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Bioactive substances isolated from marine sponge, a miniature conglomerate of various organisms

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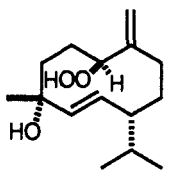
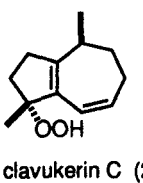
This article reviews our recent pharmacochemical investigations on the bioactive marine natural products isolated from marine sponges and also deals with our current studies of some microbial products which are obtained from the cultures of microorganisms associated with those marine sponges.

Due to their unusual living environment as compared with terrestrial organisms, marine organisms, such as sponge, octocoral, tunicate and bryozoan, metabolite and produce a variety of substances which often have various unprecedented chemical structures and exhibit significant biological activities. These bioactive constituents may offer interesting subjects of studies in view of bioorganic chemistry.

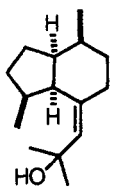
In search of new pharmaceutically valuable substances from marine organisms, we have been engaged recently in chemical studies on the constituents of octocorals and marine sponges mostly inhabiting the Okinawan coral reefs.[1] We have found various types of marine natural products, among which some possess rather uncommon moieties in their structures, such as a hydroperoxy group [found in octocorals, *e.g.* nephtheoxydiol (**1**)[2], clavukerin C (**2**)[3]] or a cyclic peroxide moiety (found in marine sponge, *vide infra*). The other findings of interest are that some soft corals metabolite several sesquiterpenes having carbon frameworks antipodal to those isolated from terrestrial plants [*e.g.* valerenenol (**3**)[4], *ent*-oplopanone (**4**)[2]] and that the Okinawan stolonifer *Clavularia viridis* produces *ent*-prostanoid compounds [*e.g.* claviridenone A (**5**)[5]]. It is also noted that a soft coral of *Xenia* sp. contains a pair of diastereomeric diterpenes [*e.g.* xeniolone



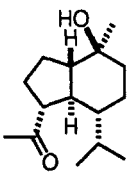
nephtheoxydiol (**1**)



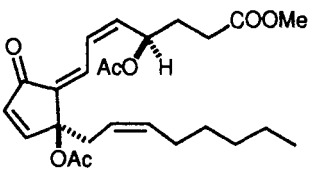
clavukerin C (**2**)



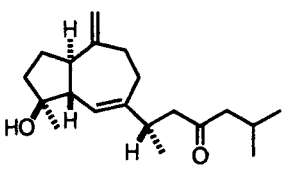
valerenenol (**3**)



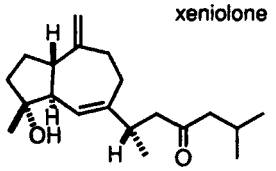
ent-oplopanone (**4**)



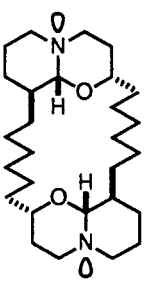
claviridenone-a (**5**)



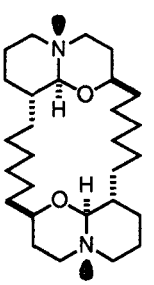
xeniolone (**6**)



isoxeniolone (**7**)



(+)-aragusupongine D (**8**)
[= xestospongin A]



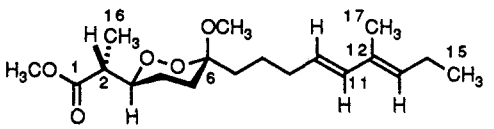
(-)-aragusupongine D (**9**)

(**6**) and isozeniolone (**7**)][6] whereas an Okinawan marine sponge of *Xestospongia* sp. was shown to contain a pair of enantiomeric alkaloids in unequal ratio [*e.g.* (+)-aragusponge D (**8**) and (-)-aragusponge D (**9**) in 4:6 ratio][7]. These alkaloids are chemically characteristic of having two 1-oxaquinolizidine moieties linked with two oligomethylene chains thus forming the macrocyclic structures.

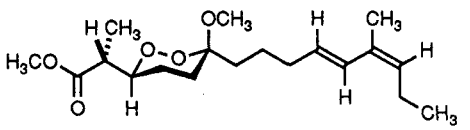
On the other hand, octocorals and marine sponges are known to hold symbiotic and/or parasitic inherent microorganisms. Among those marine organisms in coral reefs, sponges are particularly of interest because of their infra-structures. Microscopic analysis (either by means of scanning electron microscopy or with an optical microscope) has shown that marine sponge comprises blue-green alga(e), fungus(-i), or bacterium(-a) in the tissue, reminiscent of the nature of marine sponge as a miniature conglomerate of various organisms. So that, a questionnaire has arisen which organism(s) may be true producer(s) of various chemical constituents initially isolated by extraction of whole marine sponges. We have been carring forward our studies in this connection, and meanwhile investigating the chemical constituents characteristically isolated from the cultures of microorganisms which are separated from the tissue of marine sponges. This paper presents our recent investigations on the bioactive marine natural products initially isolated from marine sponges themselves and from the cultures of marine microorganisms associated with those marine sponges.

1. Antifungal Peroxyketal Acids from a Marine Sponge of *Plakortis* sp.

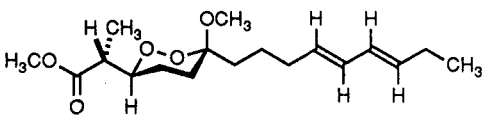
A number of cyclic peroxides have been isolated from marine organisms, and in particular, marine sponges of the genus *Plakortis* are known as rich sources of compounds having cyclic peroxide and peroxyketal structures. Through bioactivity-directed separations, we have isolated, from a marine sponge of *Plakortis* sp. collected at Zamami-jima, Okinawa Prefecture, antifungal peroxyketal acids and their methyl esters named peroxyplakoric acids A_1 methyl ester (**10**), A_2 methyl ester (**11**), A_3 methyl ester (**12**), B_1 methyl ester (**13**), and B_3 methyl ester (**14**), respectively.[8] The peroxyketal acid mixture showed strong growth inhibition for *Candida tropicals*.



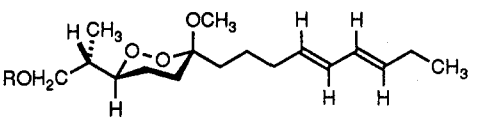
peroxyplakoric acid A_1 methyl ester (**10**)



peroxyplakoric acid A_2 methyl ester (**11**)



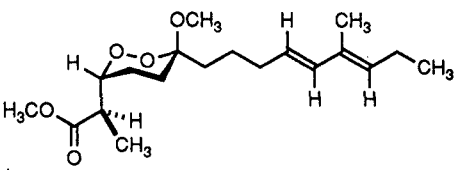
peroxyplakoric acid A_3 methyl ester (**12**)



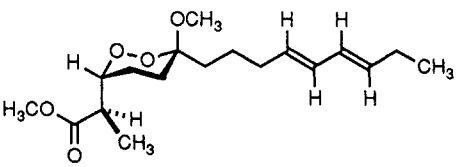
15 : R = H

16a : R = (+)-MTPA

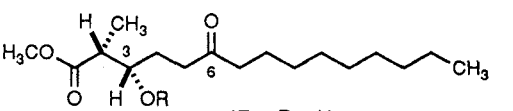
16b : R = (-)-MTPA



peroxyplakoric acid B_1 methyl ester (**13**)



peroxyplakoric acid B_3 methyl ester (**14**)



17 : R = H

18a : R = (+)-MTPA

18b : R = (-)-MTPA

However, the peroxyketal acids were too unstable to be isolated to each component by various chromatography. Thus, the peroxyketal acid mixture was treated with diazomethane to convert to the methyl esters, which were then separated by SiO_2 column and HPLC to provide five new compounds: peroxyplakoric acids A_1 methyl ester (**10**)$\sim B_3$ methyl ester (**14**). These peroxyketal acid methyl esters **10**$\sim$**14** were also obtained from the initial AcOEt soluble portion of this sponge in 0.8 $\sim$ 3.4% yields, respectively.

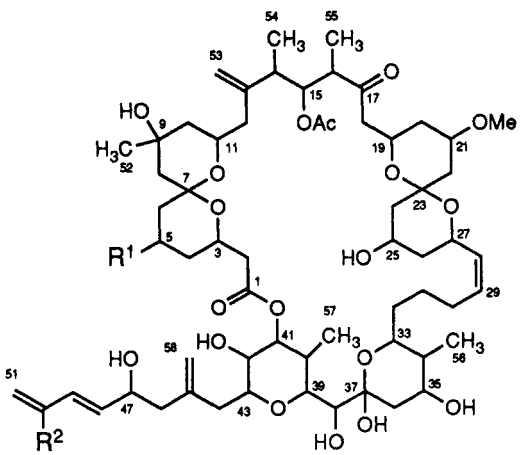
In order to determine the absolute stereostructures of **10**$\sim$**14**, the following conversions were carried out. At first, peroxyplakoric acid A_3 methyl ester (**12**) was treated with LiAlH(*t*-BuO)3 to furnish the peroxyketal alcohol **15**, which was then treated with (+)- and (-)-methoxytrifluoromethylphenylacetic acid (MTPA) and DCC in the presence of DMAP to provide the (+)-MTPA ester **16a** and the (-)-MTPA ester **16b**, respectively. The 2R configuration in **12** was demonstrated from the coupling patterns of the C-1 methylene proton signals of **16a** (observed as a pair of double-doublets) and those of **16b** (observed as a doublet).[9] Next, peroxyplakoric acid A_3 methyl ester (**12**) was subjected to catalytic hydrogenation over 10% Pd/C to furnish the 3-hydroxy-6-keto acid methyl ester **17**, which was further converted to the (+)-MTPA ester **18a** and the (-)-MTPA ester **18b**, respectively. Judging from the difference observed between the ^1H NMR spectra of **18a** and **18b**, which is attributable to the anisotropic effect of the phenyl ring, the absolute configuration at C-3 of **12** has been shown S. The absolute stereostructures of other peroxyplakoric acid methyl esters **10**, **11**, **13**, and **14** were determined in the same manner. It is noteworthy to mention that free peroxyplakoric acids inhibited the growth of *Candida tropicalis* while their methyl esters didn't.

2. Potent Cytotoxic Macrolides from the Marine Sponge *Hyrtios altum*

An acetone extract of the marine sponge *Hyrtios altum*, collected at Aragusuku-jima, Okinawa Prefecture, showed a potent cytotoxic activity (IC_{50} 0.56 μg/ml) against KB cells. Bioassay-guided separation (cytotoxicities against KB and L1210 cells) of the AcOEt soluble portion of the acetone extract provided a fr.B (5.8% from the AcOEt soluble portion)[IC_{50} 0.002 μg/ml (KB)]. The fr.B demonstrated potent antitumor activity against P388 murine leukemia (mice, *i.p.*) : T/C 155% (10 mg/kg administrated on days 1,5). Further repeated SiO_2 column chromatography and HPLC of the fr.B furnished significantly cytotoxic macrolides named altohyrtins A (**19**), B (**20**), and C (**21**), and 5-desacetyl-altohyrtin A (**22**) in 3.4×10^{-3}%, 2.2×10^{-4}%, 2.2×10^{-4}%, and 2.1×10^{-3}% yields from the AcOEt soluble portion, respectively.[10] Altohyrtins A (**19**), B (**20**), and C (**21**), and 5-desacetylaltohyrtin A (**22**) exhibited extremely potent cytotoxicities against KB (IC_{50} 0.01, 0.02, 0.4, and 0.3 ng/ml) and L1210 (IC_{50} 0.1, 0.03, 1.3, and 2.3 ng/ml) cell lines, respectively.

Altohyrtin A (**19**) showed a quasi-molecular ion peak at *m/z* 1245 $(M+Na)^+$ in the FAB MS and the molecular formula was determined as $C_{63}H_{95}O_{21}Cl$ by HR-FAB MS and NMR analysis. Ordinary acetylation (Ac_2O/pyridine, r.t.) of **19** furnished the triacetate. Four partial structures (fragment A: C-1 $\sim$ C-6 and C-38 $\sim$ C-51, fragment B: C-7 $\sim$ C-14, fragment C: C-15 $\sim$ C-23, fragment D: C-24 $\sim$ C-37) were figured out from the detailed analysis of H-H COSY, HMQC, HOHAHA, and HMBC spectra (Fig. 1). The locations of hydroxyl groups were substantiated by deuterium shifts observed in the ^{13}C NMR spectra of **19** taken in CD_3OH and CD_3OD. The signals due to seven carbons at C-9, 25, 35, 37, 38, 42, and 47 were shifted to higher field by 0.1 ppm while the signals due to other oxygenated carbons unchanged. Furthermore, the locations of hydroxyl groups were also confirmed by H-H COSY spectrum taken in d_6-DMSO, which showed correlations between geminal hydroxyl and methine protons at C-25, 35, 38, 42, and 47. Six ring structures (A$\sim$F) and the connectivities of four partial structures (frag. A$\sim$frag. D) were figured out mainly on the basis of HMBC correlations (Fig. 1).

The NOESY correlations observed for altohyrtin A (**19**) are shown in Fig. 2, which not only corroborate the connectivities of the partial structures but also lead to the partial relative stereostructures. Thus, on the basis of the accumulated evidence, the plane structure, with some partial relative configurations, of altohyrtin A has been elucidated as **19**.[10a)]



	R^1	R^2
altohyrtin A (**19**)	OAc	Cl
altohyrtin B (**20**)	OAc	Br
altohyrtin C (**21**)	OAc	H
5-desacetyl-altohyrtin A (**22**)	OH	Cl

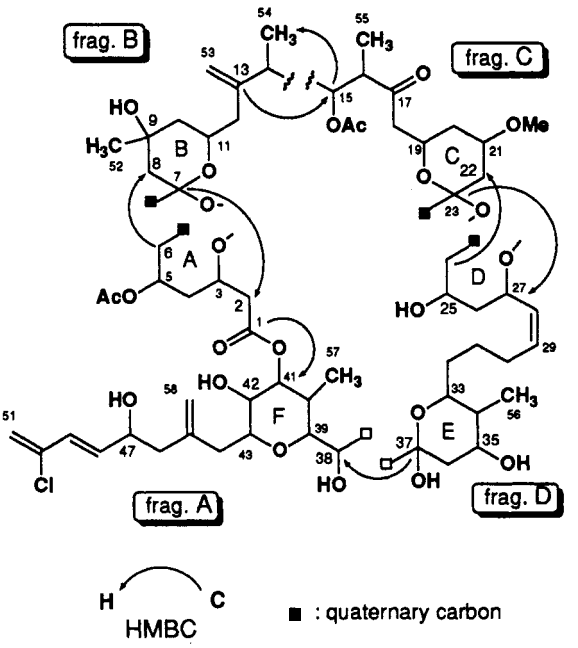


Fig. 1 HMBC Correlations of Altohyrtin A (**19**)

Altohyrtin B (**20**) and altohyrtin C (**21**) have been elucidated as a 50-bromo analog and a deschloro analog of altohyrtin A (**19**), respectively, while 5-desacetylaltohyrtin A (**22**) presumed to be a 5-desacetyl analog of **19**. Thus, the C-4, 5, and 6 carbon signals of altohyrtin A (**19**) were observed with appropriate acylation shifts as compared with those of 5-desacetylaltohyrtin A (**22**). Furthermore, the deuterium shifts were observed with 0.1-0.15 ppm for the signals due to eight carbons of **22** each bearing a hydroxyl group. Consequently, the plane structure of 5-desacetylaltohyrtin A has been determined as **22**.

As for the relative partial stereostructures of altohyrtins B (**20**) and C (**21**) and 5-desacetylaltohyrtin A (**22**), the NOESY correlations provided the quite similar result as obtained in the case of altohyrtin A (**19**). Thus, these three congeners have been presumed to have identical stereostructures with those of **19** which are supported also from the detailed comparison of chemical shifts and coupling patterns in their ^{1}H NMR spectra.[10b]

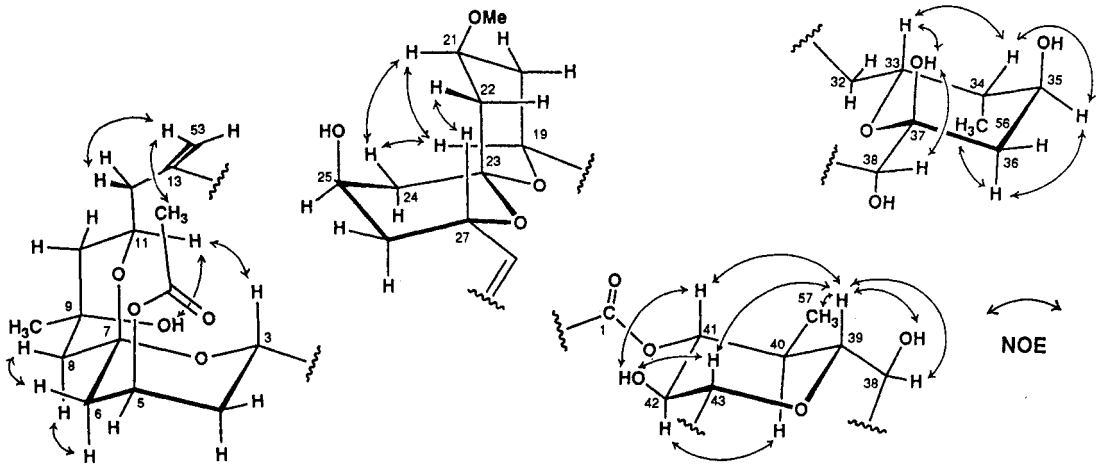


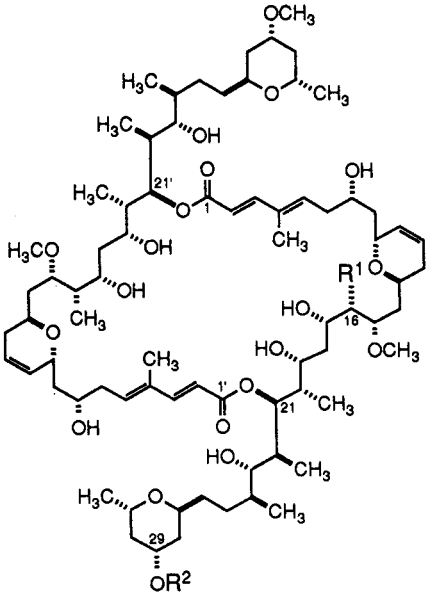
Fig. 2 NOESY Correlations Observed for Altohyrtin A (**19**)

Altohyrtin A (**19**) and its congeners (**20**, **21**, **22**) belong to a new class of antitumor marine macrolide and their absolute stereostructures are now under investigation. Altohyrtin A (**19**) was found to exhibit antitumor activity: T/C 140% for 5 μg/kg (mice) administered on days 1, 5.

3. Marine Sponge as a Miniature Conglomerate

During the course of our studies in search of antitumor marine natural products, we isolated four potent cytotoxic dimeric macrolide, named swinholides A (**23**), B (**24**), and C (**25**) and isoswinholide A (**26**),[11] from the marine sponge *Theonella swinhoei* collected at Zamami-jima, Okinawa Prefecture. From the same sponge, we also isolated five new lipophilic tridecapeptide-lactones, named theonellapeptolides Ia ~ Ie,[12] two new 3-keto-4-methylene steroids, theonellasterone and conicasterone, and a Diels-Alder type dimeric steroid, named bistheonellasterone.[13] Among these swinholide analogs, swinholides A (**23**), B (**24**), and C (**25**) were shown to exhibit potent cytotoxic activities for KB cell lines (IC_{50} 0.04, 0.04, and 0.05 μg/ml), respectively. However, isoswinholide A (**26**), which differs from **23** only in the size of the dilactone framework, was found to show weaker cytotoxicity (IC_{50} 1.1 μg/ml).

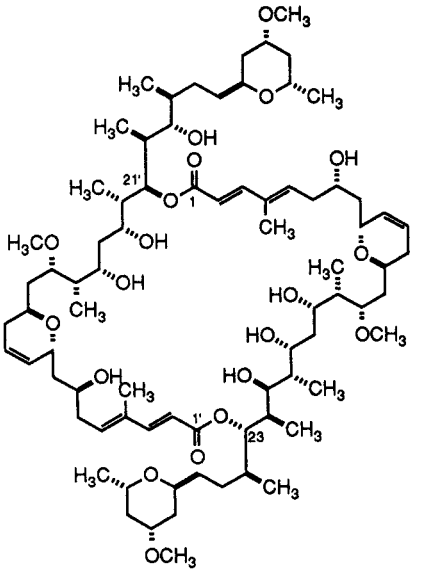
In these structural studies of swinholides, we noticed that the atomic array in the monomeric unit of swinholide A (**23**) was very similar to that in scytophycin C,[14] which was isolated by Prof. Moore and his group in 1986 from the cultured terrestrial blue-green alga *Scytonema pseudohofmanni*. In addition, the configurations at asymmetric carbons of **23** were almost similar to those of scytophycin C. Consequently, it became of interest for us whether the marine sponge *Theonella swinhoei* itself may biosynthesize swinholide analogs (**23**, **24**, **25**, **26**), since marine sponge in general is known to live, as mentioned above, like a "miniature conglomerate" which comprises various microorganisms such as a blue-green alga, fungus, and bacterium. Examinations by means of scanning electron microscopy disclosed the presence of many filamentous blue-green alga in the tissue of the sponge. Although pure culture of the alga has not yet been realized, our interest has been directed to elucidation of the possible contribution of either symbiotic and/or parasitic microorganism(s) for biosynthesis of such biologically active compounds which are initially isolated as the spongean products.



swinholide A (**23**) : $R^1 = R^2 = CH_3$

swinholide B (**24**) : $R^1 = H$, $R^2 = CH_3$

swinholide C (**25**) : $R^1 = CH_3$, $R^2 = H$



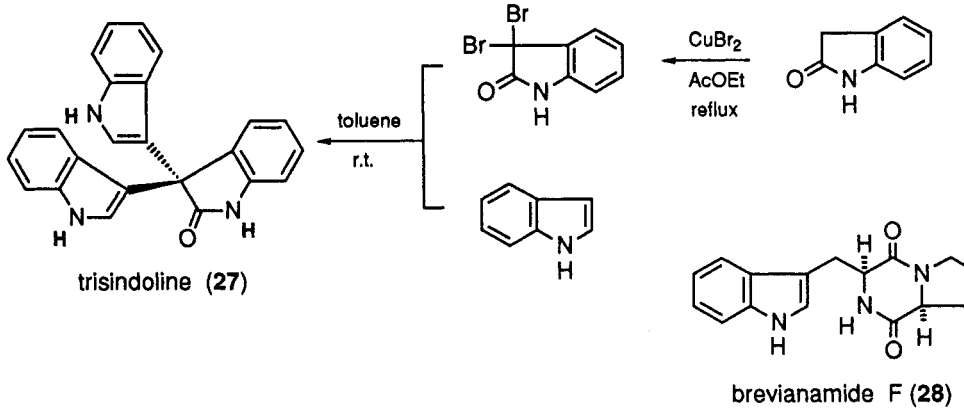
isoswinholide A (**26**)

In recent years, our investigations on the chemical constituents of marine sponges have been carried forward regarding those sponges as "miniature conglomerates". On the other hand, the chemical metabolites of marine microorganisms have also been paid much attention. In consequence, as a part of our search for new bioactive marine natural products, we have been investigating the metabolites of marine microorganisms, which are associated with marine sponges. Followings are recent examples.

4. A New Antibiotic Trisindole Derivative Produced by a Bacterium Separated from the Marine Sponge *Hyrtios altum*

A new antibiotic trisindole derivative named trisindoline (**27**) has been characterized from a culture of a marine bacterium, which was separated from the fresh marine sponge *Hyrtios altum*. As mentioned above, we isolated altohyrtin A (**19**) and its analogs from this sponge. The bacterium[15] was grown in a Zobell 2216E medium prepared with sea water. The cultivation was carried out in 5 l round flasks with vigorous shaking at 25°C for 5 days. The combined culture (80 l) was homogenized by biomixer and then partitioned with AcOEt. The AcOEt soluble portion (3.2 g) was subjected to antibiotic activity-directed separation to provide indole, trisindoline (**27**), and brevianamide F (**28**), which was previously identified from the fungus *Penicillium brevicompactum*. Trisindoline (**27**) was shown to exhibit potent antibiotic activity [15 mm diameter of growth inhibition against *Escherichia coli* at 10 μg/disk (φ=8 mm)]. The ^{1}H and ^{13}C NMR spectra of **27** substantiated the presence of two indole moieties and one oxindole moiety. The connectivity of these indole units has been presumed on the basis of C-H COSY and HMBC correlations to figure out the structure **27**.

In order to confirm the structure of trisindoline (**27**), a synthesis was carried out. A solution of oxindole in AcOEt was treated with copper (II) bromide at 80 °C for 6 h to furnish 3,3-dibromooxindole. 3,3-Dibromooxindole was then treated with indole in toluene at room temperature to furnish **27**.

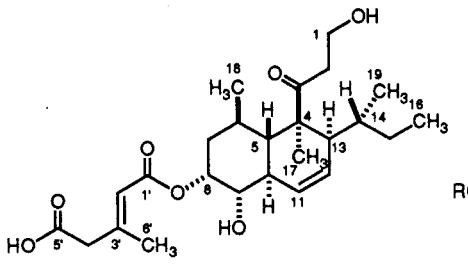


5. A New Polyketide Produced by the Imperfect Fungus *Trichoderma harzianum* Separated from the Marine Sponge *Micale cecilia*

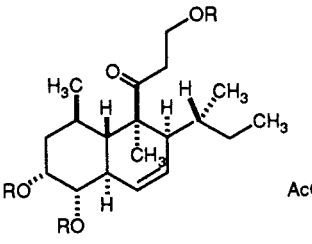
A new polyketide named trichoharzin (**29**) was isolated from a culture of the imperfect fungus *Trichoderma harzianum* Rifai, which was separated from the fresh marine sponge *Micale cecilia*. The sponge collected at Amami-jima, Kagoshima Prefecture, was first subjected to chemical analysis of the constituents which is yet on the way. Meanwhile, a fungus identified as *Trichoderma harzianum* was separated from the sponge. Since *Trichoderma harzianum* is a widespread soil fungus known to produce antibiotics active against other microscopic fungi, we compared by TLC the metabolites obtained from the culture in the Wickerham medium prepared with sea water with those obtained from the culture in the same medium prepared with fresh water. The respective cultivation was carried out in 5 *l* round flasks with vigorous shaking at 25°C for 10 days. The combined culture (30 *l*) was filtered with satin and the filtrate was partitioned with AcOEt. The AcOEt soluble portion gave 1.5 g of the extractive. The chemical constituents of extractives from both media differed significantly, and silica gel column chromatographic and HPLC separations of the

extractive from the salty medium provided a new polyketide named trichoharzin (**29**)(15 mg) as a characteristic metabolite.[16]

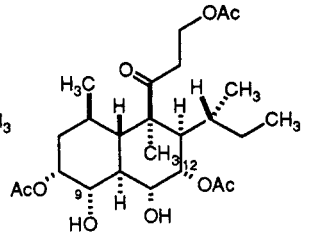
Treatment of trichoharzin (**29**) with trimethylsilyl diazomethane furnished the monomethyl ester. The detailed analyses of H-H COSY and C-H COSY spectra of **29** have first led us to figure out four partial structures. Then, the connectivities of these partial structures have been substantiated from the HMBC and COLOC correlations. Furthermore, based on the NOESY correlation, the relative stereostructure of trichoharzin (**29**) has been elucidated.



trichoharzin (**29**)



30 : R= H
31 : R= Bz



32

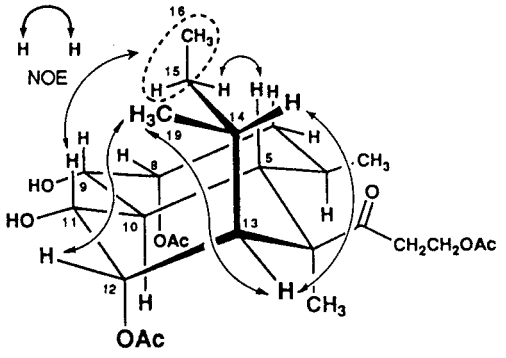


Fig. 3 NOESY Correlations Observed for Trichoharzin (**29**)

The absolute stereostructure of trichoharzin (**29**) has been determined in the following manner. Treatment of **29** with aqueous KOH-methanol furnished the triol (**30**), which was then subjected to benzoylation to yield the tribenzoate (**31**). The exciton coupling ($\Delta\varepsilon$ -51.3 at 236 nm; $\Delta\varepsilon$ +16.9 at 219 nm) observed in the CD spectrum of **31**, has assured the 8R, 9S configurations. The C-14 configuration of **31** has been elucidated as shown in Fig. 3 from the NOESY correlations of the pentaol-triacetate **32**, which was prepared from **30** *via* acetylation (giving 1,8,9-triacetate) followed by osmium tetroxide oxidation in benzene-pyridine (10:1). Consequently, the absolute stereostructure of trichoharzin (**29**) has been confirmed.

Trichoharzin (**29**) is the first octaketide with a new decalin framework. It is noteworthy to mention that the acyl moiety, 3-methylglutaconic acid residue, is hitherto known to occur only in a lichen chromone and is presumed to be derived from mevalonic acid. The biogenetic pathway of trichoharzin (**29**) may be outlined as in Fig. 4.

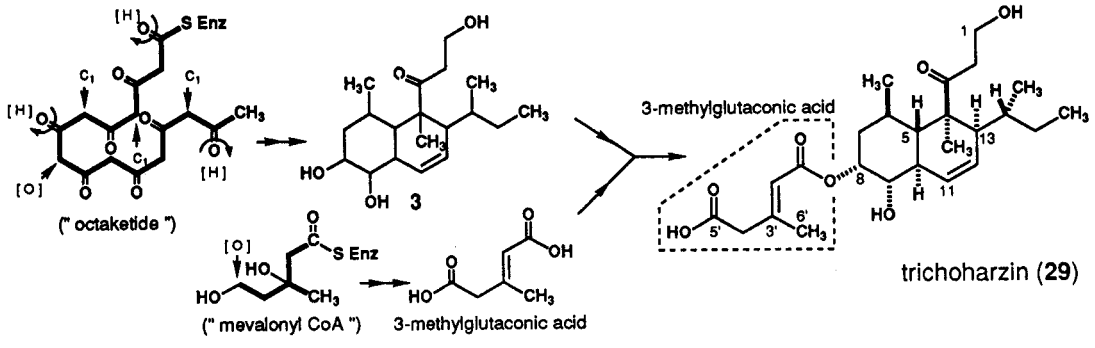


Fig. 4. Biogenetic Pathway of Trichoharzin (**29**)

As a concluding remark in this paper, examinations of the metabolites of microorganisms associated with marine sponges may be promising to find new leads with the view of "drugs from the sea". Furthermore, after detailed investigations of the culture conditions, these procedures may provide evidence demonstrating which organism is a true contributer to the constituents initially isolated from marine sponges.

REFERENCES

1) Reviews: a) I. Kitagawa, *Yakugaku Zasshi* (*J. Pharm. Soc. Japan*), **108**, 398-416 (1988); b) *Idem*, *ibid*, **112**, 1-41 (1992).
2) I. Kitagawa, Z. Cui, B. W. Son, M. Kobayashi, and Y. Kyogoku, *Chem. Pharm. Bull. (Tokyo)*, **35**, 124 (1987).
3) M. Kobayashi, B. W. Son, Y. Kyogoku, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **32**, 1667 (1984).
4) M. Kobayashi, T. Yasuzawa, Y. Kyogoku, M. Kido, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **30**, 3431 (1982).
5) I. Kitagawa, M. Kobayashi, T. Yasuzawa, B. W. Son, M. Yoshihara, and Y. Kyogoku, *Tetrahedron*, **41**, 995 (1985).
6) a) I. Kitagawa, M. Kobayashi, Z. Cui, Y. Kiyota, and M. Ohnishi, *Chem. Pharm. Bull. (Tokyo)*, **34**, 4590 (1986); b) I. Kitagawa, Z. Cui, Y. Cai, M. Kobayashi, and Y. Kyogoku, *ibid*, **34**, 4641 (1986).
7) a) M. Kobayashi, K. Kawazoe, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **37**, 1676 (1989); b) *Idem*, *Tetrahedron Lett.*, **30**, 4149 (1989).
8) M. Kobayashi, K. Kondo, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, submitted (1993).
9) F. Yasuhara, S. Yamaguchi, R. Kasai, and O. Tanaka, *Tetrahedron Lett.*, **27**, 4033 (1986).
10) a) M. Kobayashi, S. Aoki, H. Sakai, K. Kawazoe, N. Kihara, T. Sasaki, and I. Kitagawa, *Tetrahedron Lett.*, **34**, 2795 (1993); b) M. Kobayashi, S. Aoki, H. Sakai, N. Kihara, T. Sasaki, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **41**, 989 (1993).
11) a) M. Kobayashi, J. Tanaka, T. Katori, M. Matsuura, M. Yamashita, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **38**, 2409 (1990); b) M. Kobayashi, J. Tanaka, T. Katori, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **38**, 2960 (1990); c) M. Doi, T. Ishida, M. Kobayashi, and I. Kitagawa, *J. Org. Chem.*, **56**, 3629 (1991).
12) a) I. Kitagawa, N. K. Lee, M. Kobayashi, and H. Shibuya, *Tetrahedron*, **47**, 2169 (1991); b) M. Kobayashi, N. K. Lee, H. Shibuya, T. Momose, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **39**, 1177 (1991).
13) M. Kobayashi, K. Kawazoe, T. Katori, and I. Kitagawa, *Chem. Pharm. Bull. (Tokyo)*, **40**, 1773 (1992).
14) M. Ishibashi, R. E. Moore, G. M. L. Patterson, C. Xu, J. Clardy, *J. Org. Chem.*, **51**, 5300 (1986).
15) Identification of the bacterium is under way.
16) M. Kobayashi, H. Uehara, K. Matsunami, S. Aoki, and I. Kitagawa, submitted (1993).