*



celenamide A R_1=$CH_2CH(CH_3)_2$, R_2=OH
celenamide B R_1=$CH(CH_3)_2$, R_2=OH
celenamide A R_1=$CH_2CH(CH_3)_2$, R_2=H



celenamide D

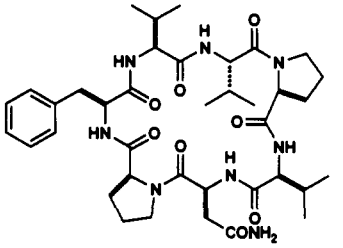
integerrimus,[73] which has an essentially identical framework. Celenamides are also similar to tunichromes, reducing blood pigments of vanadium-concentrating tunicates.[74] Synthesis of hexaacetylcelenamide A was reported.[75]

V. Axinellida

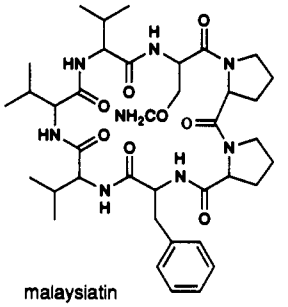
Secondary metabolites in this order have been intensively studied.[2] Most of the metabolites reported from sponges of the order Axinellida may be classified into two classes; one contains bromopyrrole derivatives related to oroidin and the other contains nitrogenous terpenoids.

A. Axinastatin 1 from *Axinella* sp., Pseudoaxinellin from *Pseudoaxinella massa*, and Malaysiatin from *Pseudoaxinyssa* sp.

Three cyclic heptapeptides were isolated from South Pacific sponges: axinastatin 1 from the Palauan *Axinella* sp.,[76] pseudoaxinellin from the Papua New Guinean sponge *Pseudoaxinella massa*,[77] and malaysiatin from a Borneo sponge *Pseudoaxinyssa* sp.[78] The same gross structure was assigned for axinastatin 1 and pseudoaxinellin on the basis of NMR data including COSY, HOHAHA, HMQC, HMBC, NOESY/ROESY spectra and FABMS/MS data in case of axinastatin 1, whereas the gross structure of malaysiatin was assigned on the basis of the same type of NMR data together with interpretation of fragment ions in the EI mass spectra. Since the ^1H and ^{13}C NMR data for the three compounds were almost identical, although the magnitudes of optical rotations differ significantly, one of the proposed structures could be erroneously assigned. This may have happened due to the overlapping of ^1H NMR signals. Absolute stereochemistry of the component amino acids in axinastatin 1 was determined by chiral GC analysis, while that in pseudoaxinellin was determined by HPLC analysis of the Marfey's derivatives.[79]



axinastatin 1
pseudoaxinellin



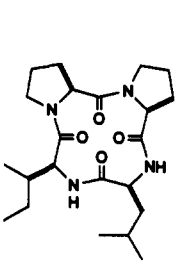
malaysiatin

VI. Halichondrida

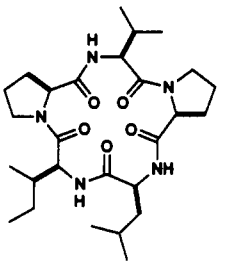
The order Halichondrida includes two families, Halichondridae and Hymeniacidonidae. The former is a source of a variety of terpenoids, while numerous bromopyrrole derivatives were isolated from the latter.[2] Macrolides, such as halichondramides[80] and halichondrins,[81] and a polyether, okadaic acid,[82] were also isolated from sponges of the genus *Halichondria*.

A. Fenestins from *Leucophloeus fenestrata*

A cyclic tetrapeptide, fenestin A, and a cyclic pentapeptide, fenestin B, were isolated from *Leucophloeus fenestrata*.[83] The structure of fenestin A [*cyclo*(L-Pro-L-Pro-L-Leu-L-Ile)] was determined by a combination of analysis of fragment ions in the CI mass spectra and long-range COSY data. The stereochemistry of the amino acid residues was determined by HPLC analysis of the hydrolyzate after derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate. The structure of fenestin B was determined in the same way. The authors investigated three collections of *L. fenestrata* from Thailand and from Fiji, all of which contained sesquiterpene isocyanates, and found fenestins only in one of the collections. Therefore, they suggested that fenestins are produced by symbiotic microorganisms. A total synthesis of fenestin B revealed that the structures proposed for fenestins A and B were incorrect.[84]



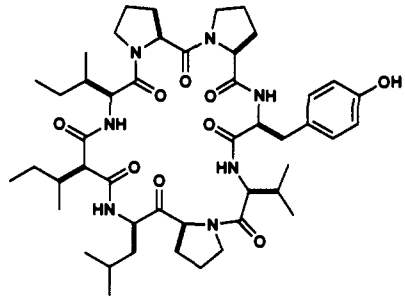
fenestin A



fenestin B

B. Hymenistatin 1 from a *Hymeniacidon* sp.

Hymenistatin 1 is a cyclic octapeptide isolated from a Palauan *Hymeniacidon* sponge.[85] The amino acid composition of hymenistatin 1 was deduced by interpretation of the NMR data, while the chirality of component amino acids was determined by chiral GC analysis on a Chirasil-Val III column. The sequence of the amino acid residues was determined by the NOE data, which was confirmed by the FABMS/MS spectra. In the FABMS/MS spectrum protonation of each proline nitrogen afforded three different series of ions. Hymenistatin 1 was cytotoxic against P388 leukemia cells with an IC_{50} of 0.26 μg/mL.



hymenistatin 1

VII. Concluding Remarks

Most marine natural product chemists have attempted to isolate peptides from sponges by using a specific bioassay, which may be the reason why most peptides mentioned above are cyclic and lipophilic. We might have missed linear or more polar peptides during isolation. The most significant feature of sponge peptides is the presence of unusual amino acids in the molecules as represented by Aboa, α-keto amino acids, and vinylogous amino acids, which may conceivably be connected to symbiotic microorganisms, particularly blue-green algae (cyanobacteria). In fact, some peptides from both sponges and blue-green algae share some common features in constituent amino acids.

If blue-green algae participate in synthesis of peptides in sponges, what are their roles? Why are some peptides contained in unexpectedly large quantities, while some are present in trace amounts? Why are some peptides highly bioactive? Defensive roles of bioactive metabolites in sponges have been suggested. However, hard-bodied species or those which are overgrown by epiphytes often contain highly bioactive peptides. We may have to wait for the answer until culture of sponge cells, or culture of symbiotic microbes become possible.

Finally, sponge peptides appear to be important potential drugs; cyclotheonamides serve as a model compound for antithrombin drugs; discodermins are potential antitumor promoting drugs; and calyculins are useful biochemical reagents.

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