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, metabolize 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. 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)], clavukerin C (2) or a cyclic peroxide moiety (found in marine sponge, vide infra). The other findings of interest are that some soft corals metabolize several sesquiterpenes having carbon frameworks antipodal to those isolated from terrestrial plants [e.g. valerenenol (3), ent-oplopanone (4)] and that the Okinawan stolonifer Clavularia viridis produces ent-prostanoid compounds [e.g. claviridenone A (5)]. It is also noted that a soft coral of Xenia sp. contains a pair of diastereomeric diterpenes [e.g. xeniolone...]

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[References and structures of compounds mentioned are included in the text and images.]

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and isozeniolone (7)\(^5\) 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 \(\text{1-oxaquinolizidine moieties linked with two oligomethylene chains thus forming the macrocyclic structures.}\)

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 carrying 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 peroxylketal structures. Through bioactivity-directed separations, we have isolated, from a marine sponge of *Plakortis* sp. collected at Zamami-jima, Okinawa Prefecture, antifungal peroxylketal acids and their methyl esters named peroxylakoric 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 peroxylketal acid mixture showed strong growth inhibition for *Candida tropicalis*.
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 SiO2 column and HPLC to provide five new compounds: peroxyplakoric acids A1 methyl ester (10)~B3 methyl ester (14). These peroxyketal acid methyl esters 10~14 were also obtained from the initial AcOEt soluble portion of this sponge in 0.8~3.4% yields, respectively.

In order to determine the absolute stereostructures of 10~14, the following conversions were carried out. At first, peroxyplakoric acid A3 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).3 Next, peroxyplakoric acid A3 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 (IC50 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) [IC50 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 SiO2 column chromatography and HPLC of the fr.B furnished significantly cytotoxic macrocles named altohyrtins A (19), B (20), and C (21), and 5-desacetyl-altohyrtin A (22) in 3.4x10-3%, 2.2x10-4%, 2.2x10-4%, and 2.1x10-3% yields from the AcOEt soluble portion, respectively.10 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 C63H95O21Cl by HR-FAB MS and NMR analysis. Ordinary acetylation (Ac2O/pyridine, r.t.) of 19 furnished the triacetate. Four partial structures (fragment A: C-1~C-6 and C-38~C-51, fragment B: C-7~C-14, fragment C: C-15~C-23, fragment D: C-24~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 13C NMR spectra of 19 taken in CD3OD. 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 d6-DMSO, which showed correlations between geminal hydroxyl and methine protons at C-25, 35, 38, 42, and 47. Six ring structures (A~F) and the connectivities of four partial structures (frag. A~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)
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.\(^{10b}\)

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

**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), 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, two new 3-keto-4-methylene steroids, theonellasterone and conicasterone, and a Diels-Alder type dimeric steroid, named bistheonellasterone. Among these swinholide analogs, swinholides A (23), B (24), and C (25) were shown to exhibit potent cytotoxic activities for KB cell lines (IC50 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 (IC50 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, which was isolated by Prof. Moore and his group in 1986 from the cultured terrestrial blue-green alga Scytonema pseudohoffmanni. 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.
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 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 1H and 13C 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.\(^6\)

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.

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 benzylation 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.
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 contributor to the constituents initially isolated from marine sponges.

REFERENCES
15) Identification of the bacterium is under way.