Bioactive Metabolites of Symbiotic Marine Microorganisms

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I. Introduction

Marine microorganisms are of considerable current interest as a new promising source of bioactive substances. Recently marine microorganisms have proven to produce a variety of chemically interesting and biologically significant secondary metabolites and some of them are expected to serve as lead compounds for drug development or pharmacological tools for basic studies of life sciences.[1] Recent studies have also suggested that some bioactive compounds isolated from marine invertebrates such as sponges, coelenterates, mollusks, or protochordates[2,3] are truly originating from symbiotic microorganisms (*e.g.* bacteria, fungi, blue-green algae, dinoflagellates, or haptophytes).

It was a striking topic when the real origin of tetrodotoxin[4] was demonstrated to be marine bacteria isolated from a variety of marine organisms such as a red alga,[5] a xanthid crab,[6] or the skin of a pufferfish.[7] Saxitoxin,[4] a well-known paralytic shellfish poison, is produced by dinoflagellates of the genus *Gonyaulax* and the blue-green alga *Aphamizomenon flos-aquae*.[8] A Gram-positive bacteria (Aalxll strain),[9] which is associated with the Japanese ivory shell *Babylonia japonica*, was shown to be responsible for the production of surugatoxins, the causative toxins of a food poisoning outbreak in 1965 at Suruga Bay.[10] Okadaic acid, a potent inhibitor of protein phosphatases first isolated from a sponge *Halichondria okadai*,[11] is produced by a dinoflagellate *Prorocentrum lima*.[12]

Since it is quite impossible to collect unlimited amounts of marine animals or plants, isolation and cultivation of symbiotic microorganisms are of great importance for sufficient and constant supply of bioactive compounds. This review describes the recent studies on bioactive substances isolated from symbiotic microorganisms associated with marine animals or plants. In sections II and III bioactive natural products isolated from symbiotic marine bacteria and fungi are surveyed, while sections IV and V mainly deal with our

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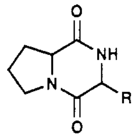
recent work on bioactive substances from symbiotic marine dinoflagellates and haptophytes.

II. Bacteria

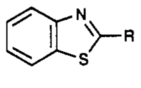
A. Bacteria Isolated from Sponges

Bioactive metabolites of marine sponges have been extensively studied by marine natural products chemists. 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 contained quite different metabolites. These facts as well as the structural characteristics of the compounds strongly suggested that microorganisms living in or on sponges are responsible for the production of many bioactive compounds. Recently some of secondary metabolites which had been isolated from extracts of sponges were obtained from the culture media of marine bacteria isolated from sponges.

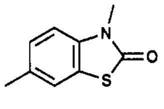
Cardellina and co-workers examined the microorganisms growing on or in sponges from Bermudian waters. From a specimen of the sponge *Tedania ignis* a bright orange-pigmented, Gram-positive bacterium was isolated and identified as a member of the genus *Micrococcus*. Culturing of the *Micrococcus* sp. was successfully carried out and from the culture media three diketopiperazines (1–3) were isolated by chromatographic techniques, including centrifugal countercurrent chromatography.[13] Compounds 1–3 proved to be identical to those previously isolated from extracts of the sponge *Tedania ignis*.[14] From the fermentation culture extracts of the same *Micrococcus* sp. four benzothiazoles (4–7) were obtained.[15] These benzothiazoles were previously known as volatile constituents of cranberries, an aroma constituent of tea leaves, a fermentation product of yeast, or synthesized compounds.



1 R=$CH_2CH(CH_3)_2$
2 R=$CH(CH_3)_2$
3 R=CH_3



4 R=SH
5 R=CH_3
6 R=OH



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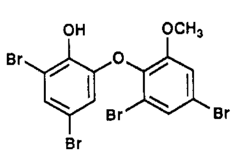
Elyakov and co-workers reported that a *Vibrio* sp. of bacteria isolated from the sponge *Dysidea* sp. collected at Eastern Samoa biosynthesized brominated diphenyl ethers on the basis of GC–MS analyses using 3,5-dibromo-2-(3′,5′-dibromo-2′-methoxyphenoxy)phenol (8) as a standard. Compound 8 was previously obtained from extracts of the sponge *Dysidea* sp.[16]



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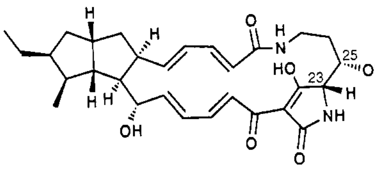
From a Japanese sponge *Halichondria okadai* a bacterium of a member of the genus *Alteromonas* was isolated and cultured in the laboratory. From the cultured mycelium of this bacterium we have isolated a unique macrocyclic lactam, alteramide A (9), possessing cytotoxic activity [IC_{50} (50% inhibitory concentration) values against P388, L1210, and KB cells, 0.1, 1.7, and 5.0 μg/mL, respectively].[17] The structure of compound 9 was elucidated by extensive analyses of the spectral data (*e.g.* ^1H–^1H COSY, HMQC, HMBC, NOESY, and ROESY) as well as the chemical degradations into small fragments. Treatment of 9 with ozone followed by $NaBH_4$ reduction and acetylation afforded the bicyclo[3.3.0]octane derivative 10, while β-hydroxyornithine (11) was obtained from 9 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 10 and the absolute configurations of C-23 and C-25 positions of 9 were defined through chiral HPLC examination of 11. Compound 9 possesses two diene groups which were vulnerable to an intramolecular photochemical [4 + 4] cycloaddition to give a hexacyclic compound 12. It should be noted that the structure of alteramide A (9) appears to be biogenetically related to those of an antibiotic ikarugamycin (13) produced by a terrestrial *Streptomyces* sp.[18] and an antifungal and cytotoxic metabolite discodermide (14) isolated from a Caribbean marine sponge *Discodermia dissoluta*.[19] Since compound 9 was obtained from a bacterium isolated from a marine sponge, discodermide (14) is strongly suggested to be of microbial origin.



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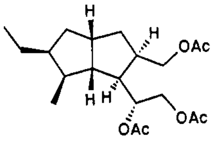

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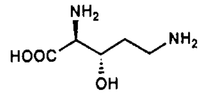

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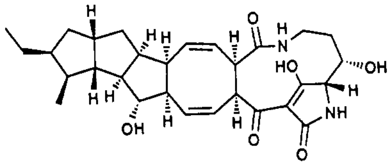

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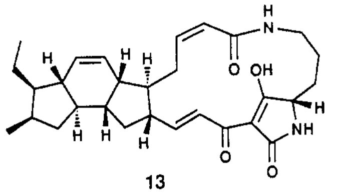

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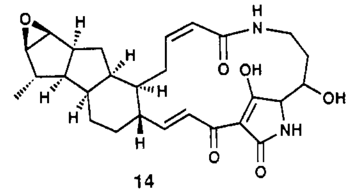

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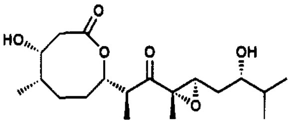
B. Others

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.[20] From the culture broth of the *Streptomyces* sp. two closely related novel compounds, octalactins A (**15**) and B (**16**), were isolated and their structures, including relative stereochemistries, were firmly established, with **15** being defined by the 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 molecular mechanics calculation. Octalactin A (**15**) exhibited strong cytotoxic activity toward B-16-F10 murine melanoma and HCT-116 human colon tumor cell lines with the IC$_{50}$ values of 0.0072 and 0.5 μg/mL, respectively, while quite surprisingly octalactin B (**16**) was completely inactive in the cytotoxicity assay.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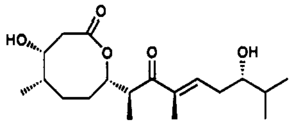
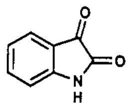

16

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.) which was consistently isolated from healthy embryos effectively inhibited the growth of the fungus *L. callinectes* in vitro. This bacterial strain was shown to produce and release an antifungal compound, which was identified as 2,3-indolinedione (**17**, known as isatin).[21] Bacteria-free embryos which were exposed to the fungus quickly died, whereas embryos reinoculated with the bacteria or treated only with 2,3-indolinedione (**17**) thrived. Thus it was clearly revealed that the commensal *Alteromonas* sp. bacteria protect shrimp embryos from fungal infection by producing and liberating the antifungal metabolite **17**.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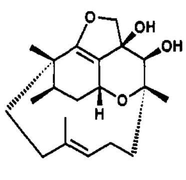
III. Fungi

A. Fungi Isolated from Marine Animals

Chemical studies on the bioactive metabolites of fungal isolates from the marine environments have been limited to date. Marine fungi, however, can be expected as a potential source of new bioactive substances from the following studies. Researchers of the Sankyo group have isolated the novel platelet-activating factor (PAF) antagonists, phomactins A (**18**),[22] B (**19**), B$_1$ (**20**), and B$_2$ (**21**),[23] 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 (**18**) as well as a diketone derived from phomactin B (**19**). Phomactin B$_1$ (**20**) and B$_2$ (**21**) were chemically correlated with phomactin B (**19**). Compounds **18**–**21** inhibited PAF-induced platelet aggregation (IC$_{50}$ 1.0 × 10^{-5} M, 1.7 × 10^{-5} M, 0.98 × 10^{-5} M, and 0.16 × 10^{-5} M, respectively) and binding of PAF to its receptors (IC$_{50}$ 0.23 × 10^{-5} M, >10^{-5} M, 2.0 × 10^{-5} M, and 0.54 × 10^{-5} M, respectively). Phomactin A (**18**), however, exhibited no effect on adenosine diphosphate-, arachidonic acid-, and collagen-induced platelet aggregation. Phomactin A (**18**) was thus considered to be a new type of specific PAF antagonist.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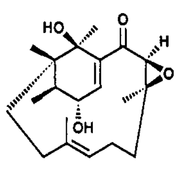
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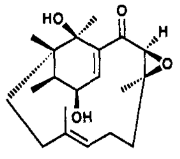
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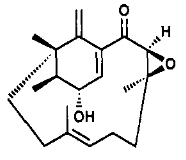
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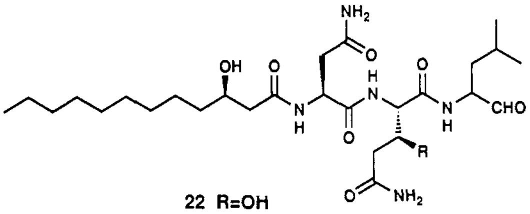
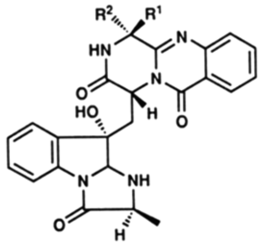
Two lipophilic tripeptides, fellutamides A (**22**) and B (**23**),[24] have been isolated by us from the cultured mycelium of a fungus *Penicillium fellutanum* Biourge which was isolated from the gastrointestine of the marine fish *Apogon endekataenia* Bleeker, collected off Manazuru beach, Kanagawa prefecture, Japan. The structures of fellutamides were elucidated by applying several types of two-dimensional 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 (**22**) and B (**23**) were potently cytotoxic against murine leukemia P388 (IC$_{50}$ 0.2 and 0.1 μg/mL, respectively) and L1210 (IC$_{50}$ 0.8 and 0.7 μg/mL, respectively) cells and human epidermoid carcinoma KB (IC$_{50}$ 0.5 and 0.7 μg/mL, respectively) cells in vitro. The fungus *Penicillium fellutanum* is not a marine-specific one, since the same species have been isolated from terrestrial sources. These peptides **22** and **23** which are structurally related to the leupeptines[25] exhibited stimulating activity on nerve growth factor (NGF) synthesis.[26]

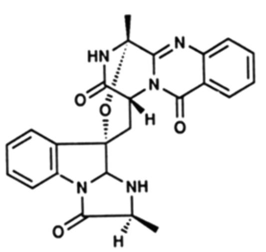

22 R=OH
23 R=H

Numata and co-workers have isolated three quinazoline derivatives, fumiquinazolines A (**24**), B (**25**), and

C (**26**),[27] from the cultured mycelium of a strain of *Aspergillus fumigatus* which existed in the gastrointestinal tract of the saltwater fish *Pseudolabrus japonicus*.



24 R^1=Me, R^2=H
25 R^1=H, R^2=Me

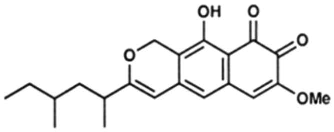


26

Their structures were based on spectral and chemical evidences as well as X-ray diffraction analysis of **26**. The absolute stereostructure of **26** was revealed by the production of L-(+)-alanine through its acid hydrolysis. Compounds **24–26** exhibited moderate cytotoxic activity against the cultured P388 lymphocytic leukemia cells.

B. Fungi Isolated from Marine Plants

A polyketide metabolite, obionin A (**27**),[28] has been isolated by Gloer and co-workers from the liquid culture of the marine fungus *Leptosphaeria obiones* (Crouan et Crouan) Saccoro, a halotolerant ascomycete originally isolated from the salt marsh grass *Spartina alterniflora* in the coastal marshlands of Sapelo Island, GA. The culture of *L. obiones* originally exhibited potent brine shrimp toxicity. Obionin A (**27**), however, did not account for that activity. Since an ichthyotoxic hydroquinone metabolite was reported from a brown alga which oxidizes in air to the less active *o*-quinone,[29] a similar phenomenon might have occurred for the extract of the *L. obiones* culture. Obionin A (**27**) showed inhibitory activity against binding of the dopamine D-1 selective ligand ^{3}H-SCH 23390 to bovine corpus striatum membrane with an IC_{50} value of 2.5 μg/mL.



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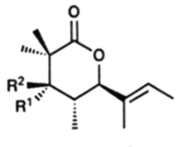
Gloer and co-workers also reported isolation of two isomeric δ-lactones, helicascolides A (**28**) and B (**29**),[30] from the Hawaiian mangrove ascomycete *Helicascus kanaloanus* Kohlmeyer. Their structures were based primarily on NMR studies and the absolute configuration was deduced from CD spectral data. Secondary metabolites with these structural features have never been obtained from terrestrial fungi. Marine fungi, therefore, may have potential capabilities to produce different types of compounds from those of terrestrial sources.



28 R^1=OH, R^2=H
29 R^1=H, R^2=OH

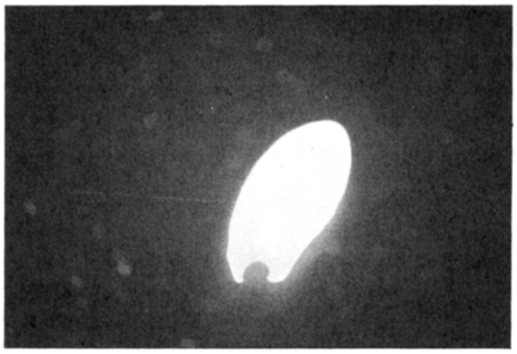


Figure 1. Host: flatworm *Amphiscolops magniviridis* (5 mm in length).

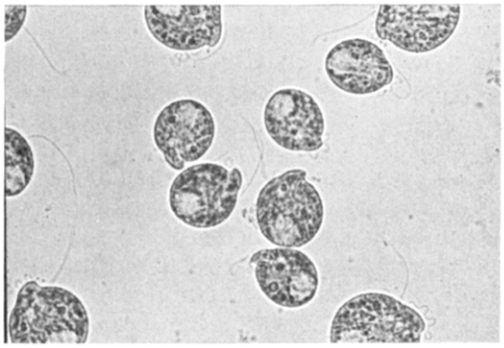


Figure 2. Symbiont: dinoflagellate *Amphidinium* sp. (Y-26, 20 μm in length).

IV. Dinoflagellates

A. Symbiotic Dinoflagellates of Flatworms

During the course of our studies on the search for bioactive substances from Okinawan marine organisms,[31-33] we initiated a project on bioactive natural products of symbiotic marine microalgae about seven years ago. With help from previous studies by Yamasu who systematically collected symbiotic microalgae from marine invertebrates of Okinawan coastal waters,[34,35] large-scale cultures of the microalgae were carried out in our laboratory. Figures 1 and 2 represents the host (flatworm) and the symbiont (dinoflagellate), respectively.

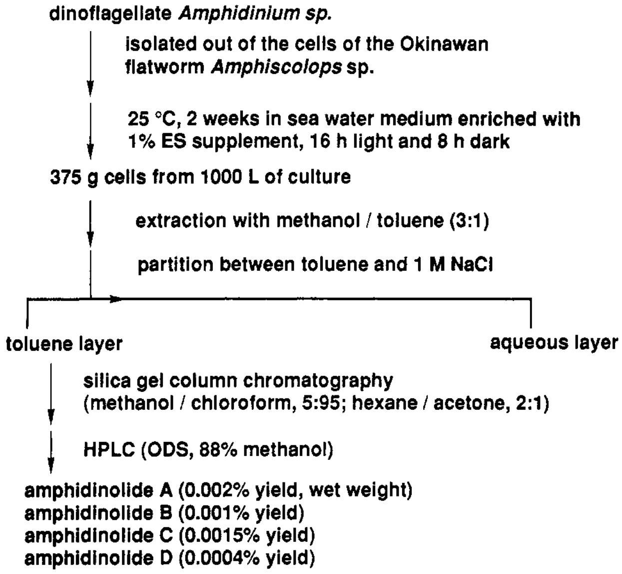
The first microalga we utilized for research was a dinoflagellate belonging to the genus *Amphidinium* (strain number Y-5, 25 μm in length and 20 μm in width) isolated from the inner tissue of the host, a flatworm of the genus *Amphiscolops* Graff, 1905 (500 μm in length and 220 μm in width, green color), which was living on algae or sea weeds such as *Enteromorpha* and *Jania* spp. and collected at Chatan beach, Okinawa Island.[36] The unialgal cultures of *Amphidinium* sp. (Y-5) were grown in 3-L glass bottles containing 2 L of sea water medium enriched with Provasoli's ES (Erd-Schreiber) supplement (Table I).[37,38] 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 of light and 8 h of 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 of culture. Harvested cells were extracted with methanol/toluene (3:1) followed by partitioning between toluene and water. The toluene-soluble fraction was subjected

Table I. Contents of Provasoli's ES supplement[a]

distilled H_2O	100 mL
$NaNO_3$	350 mg
sodium glycerophosphate	50 mg
Fe (as EDTA;[b] 1:1 mol)	2.5 mg
PII solution of metals[c]	25 mL
vitamin B_{12}	10 μg
vitamin B_1	0.5 mg
biotin	5 μg
TRIS[d]	500 mg
pH	7.8

[a] This was filtered with sterile membrane filter and added to sterile sea water to make a 1% solution. [b] Ethylenediaminetetraacetic acid. [c] The solution consists of Fe (as Cl^-, 1 mg), B (H_3BO_3, 20 mg), Mn (as Cl^-, 4 mg), Zn (as Cl^-, 500 μg), Co (as Cl^-, 100 μg), Na_2-EDTA (100 mg), and distilled H_2O (100 mL). [d] Tris(hydroxymethyl)aminomethane.

Scheme I. Isolation Procedure for Amphidinolides A–D (30–33)



dinoflagellate *Amphidinium sp.*

isolated out of the cells of the Okinawan flatworm *Amphiscolops* sp.

25 °C, 2 weeks in sea water medium enriched with 1% ES supplement, 16 h light and 8 h dark

375 g cells from 1000 L of culture

extraction with methanol / toluene (3:1)

partition between toluene and 1 M NaCl

silica gel column chromatography (methanol / chloroform, 5:95; hexane / acetone, 2:1)

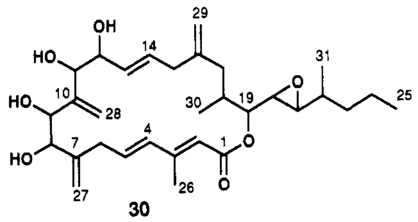
HPLC (ODS, 88% methanol)

amphidinolide A (0.002% yield, wet weight)
amphidinolide B (0.001% yield)
amphidinolide C (0.0015% yield)
amphidinolide D (0.0004% yield)

to repeated silica gel flash chromatography followed by reversed-phase HPLC resulting in isolation of four cytotoxic macrolides amphidinolides A (**30**),[39] B (**31**),[40] C (**32**),[41] and D (**33**)[42] (Scheme I).

Three other species of dinoflagellates of the genus *Amphidinium* (strain number 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 the extracts of the cultured cells of these strains of *Amphidinium* spp. four other cytotoxic macrolides amphidinolides E (**34**),[43] F (**35**),[44] G (**36**),[45] and H (**37**)[45] were isolated. The isolated yields and cytotoxicity data of amphidinolides A–H (**30–37**) are presented in Table II. In addition to potent cytotoxic activity, amphidinolides B (**31**) and C (**32**) were shown to activate rabbit skeletal muscle actomyosin ATPase activity.[46] The chemical structures of these macrolides (**30–37**) were elucidated mainly on the basis of extensive spectroscopic studies including several types of two-dimensional NMR experiments[47] (*e.g.* ^1H–^1H COSY, ^1H–^{13}C COSY, HMBC, HMQC, and NOESY spectra).

Amphidinolide A (**30**), $C_{31}H_{46}O_7$, was suggested to possess an α,β,γ,δ-dienoate chromophore from its UV absorption spectrum (λ_{max} 265 nm). ^1H and ^{13}C NMR data revealed the presence of one epoxide and three



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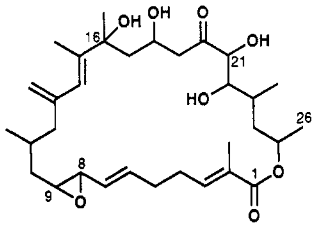
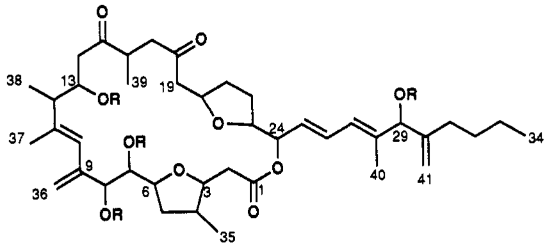
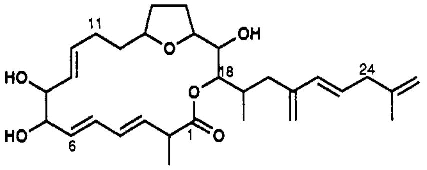


31
33 C-21 epimer



32 R=H
38 R=Ac



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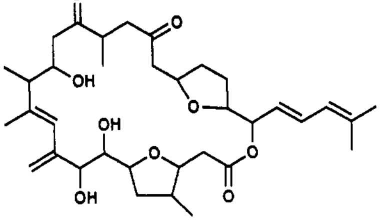

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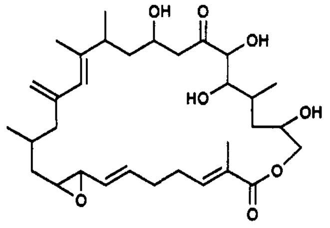

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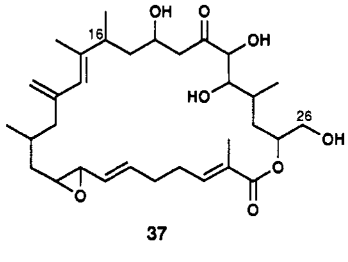

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Table II. Isolated Yields and Cytotoxicity Data of Amphidinolides

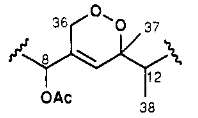
	isolated yields ($\times 10^{-4}$%)[a] strain				cytotoxicity (IC_{50}, μg/mL)	
compounds	Y-5	Y-5′	Y-25	Y-26	L1210	KB
amphidinolide A	20	4	–[b]	–	2.0	5.7
amphidinolide B	10	–	–	0.8	0.00014	0.0042
amphidinolide C	15	–	–	0.3	0.0058	0.0046
amphidinolide D	4	–	–	–	0.019	0.08
amphidinolide E	–	3	–	–	2.0	10
amphidinolide F	–	–	–	0.1	1.5	3.2
amphidinolide G	–	–	20	–	0.0054	0.0059
amphidinolide H	–	–	17	–	0.00048	0.00052

[a] On the basis of wet weight of the harvested cells. [b] "–" denotes "not isolated".

exo-methylene groups. Detailed analysis of the ^1H–^1H COSY spectrum allowed assignment of all proton signals and clearly established the proton connectivities from H-2 to H_3-25, 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.[48]

Amphidinolide B (**31**), $C_{32}H_{50}O_8$, 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. ^1H and ^{13}C NMR data also suggested the presence of one *exo*-methylene, 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 (**32**), $C_{41}H_{62}O_{10}$, exhibited the 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 two-dimensional NMR experiments were carried out on the tetraacetate (**38**) prepared from **32**. 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. 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 **38** was slowly oxidized by air to afford a [4 + 2] cycloaddition product (**39**), the spectral data of which provided additional proof for the structure of amphidinolide C (**32**).



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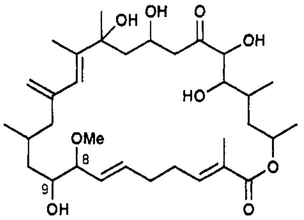
Amphidinolide E (**34**), $C_{30}H_{44}O_6$, showed a UV absorption maximum at λ_{max} 230 nm, corresponding to a diene chromophore. The presence of two *exo*-methylenes and one tetrahydrofuran ring was inferred by analyzing the ^1H and ^{13}C NMR data. ^1H–^1H COSY data afforded information on the proton connectivities for three partial structures (C-2–C-14, C-15–C-19, and C-20–C-27). Geometries of the four disubstituted double bonds were deduced on the basis of the ^1H–^1H coupling constants determined by a *J*-resolved two-dimensional NMR experiment. The partial structures were revealed to be linked to each other by ^1H 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 (**33**), G (**36**), and H (**37**), whose molecular formulas are all $C_{32}H_{50}O_8$, revealed that they are structurally related closely to amphidinolide B (**31**): amphidinolides B (**31**) and D (**33**) are stereoisomers at C-21; amphidinolides G (**36**) and H (**37**) are regioisomers of the lactone-terminal positions (C-25 or C-26); amphidinolides B (**31**) and H (**37**) are only different in the position of one hydroxyl group (C-16 or C-26). Amphidinolide F (**35**), $C_{35}H_{52}O_9$, proved to be analogous to amphidinolide C (**32**). The structural difference between amphidinolides F (**35**) and C (**32**) was found in the length of the alkyl side chain, the former possibly being a biogenetic precursor of the latter.

Structural features of the amphidinolides are unique. Amphidinolides show a variation in the size of the macrocyclic lactone rings [19-membered, amphidinolide E (**34**); 20-membered, amphidinolide A (**30**); 26-membered, amphidinolides B (**31**), D (**33**), and H (**37**); 25-membered, amphidinolides C (**32**) and F (**35**); 27-membered, amphidinolide G (**36**)]. It should be noted that cytotoxic activity of amphidinolide B (**31**) and its related compounds (**33**, **36**, and **37**) are extremely strong (Table II). Amphidinolide D (**33**), an epimer of amphidinolide B (**31**) 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 (**31**) to generate compound **40**,[42] the IC_{50} value of which was 0.081 μg/mL against L1210 cell, being considerably weaker than that of **31** (1/600). These results implied that the stereochemistry at C-21 and the presence of an epoxide at C-8/C-9 position are quite important for the cytotoxic activity of these compounds, presumably due to the significant change of the



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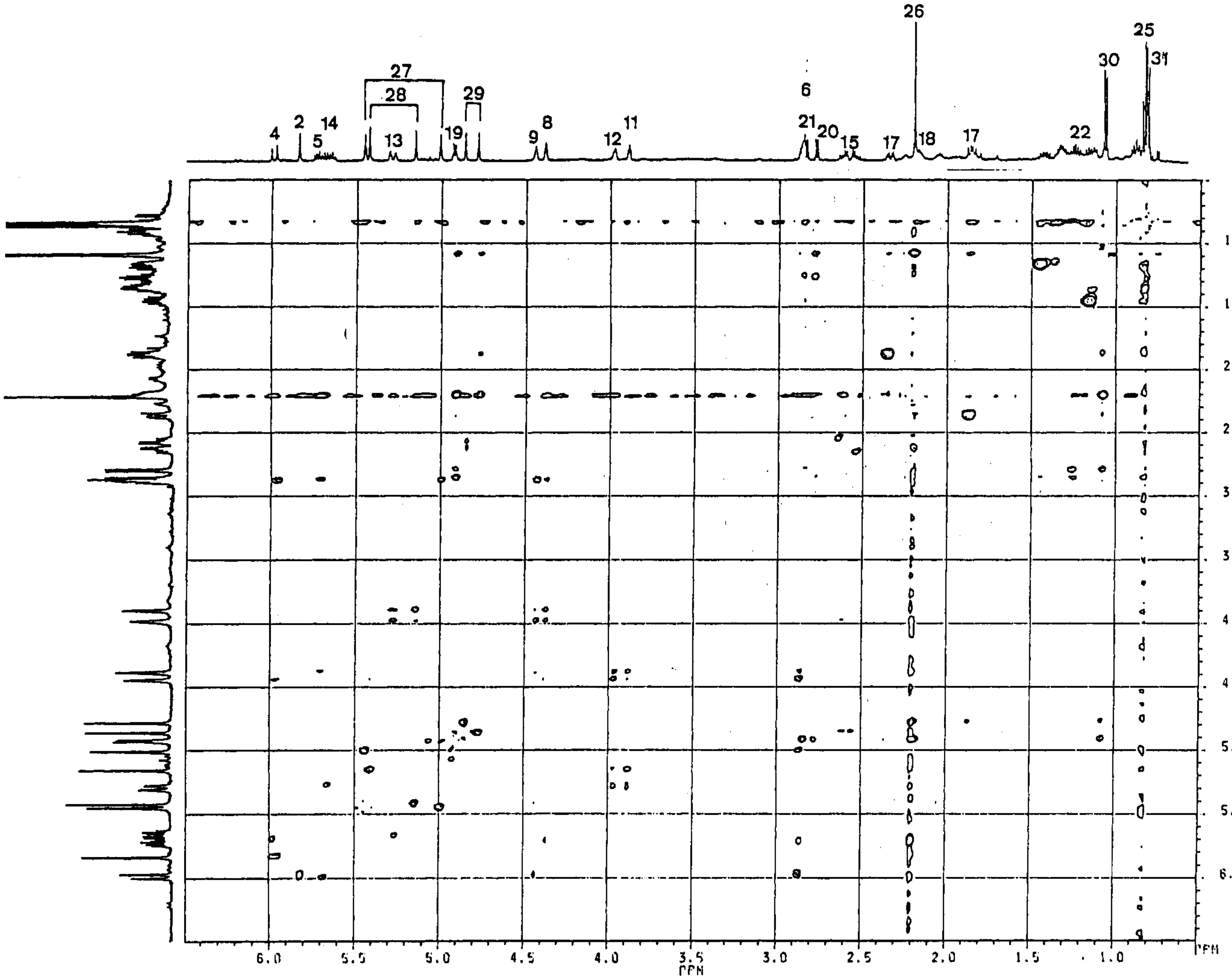
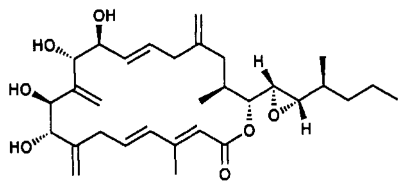


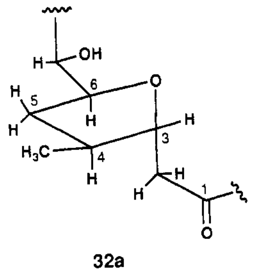
Figure 3. Phase-sensitive NOESY spectrum of Amphidinolide A (30) (500 MHz, C_6D_6).

molecular conformation. The cytotoxicities of amphidinolides C (**32**) and F (**35**) 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. It seems to be also noteworthy that amphidinolides A (**30**), B (**31**), C (**32**), and E (**34**) are quite unlike one another in molecular constitution and substitution patterns although they were obtained from the same species of dinoflagellate *Amphidinium* sp. (Y-5 or Y-5′). Their biogenetical relationships may raise an interesting subject.

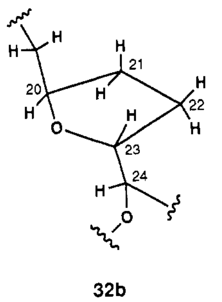
A very important question remaining undefined is the stereochemistry of amphidinolides. All attempts to obtain crystals of the amphidinolides suitable for X-ray analysis have been unsuccessful. Efforts to elucidate the stereochemistry of amphidinolide A (**30**), however, were made by analyzing the phase-sensitive NOESY spectra of this 20-membered macrolide with nine chiral centers (Figure 3). Interpretation of the relative stereochemistry of conformationally mobile molecules by spectral data alone is still not easy and very few reports have ever been published on this subject.[49] The NOE data, however, may provide some suggestive information for the relative configurations and molecular conformations of such molecules.[50] We have proposed a possible stereostructure of amphidinolide A as **30a** containing the relative configurations of the nine chiral centers of **30** and most sufficiently satisfying the NOESY data obtained in several solvent systems.[51] NOESY experiments on amphidinolide C (**32**) were also carried out (Figure 4) and the relative stereochemistry around the two tetrahydrofuran ring moieties were proposed as **32a** and **32b**. For unambiguous assignment of the stereochemistry of amphidinolides the following studies are required: (a) further large-scale culturing of the dinoflagellates *Amphidin-*



30a



32a



32b

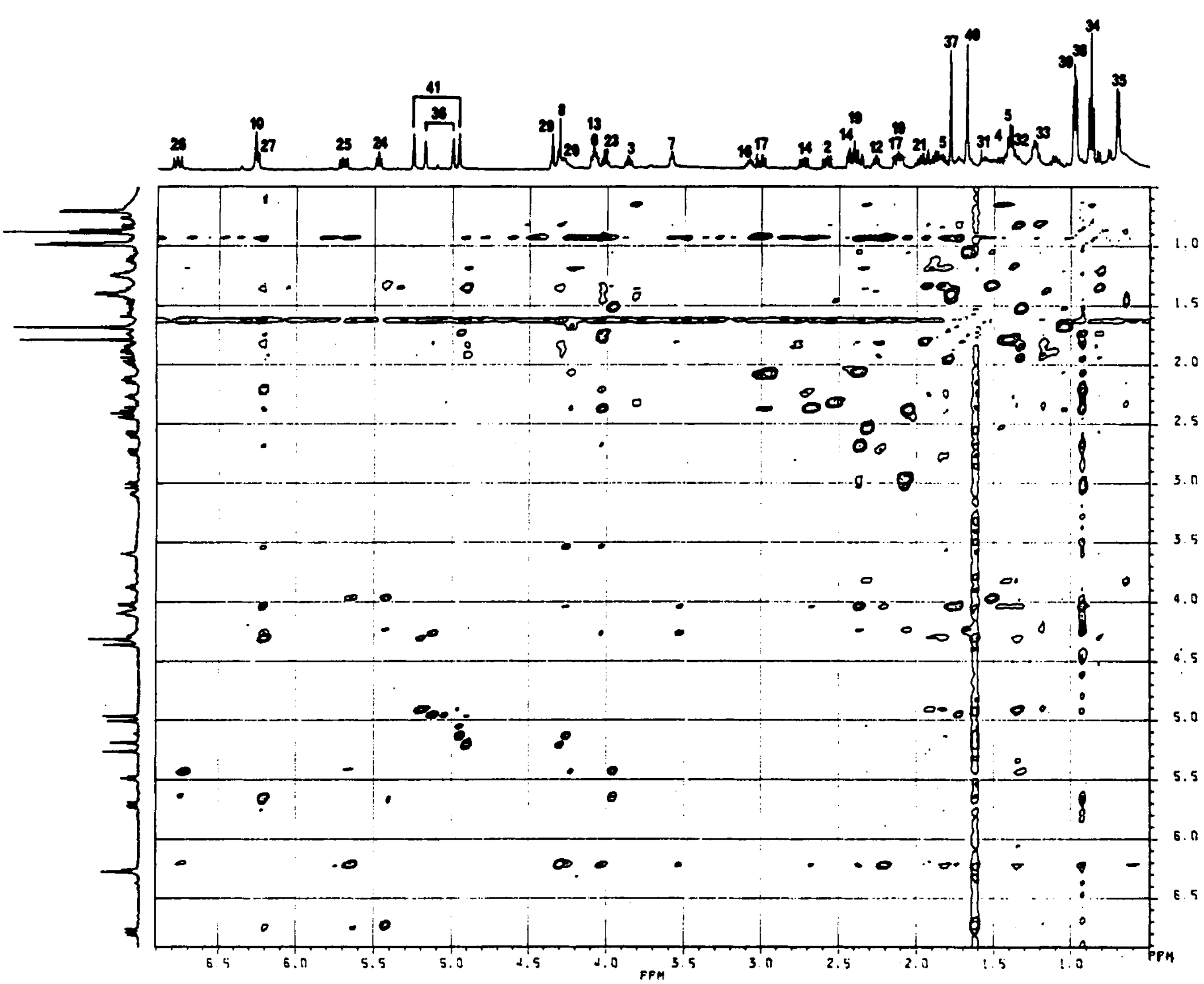
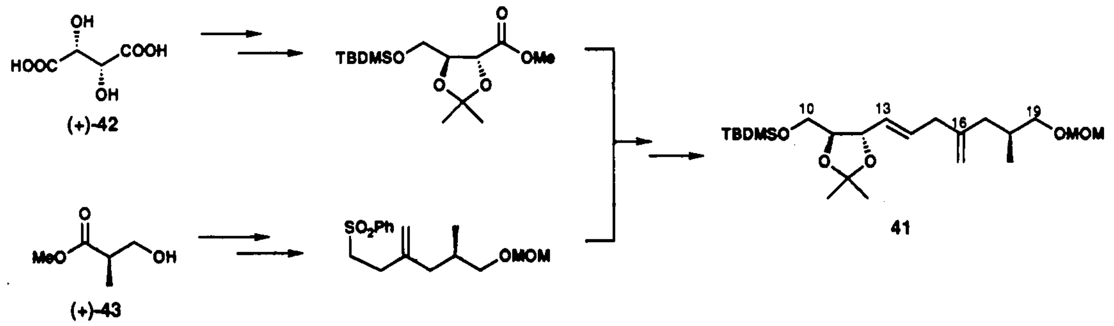


Figure 4. Phase-sensitive NOESY spectrum of Amphidinolide C (32) (500 MHz, C_6D_6).

Scheme II. Outline of the Synthesis of a Potential C-10–C-19 Fragment (41) of Amphidinolide A (30)



ium spp. to obtain an adequate quantity of these macrolides; (b) degradation experiments (ozonolysis or periodate oxidation) of these macrolides to lead to several small fragments; (c) stereoselective synthesis of all possible stereoisomers of the small fragments in optically active forms. These challenging problems are currently under investigation by us. Williard and coworkers independently described synthetic efforts on amphidinolide A (30) in order to make an unambiguous assignment of its stereochemistry.[52] A potential C-10–C-19 fragment (41) of amphidinolide A (30) was prepared by a stereospecific route (Scheme II). It may be possible to prepare alternative optically pure stereoisomers of the diene 41 by utilizing similar reaction sequences starting with (+)- or (−)-tartaric acid (42) and (+)- or (−)-methyl 3-hydroxy-2-methylpropionate (43), each of which is commercially available.

The quantity of these macrolides in the extracts of the cultured cells varied a little during the course of time. Extraction of the harvested cells was carried out repeatedly after about 300–400 g of cells (wet weight) were accumulated to give different batches of extracts of the same strain of the alga. From a batch of the extract of *Amphidinium* sp., strain Y-5, which was relatively rich in amphidinolides B (31) and D (33), we once isolated another component of interest, designated as Y5-726, which is most likely a macrolide related to the amphidinolides. Y5-726 was separated from the

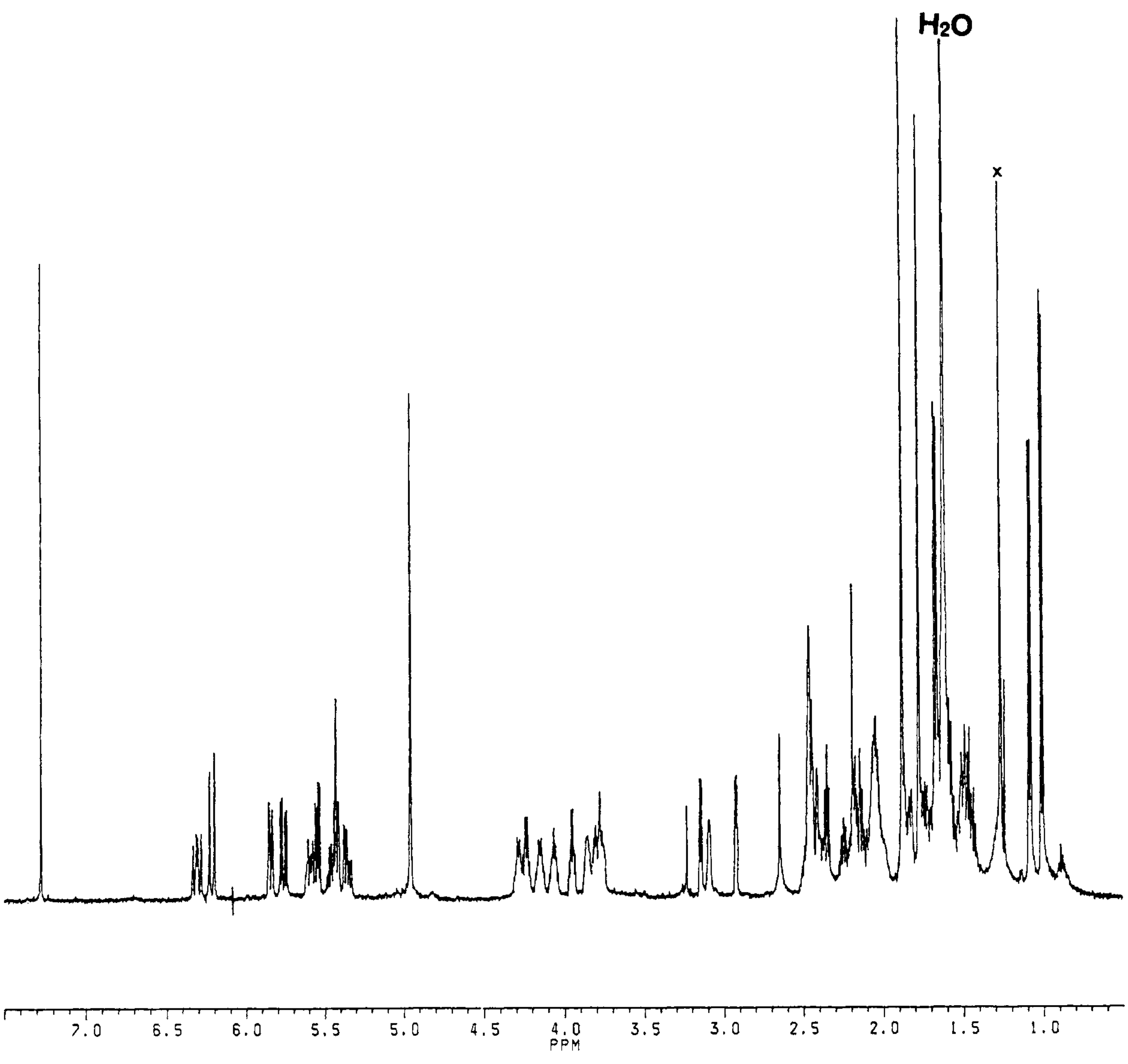
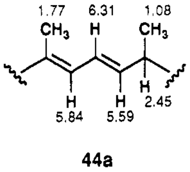
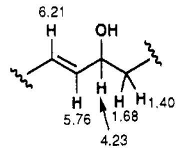


Figure 5. ^{1}H NMR spectrum of Y5-726 (500 MHz, CDCl$_3$).

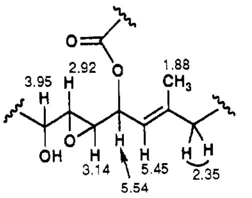
mixture of **31** and **33** by reversed-phase HPLC using 87% methanol as eluent. It was isolated in 0.0005% yield (wet weight) as colorless amorphous solid, $[\alpha]^{26}_{D}$ +4.5° (*c* 1, CHCl$_3$); UV (MeOH) 238 nm (ϵ 29 000); IR (film) 3400, 2930, 1730, 1440, and 1050 cm^{-1}. The molecular weight of Y5-726 was suggested to be 726 by the FABMS data [glycerol matrix *m*/*z* 727 $(M + H)^+$ and 709 $(M + H - H_2O)^+$; diethanolamine (DEA) matrix *m*/*z* 832 $(M + DEA + H)^+$]. The ^{1}H NMR spectrum of Y5-726 (Figure 5) showed signals due to three secondary methyls [δ 1.00 (3 H, d, J = 6.5 Hz), 1.08 (3 H, d, J = 7 Hz), 1.67 (3 H, d, J = 6.5 Hz)], two vinyl methyls [δ 1.77 (3 H, s) and 1.88 (3 H, s)], two possible epoxide-bearing methine protons [δ 2.92 (1 H, dd, J = 4.5 and 2 Hz) and 3.14 (1 H, dd, J = 5.5 and 2 Hz)], eight oxymethine protons [δ 3.77 (2 H, m), 3.86 (1 H, br s), 3.95 (1 H, dd, J = 12 and 4.5 Hz), 4.07 (1 H, m), 4.17 (1 H, m), 4.23 (1 H, dd, J = 13 and 7 Hz), and 4.30 (1 H, m)], two possible *exo*-methylene protons [δ 4.95 (2 H, s)], and eight olefinic and one possible lactone-terminal protons [δ 5.36 (1 H, dd, J = 15 and 9 Hz), 5.45 (2 H, m), 5.54 (1 H, dd, J = 9.5 and 5.5 Hz), 5.59 (1 H, m), 5.76 (1 H, dd, J = 15.5 and 6.5 Hz), 5.84 (1 H, d, J = 11 Hz), 6.21 (1 H, d, J = 15.5 Hz), and 6.31 (1 H, dd, J = 15 and 11 Hz)]. The incomplete ^{1}H–^{1}H COSY spectrum of Y5-726 was recorded (Figure 6) and suggested the presence of partial structures of **44a–c**.



44a



44b



44c

Further structural studies of Y5-726 were unfortunately precluded since the compound decomposed extensively during storage as a CDCl$_3$ solution in a refrigerator, probably due to contact with traces of hydrochloric acid in the CDCl$_3$ solvent. Unfortunately, Y5-726 was not isolated from the extracts of subsequent *Amphidinium* sp. cultures.

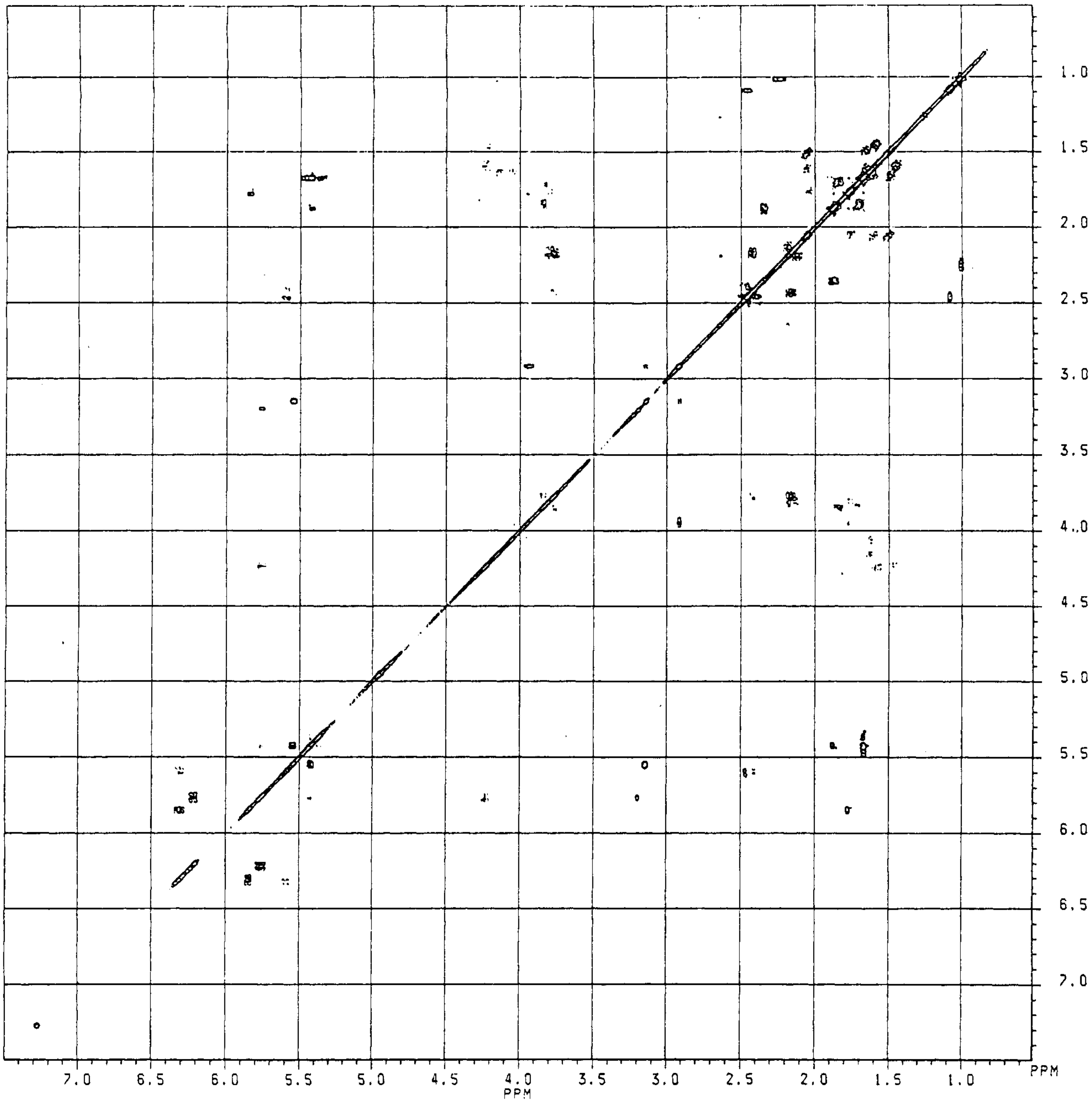
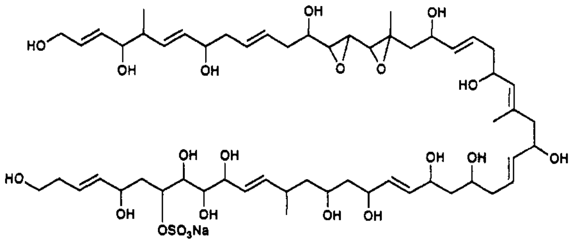
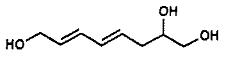


Figure 6. ^1H–^1H COSY spectrum of Y5-726 (500 MHz, $CDCl_3$).

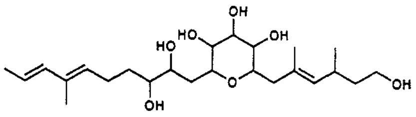
Nakamura and co-workers isolated the novel vasoconstrictive substances, named zooxanthellatoxins A and B,[53,54] from a cultured zooxanthella (typical symbiotic dinoflagellate) of the genus *Symbiodinium*, which was isolated from an Okinawan flatworm *Amphiscolops* sp. Zooxanthellatoxins A and B (negative FABMS, *m/z* 2873 and 2835, respectively) proved to be sulfate esters of highly unsaturated polyols and induce the contractile response of the rabbit aorta at 7 × 10^{-7} M each. This contractile response was not affected by tetrodotoxin (a sodium channel blocker), histamine antagonists, or serotonin antagonists, while it was markedly inhibited by calcium antagonists such as verapamil or magnesium chloride. Thus this vasoconstrictive activity was suggested to result from an increase of the calcium permeability across the membrane. The structure of zooxanthellatoxin A was investigated through the periodate oxidation to give several fragments, the structures of which were elucidated (*e.g.* **45a–d**) by extensive spectral analyses.



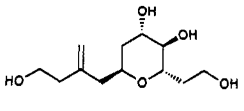
45a



45b



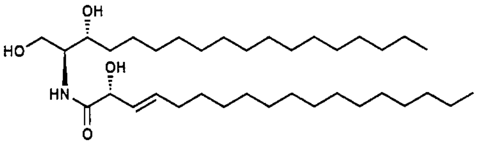
45c



45d

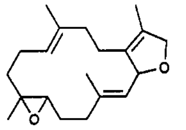
B. Others

A symbiotic dinoflagellate of the genus *Symbiodinium* was isolated from the inside of gill cells of the Okinawan bivalve *Fragum* sp. The procedure of culturing this dinoflagellate in the laboratory followed that of the dinoflagellate *Amphidinium* spp. described above. From the toluene-soluble fraction of the extract of the havested cells a sphingosine derivative, symbioramide (46),[55] was isolated by using silica gel column chromatographies. The structure of symbioramide (46) was elucidated on the basis of spectral and chemical means. The ^1H and ^{13}C NMR data suggested that compound **46** belongs to ceramides. Acid hydrolysis of **46** afforded methyl 2-hydroxyoctadec-3(*E*)-enoate and 2(*S*)-amino-3(*R*)-hydroxyoctadecan-1-ol. The absolute configurations at C-2 and C-3 positions of the sphingosine part of **46** were thus determined. Symbioramide (**46**) exhibited weak cytotoxic activity against L1210 cells in vitro with an IC_{50} value of 9.5 μg/mL. Additionally, compound **46** was found to be a sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activator. The Ca^{2+}-ATPase in SR membrane plays an key role in muscle relaxation by energizing Ca^{2+} pumping from the cytoplasm into the lumen of SR.[56] Symbioramide (**46**) at 10^{-4} M activated SR Ca^{2+}-ATPase activity by 30%. This is the first example of SR Ca^{2+}-ATPase activator of marine origin. Symbioramide (**46**) may serve as a valuable chemical tool for studying the reguratory mechanisms of SR Ca^{2+}-pumping systems. The α-hydroxy-β,γ-dehydro fatty acid contained in symbioramide (**46**) is rare from natural sources. Previously ceramides of α-hydroxy fatty acids were obtained from the sponge *Dysidea etheria*.[57] 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 2-hydroxyoctadec-3(*E*)-enoic acid moiety of symbioramide (**46**), which we left unassigned, was established as *R* on the basis of the total synthesis of symbioramide (**46**) achieved by Hino and co-workers.[58]



46

Soft corals are known to possess symbiotic microalgae. We proviously isolated a diterpene, 16-deoxysarcophine (47),[59] 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 (**47**) was detected by HPLC analysis of the extracts of the dinoflagellate *Symbiodinium* sp.



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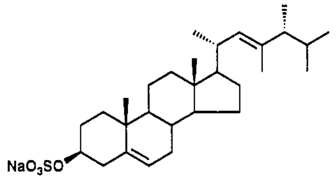
V. Other Microalgae

The Haptophyceae are microscopic, unicellular algae, which are widely distributed in the oceans and often constitute a major proportion of marine phytoplankton. We encountered a haptophyte of the genus *Hymenomonas*, which was isolated from an unidentified cylindrical stony coral collected at Sesoko Island, Okinawa. The alga was mass cultured by the same procedure as that of the *Amphidinium* spp. of dinoflagellates. 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 (48),[60] with potent Ca^{2+}-releasing activity in SR.[61] The structure of **48** was elucidated by conversion of **48** 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 (**48**) 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 (**49** and **50**, respectively), while the chloroform layer contained mainly octadecatetraenoic acid (**51**) along with a small amount of monogalactosylmonoacylglycerol (**52**). In the SR, the Ca^{2+}-releasing activity of **48** was 10 times more potent than that of caffeine, a well-known Ca^{2+} releaser. The glycolipids **49**, **50**, and **52** exhibited[60] inhibition of Na^+,K^+-ATPase activity[62] with an IC_{50} value of 2 × 10^{-5} M each.



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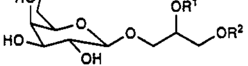
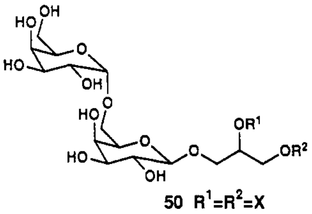
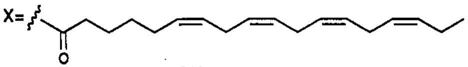


49 $R^1=R^2=X$
52 $R^1=H$, $R^2=X$



50 $R^1=R^2=X$

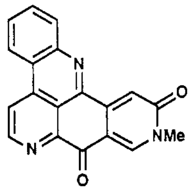


51 X-OH

VI. Marine Natural Products Assumed To Be of Microbial Origins

Recently, many fused tetra- and pentacyclic aromatic alkaloids have been isolated from a variety of marine organisms (Chart I),[4] *e.g.*, from sponges, amphimedine

Chart I



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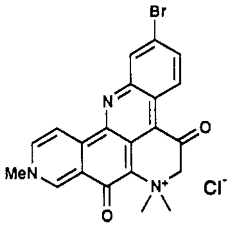

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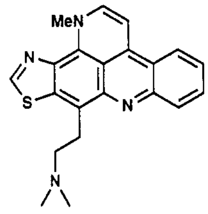

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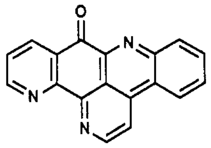

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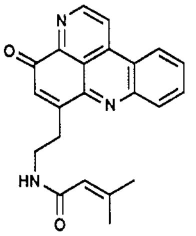

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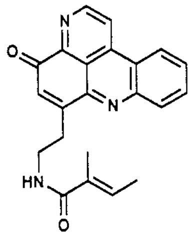

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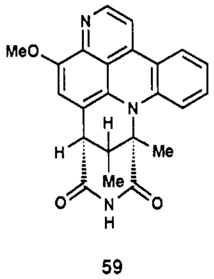

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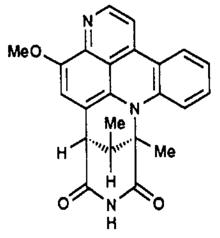

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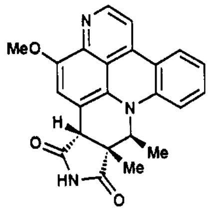

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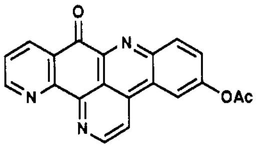

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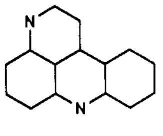
(**53**, *Amphimedon* sp.),[63] petrosamine (**54**, *Petrosia* sp.),[64] and dercitin (**55**, *Dercitus* sp.);[65,66] from tunicates, ascididemin (**56**, *Didemnum* sp.),[67] cystodytins (**57** and **58**, *Cystodytes* sp.),[68,69] and segolines (**59**–**61**, *Eudistoma* sp.);[70,71] and from a sea anemone, calliactine (**62**, *Calliactis* sp.).[72,73] These alkaloids, obtained from such diverse sources, might be assumed to originate from symbiotic microorganisms, since they possess a common tetracyclic ring system (**63**, a pyrido[4,3,2-*mn*]acridine skeleton or benzo-3,6-diazaphenanthroline ring) and are closely related to one another. For example, segoline B (**60**) and isosegoline A (**61**) appear to correspond to regioisomers which were assumed to be generated through intramolecular [4 + 2] cycloaddition reaction from cystodytin B (**58**).



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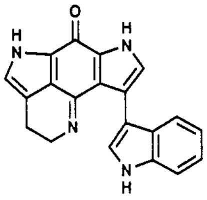
From a compound tunicate *Clavelina* sp. collected at Wakaya Island, Fiji, a pyrroloiminoquinone alkaloid, wakayin (**64**),[74] was isolated and this alkaloid exhibited cytotoxic, antimicrobial, topoisomerase II inhibitory, and DNA-damaging activities. The structure of compound **64** appears to be closely related to those of discorhabdin C (**65**)[75–77] and prianosin A (**66**),[78–80] which were isolated from marine sponges *Latrunculia* sp. and *Prianos melanos*, respectively. Wakayin (**64**) may be biogenetically derived from two molecules of tryptophan, while compounds **65** and **66** seem to be biosynthesized from tyrosine and tryptophan. Since similar biogenetic origins could be assumed for these compounds, these metabolites may be produced by symbiotic microorganisms.



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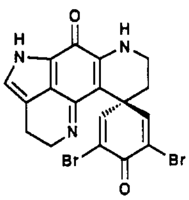

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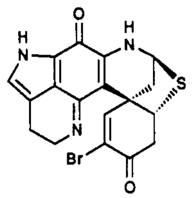

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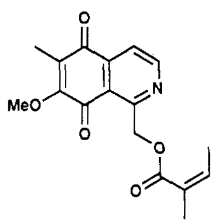
Several isoquinoline quinone alkaloids, such as renierone (**67**),[81] mimosamycin (**68**),[81] renieramycin E (**69**),[82] and renierol (**70**) (Chart II),[83] have been isolated from marine sponges of the genera *Reniera* and *Xestospongia*. From a tunicate *Ecteinascidia turbinata* similar tetrahydroisoquinoline alkaloids, ecteinascidins (*e.g.*, **71**),[84,85] were isolated. These alkaloids were likely to be biosynthesized by symbiotic microorganisms, since mimosamycin (**68**) was first isolated from *Streptomyces lavendulae* no. 314.[86] and this terrestrial microorganism also yielded saframycin B (**72**),[87] which

Chart II



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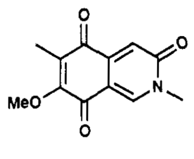

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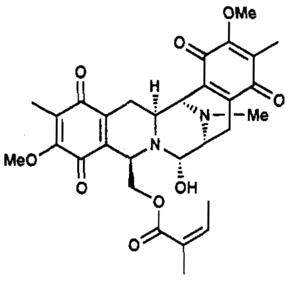

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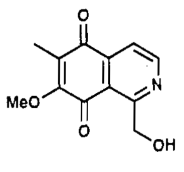

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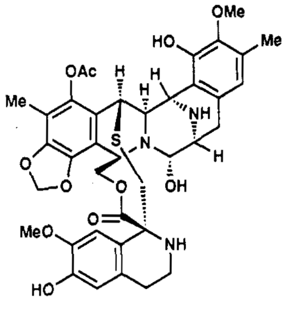

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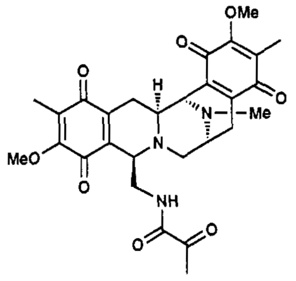

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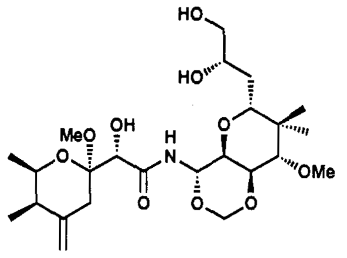
Chart III



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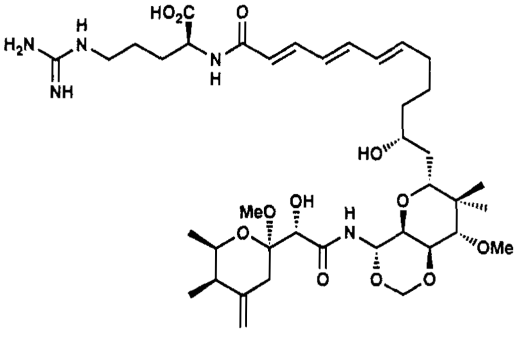

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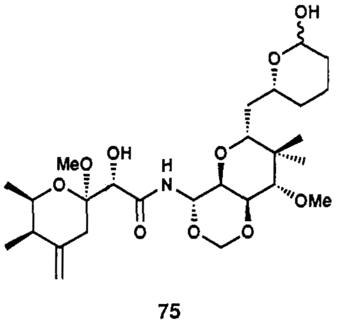

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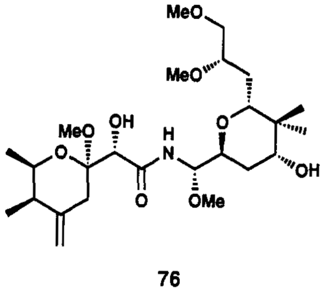

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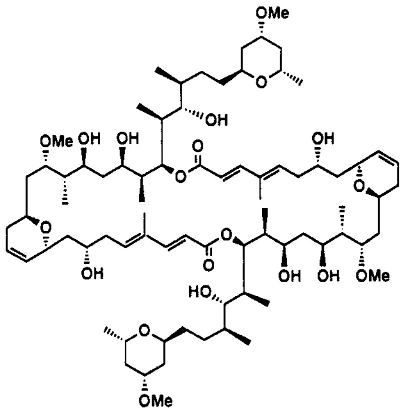
is similar in structure to renieramycins and ecteinacidins.

From sponges of the genus *Mycale* and *Theonella*, several polyether metabolites such as mycalamide A (73),[88,89] onnamide A (74),[90,91] and theopederin A (75)[92] were isolated. Their structures have striking resemblances to pederin (76) (Chart III),[93] a vesicatory principle from blister beetles of the genus *Paederus*. It seems quite interesting that polyethers obtained from marine sponges and terrestrial insects bear such strong structural similarities.

Swinholide A (77)[94–98] and bistheonelide A (78)[99–102] are potent cytotoxic macrodiolides isolated from marine sponges of the genus *Theonella*. From the egg masses of the nudibranch *Hexabranchus sanguineus* oxazole-containing macrolides, ulapualide A (79)[103] and kabiramide C (80),[104,105] were isolated. The latter was also obtained from a sponge *Halichondria* sp. which contained a related macrolide halichondramide (81)[106,107] as the major constituent. These macrolides (77–81) were structurally related to scytophycin B (82)[108] and tolytoxin A (83)[109] which were isolated from the laboratory-cultured terrestrial blue-green algae *Scytonema pseudohofmanni* and *Tolypothrix conglutinata* var. *colorata*, respectively. Three contiguous oxazole rings of ulapualide A (79) and kabiramide C



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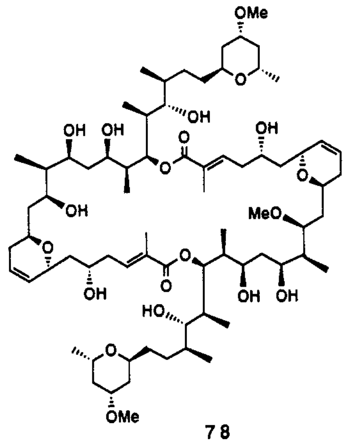

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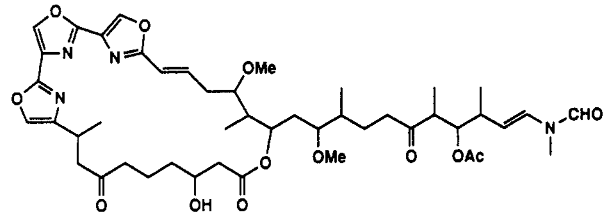

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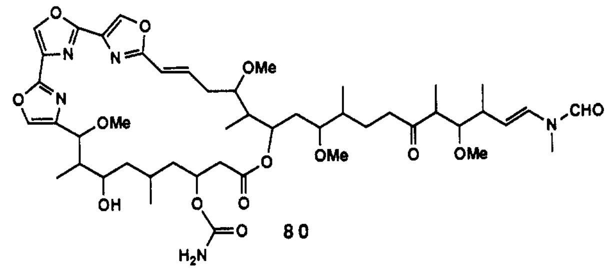

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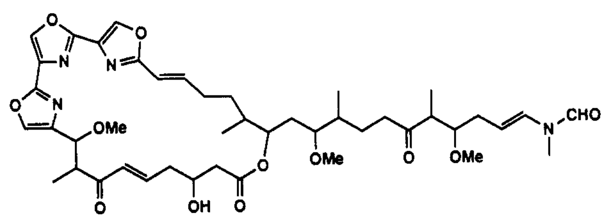

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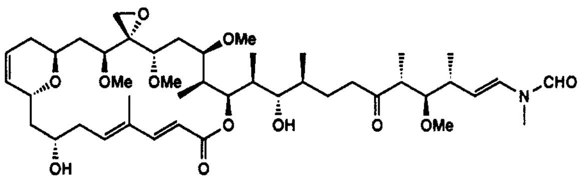

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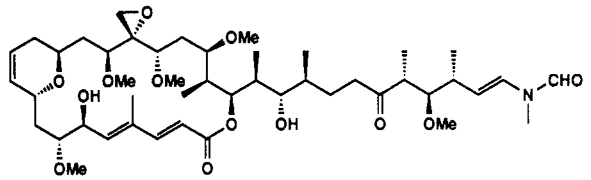

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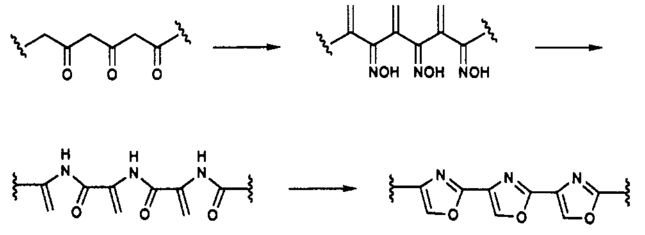
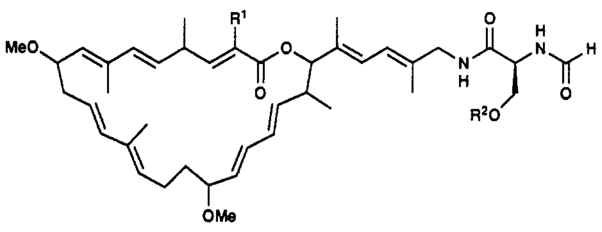
Scheme III. A Hypothetical Biogenetic Pathway for Three Oxazole Systems in Ulapualide A and Kabiramide C



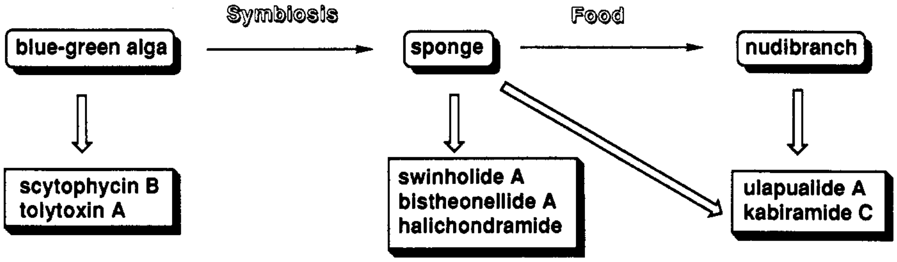
(80) may be assumed as being biosynthesized through naturally-occurring Beckmann rearrangements (Scheme III) from a precursor of 22-membered lactone ring, this ring size being the same as that of scytophycin B (82) or tolytoxin A (83). Nudibranchs are known to feed on sponges, whereas sponges are symbiotically associated with blue-green algae. Thus the real origins of these macrolides are strongly suggested to be blue-green algae (Scheme IV). Electron microscopic analysis demonstrated that a symbiotic blue-green alga inhabited the marine sponge *Theonella swinhoei* that contained swinholides.[97]

There are a number of secondary metabolites isolated from marine sponges or tunicates bearing the structural characteristics which were suggestive that those metabolites were produced by symbiotic microorganisms. Among them, macrolides and peptides are typical instances. From an Okinawan tunicate *Eudistoma* cf. *rigida* four cytotoxic macrolides, iejimalides A–D (84–87),[110,111] were isolated. Since the iejimalides possess a common structural feature consisting of a 24-membered macrocyclic lactone ring, they are presumably of microbial origin. Sponges belonging to the genus *Theonella* proved to contain numerous interesting bioactive substances. In particular, many cyclic peptides or peptide lactones were reported to be obtained from *Theonella* sponges and their structural features, including unusual amino acids or D-amino acids, suggest that they are likely to be originating from symbiotic microorganisms. Theonellapeptolide Id (88),[112–116] a peptide lactone possessing Na$^+$,K$^+$-ATPase inhibitory

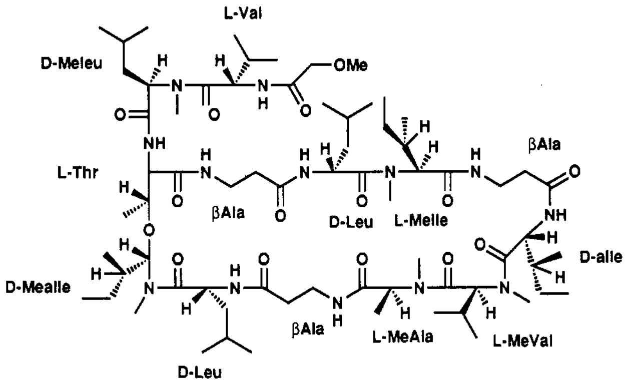


84 R^1=H, R^2=H
85 R^1=Me, R^2=H
86 R^1=H, R^2=SO$_3$Na
87 R^1=Me, R^2=SO$_3$Na

Scheme IV. Swinholide-Related Macrolides and Organisms Containing Them



activity, was isolated from Okinawan *Theonella* sponges. This peptide 88 is a rare example comprised of *N*-methyl and D-amino acids in a high ratio. Konbamide (89),[117] a calmodulin antagonist, and keramamide A (90),[118] a SR Ca^{2+}-ATPase inhibitor, were isolated from Okinawan *Theonella* sponges and are structurally unique cyclic peptides with an ureido bond. Peptides containing ureido bonds were rare, but have previously been reported from terrestrial microorganisms.[119] Keramamides B–D (91–93),[120] potent inhibitors of the superoxide generation response of the human neutrophiles elicited with fMLP, and keramamide F (94),[121] a cytotoxic metabolite, were also isolated from Okinawan *Theonella* sponges. These cyclic peptides (91–94) contained several unusual amino acids (*e.g.* halogenated tryptophan derivatives and α-oxo-β-amino acids).



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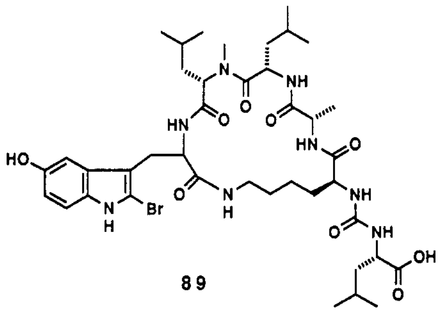

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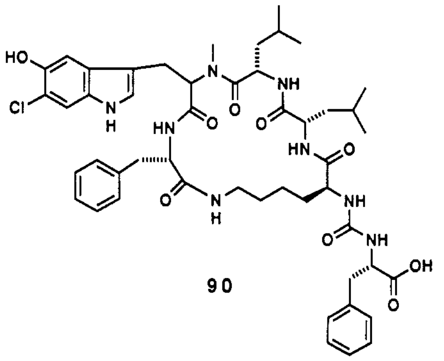

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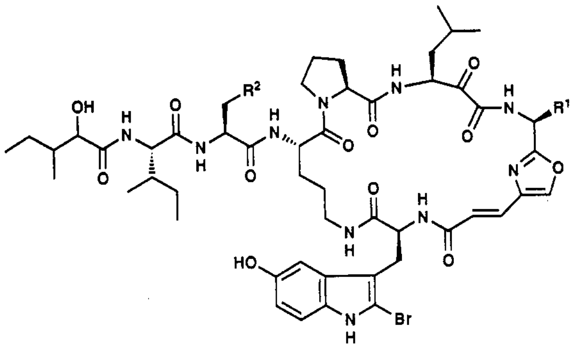
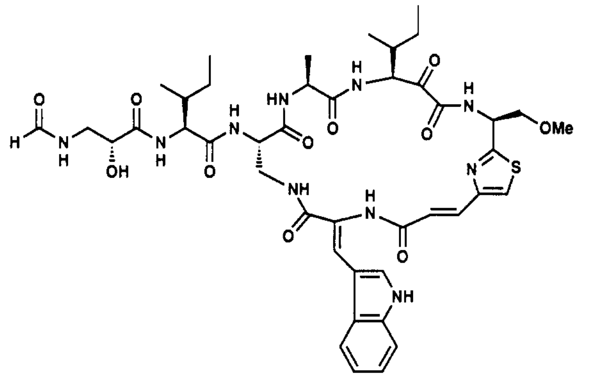


91 $R^1=CH_2CH_3$, $R^2=CH_2CH_3$
92 $R^1=CH_2CH_3$, $R^2=CH_3$
93 $R^1=CH_3$, $R^2=CH_3$



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VII. Conclusion

Symbiotic marine microorganisms have proven to be a new promising source for bioactive substances. Countless numbers of species of microorganisms are living in the sea. Research to date indicates that further studies on marine microorganisms may be confidently expected to yield new bioactive compounds that are applicable to drug development or useful for basic research in life sciences.

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