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Recent insights into natural venoms*

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Abstract: Toxic substances that occur in nature have various structures and functions. In fact, the very novelty of their structures and functions sometimes extends far beyond the realm of human imagination, and the capabilities of these compounds are still largely untapped despite the major advances of modern science. In this report we focus on the most recent developments in this field, with a particular emphasis on natural venoms, marine sunscreen, and marine huge molecules.

Keywords: arginine kinase-like protein; blarina toxin; cyclic peptides; halichondrin B; long carbon-chain polyols; marine sunscreen; natural venom; palytoxin.

INTRODUCTION

Diverse natural products [1–14] with extraordinary chemical structures and significant biological activities have been isolated and characterized from both marine and terrestrial organisms [15,16], and they have been studied for a long time [17]. Discovery of new compounds with potent activity can develop and even create scientific fields. Toxic substances that occur in nature have a variety of structures and functions. In fact, their novel structures and specificity of function sometimes extend far beyond the realm of human imagination, and the capabilities of these compounds are still largely untapped despite the major advances of modern science. In this report we focus on the most recent developments in this field, with a particular emphasis on natural venoms. The discovery of new basic compounds holds the key for the advancement of materials science. First, as specific and successful examples, we will describe the recent findings regarding palytoxin (**1**) and halichondrin B (**2**). Next, as examples of the power of these naturally occurring organic molecules, we will discuss (1) natural venoms, (2) marine sunscreen, and (3) marine huge molecules.

Palytoxin

In 2010, the Nobel Prize [18] in Chemistry was awarded to Richard F. Heck, Ei-ichi Negishi, and Akira Suzuki. In his Nobel Lecture presented in Sweden and entitled, "Cross-Coupling Reactions of Organoboranes: An Easy Way for Carbon-Carbon Binding", Suzuki pointed out that this reaction was

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used by Y. Kishi et al. in their synthesis of palytoxin-COOH, and the impact of this accomplishment was noted worldwide [19].

We determined the molecular structure of palytoxin (**1**) (Fig. 1) in 1981 [20] and 1982 [21]. It is considered to be a powerful marine toxin that is 50 times more toxic than tetrodotoxin found in puffer fish (fugu). The synthesis of this molecule was studied by Y. Kishi et al. at Harvard University, and at that time the C75–C76 olefin–olefin bond was formed using the Suzuki coupling reaction. This high-yield reaction between an alkenyl borane and an iodoalkene is completed in the presence of thallium hydroxide using a palladium catalyst. This discovery catapulted the Suzuki coupling reaction onto the world stage, and the existence of complex natural molecules serves as a prime example of how greatly this reaction has contributed to developments in the field of organic reactions. We have even heard that a model of this molecule is on display at the Nobel Museum in Stockholm.

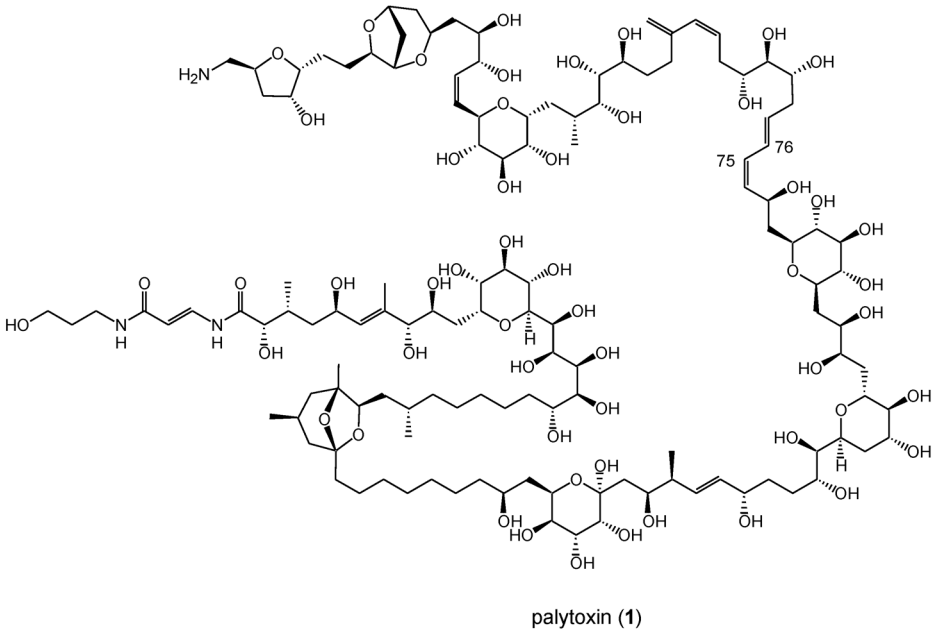


Fig. 1 Structure of palytoxin (**1**).

There was competition among groups at the University of Hawaii [22] and Nagoya University, and researchers at Harvard University to determine the chemical structure of palytoxin (**1**). The nature and function of this molecule with 64 stereocenters is still not fully understood. One question is whether there is a limit to the length of the long carbon chains [23,24] in such molecules, and another is the role of this molecule in vivo. The coelenterate *Palythoa tuberculosa* produces palytoxin (**1**), but its biosynthetic pathway has not been determined with great certainty. The molecule itself exists as a dimer in aqueous solution and is a powerful toxin that inhibits Na^+, K^+-ATPase [25–28]. Acylation of the amino terminus converts the dimer to a monomer, which reduces the toxicity to less than 1/100 that of the dimer. This was indeed a challenge because even the biggest natural product is much smaller than proteins, and to our knowledge this had never been tried before. Synchrotron radiation small-angle X-ray scattering (SAXS) was used at the SPring-8 synchrotron radiation machine [29,30]. Briefly, in aqueous solution, palytoxin has a horseshoe-like shape and exists as a dimer (Fig. 2). Interestingly, *N*-acetylpalytoxin exists as a monomer with a similar horseshoe-like shape, in which the amino group at the end of the molecule is acetylated, and its toxicity is less than 1/100 that of palytoxin.

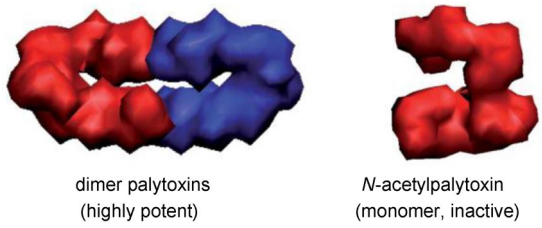
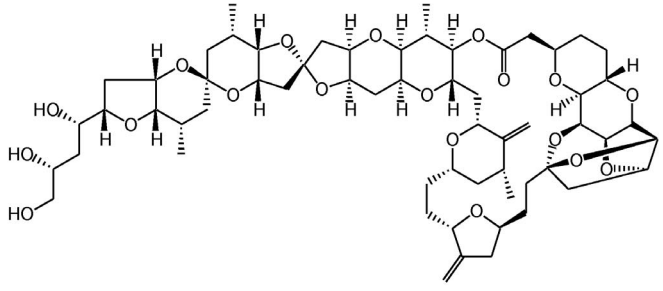


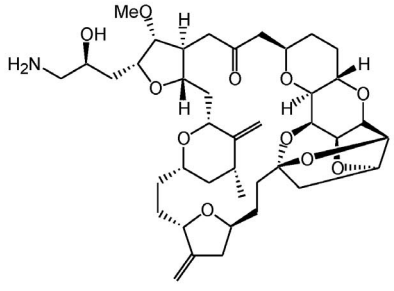
Fig. 2 Low-resolution 3D models of palytoxin and *N*-acetylpalytoxin in aqueous solution.

Halichondrin B

A similar long-carbon chain molecule has also been discovered in other marine organisms. The antitumor agent halichondrin B (**2**) was isolated from a black sponge (*Halichondria okadai*) (Fig. 3) [31,32]. A characteristic feature of this molecule is its ball-shaped polyether moiety in the right half moiety, 2,6,9-trioxatricyclo[3.3.2.$0^{3,7}$]decane. Halichondrin B (**2**) exhibits excellent antitumor activity in vivo and is expected to have extensive applications. M. Munro et al. attempted to produce enough of this molecule for clinical trials by culturing the sponges, but they did not succeed [33]. A group led by Y. Kishi completed the synthesis of the entire molecule [34]. The activity of synthetic intermediates was carefully tested at the Eisai Research Institute of Boston, which was headed by Y. Kishi at the time. From that research, the right half of the molecule was concluded to be the pharmacophore, and developmental work was conducted to fine-tune that moiety into the drug eribulin (**3**) [35].



halichondrin B (**2**)



eribulin (**3**)

Fig. 3 Structures of halichondrin B (**2**) and eribulin (**3**) and the black sponge *Halichondria okadai* in a tidal pool.

Finally, the U.S. Food and Drug Administration approved Halaven® (eribulin mesylate) for the treatment of metastatic breast cancer on 16 November 2010. Overall, 25 years had elapsed from the discovery of halichondrin B (**2**) until it was developed into a drug.

Let us consider the significance of the development of Halaven. First, the use of a 62-step chemical synthetic process to produce a molecule with 19 stereocenters as a pharmaceutical product is already quite an accomplishment. This demonstrates the rapid development of modern organic chemistry and the powerful impetus for making new drugs. Moreover, the rapid development of process chemistry that enables the supply of several kilograms a year is also apparent. An understanding of scientific facts and principles at an astonishingly high level is indispensable for drug discovery, and such processes should not be regarded simply as business endeavors [36].

Eribulin (**3**) is a microtubule dynamics inhibitor, and its mode of action differs from taxanes and vinblastine, which inhibit both the growth and the shortening of microtubules [37,38]. Interestingly, eribulin causes the nonproductive aggregation of tubulin.

NATURAL VENOMS

We will now focus on the most recent developments in the field of natural venoms. The toxic constituents produced by relatively lower animals have been well studied. Meanwhile, venomous mammals are extremely rare: only a few members of shrew and platypus have been demonstrated to produce toxic venom. However, owing to their instability as well as the difficulty of collecting fresh saliva and salivary gland specimens in sufficiently large amounts, their unique venoms have not been well investigated. The recent development of platypus and shrew venoms in this issue has been described by Dr. Masaki Kita [2,39–41].

Spider wasp venom

The female solitary spider wasp, a member of the spider wasp family Pompilidae, hunts spiders and lays her eggs on them, just as described in the classic *Souvenirs Entomologiques* (*Book of Insects* in English) by Jean-Henri Fabre (Fig. 4). The spiders are paralyzed by an anesthetic, and they remain alive without decomposing to become food for larvae that hatch in the nest. Fabre describes the spider wasp as an expert anesthesiologist. However, is this really a case of inserting the stinger into a nerve and administering an anesthetic? To answer this question, we have started research by collecting spider wasps [42].

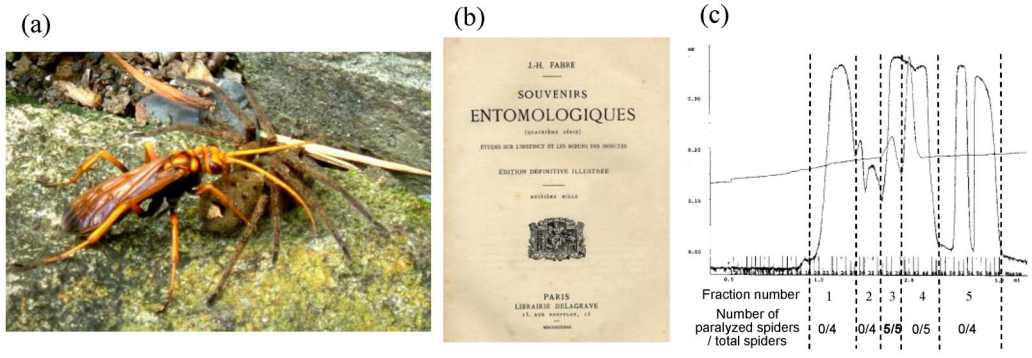


Fig. 4 (a) Spider wasp, *Cyphononyx dorsalis*; (b) Fabre's *Souvenirs Entomologiques*; (c) gel permeation chromatogram of *C. dorsalis* venom on a Superose PC 3.2/30 column.

As a characterizing feature of this paralysis, we noticed that when the spider is anesthetized, the joints of the legs lose their function entirely. Thus, the legs lose their strength and cannot be moved, but the pedipalps remain responsive and the spider is still alive. The spider is unable to move and dies in a few weeks. We collected the venom of 200 to 300 solitary spider wasps *Cyphononyx dorsalis* from June through early August. We placed the wasp on ice to inactivate it, and then removed the stinger and venom sac. We collected and stored 1 to 2 μl of venom from each individual. The important problems in this study were: (1) how to inject a microliter of venom into the prey, and (2) what to select as the targeted larva food. We solved problem 1 by devising a microliter syringe, and problem 2 by using only spiders. We injected 2 μL of crude venom into each spider. We observed a pronounced paralytic effect. A much greater effect was found than when water was used as a control. However, when crickets were injected instead of spiders, no such effect was observed.

We then decided to begin purifying the crude venom. The crude venom of 25 female wasps was separated by gel filtration and purified using a SMART System (Amersham Pharmacia Biotech Co.). The activity was concentrated in a single peak (Fig. 4c). The purified fraction was developed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and in-gel proteolytic digestion was then performed and mass spectrometry was used to analyze the results.

From mass spectrometry of the resulting lysate, we determined that this is an arginine kinase-like protein based on the structures of the fragments that were identified. Next, we cloned the arginine kinase-like protein from the solitary spider wasp. Following conventional methods, we extracted the total RNA, amplified DNA coding for arginine kinase by reverse transcriptase-polymerase chain reaction (RT-PCR), and successfully identified a 40-kDa amino acid sequence (Fig. 5). In Fig. 5, the underline parts in the sequence correspond to the parts identified by mass spectrometry. We next produced a recombinant arginine kinase using *E. coli*. Consequently, we obtained a target substance corresponding to the 27.9-kDa region from the first methionine to leucine 355. We identified the molecular weight and pI value of this substance using 2D electrophoresis. The recombinant arginine kinase purified by affinity chromatography expressed the characteristic paralyzing activity in spiders (Table 1). Interestingly, similar activity was found in an arginine kinase from sea cucumber *Stichopus japonica*. Arginine kinase is involved in energy supply by catalyzing the ATP buffering, but there had been no previous report for another function of arginine kinase, such as toxic or paralytic activity.

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  1 MVDQAVLDKL ESGYAKLAAS DSKSLLKKYL TKEIFDQLKT KKTSFGSTLL DVIQSGLENH DSGVGIYAPD
 71 AESYTVFADL FDPIIEDYHG GFKKTDKHPP KDFGDVDSMG NLDPAGEFIV STRVRCGRSL DGYPFNPCLT
141 EAQYKEMEEK VSSTLSGLEG ELKGTFYPLT GMSKEVQQKL IDDHFLFKEG DRFLQAANAC RFWPTGRGIF
211 HNDAKTFLVW CNEEDHLRII SMQMGGDLGQ VYRRLVNAVN EIEKRLPFSH NDRLGFLTFC PTNLGTTVRA
281 SVHIKVPKLA ANKAKLEEVA AKFNLQVRGT RGEHTEAEGG IYDISNKRRL GLTEYQAVKE MHDGIAELIK
351 IEKEL
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Fig. 5 Amino acid sequence of *C. dorsalis* arginine kinase determined by RT-PCR. The sequences determined by MS analysis are underlined. The first Met109 is boxed.

Table 1 Paralytic activity of recombinant protein of *C. dorsalis* arginine kinase and *S. japonica* arginine kinase.

	Protein weight (μg/unit) weight of spider (100 mg)	Number of paralyzed spider/total spiders
C. dorsalis arginine kinase	1–2.5	2/5
	3–4	3/5
S. japonica arginine kinase	10	3/5
Water (negative control)	–	0/5

As mentioned earlier, the interesting ecological phenomenon described in Fabre's *Book of Insects* has now been revealed. Further elucidation of the mechanism is still required, and we are currently undertaking this research.

Marine fireworm

Toxic marine annelids were first referred to in the literature as sea scolopendra in *de Materia* (A.D. 50) by Dioscorides, a physician of the Roman Empire. The marine animals that are commonly known as fireworms are dangerous to humans, since careless handling with bare hands can result in serious dermatitis. However, the actual toxic substance of these animals has remained unknown.

Specimens of *Eurythoa complanata* (225 g) were collected from a tidal wetland on the Awase beach in Okinawa. The fresh worms were extracted with 80 % aqueous ethanol. The concentrated extract was separated by bioassay-guided purification, using an inflammation-inducing bioassay. The inflammatory activities were evaluated by measuring the thickness of the footpad of mice over time compared to a negative control. Finally, we obtained new compounds, complanine (**4**) and neocomplanines A (**5**) and B (**6**) (Fig. 6) [43,44].

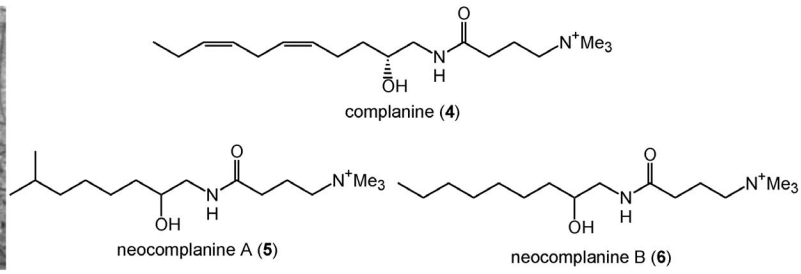
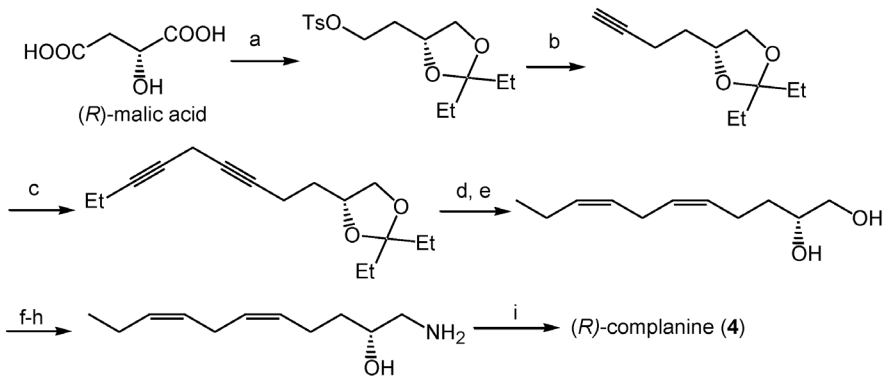


Fig. 6 Polycheate worm *Eurythoe complanata* and structures of complanines.

The novel trimethylammonium cationic structure could be characterized by an unsaturated carbon chain and an amino alcohol *N*-acylated with a GABA derivative. The stereocenter of complanine was elucidated by synthesis of the natural enantiomer to have an *R* configuration, as depicted in Scheme 1. Mechanism of action has been clarified that complanine activates protein kinase C (PKC) in the presence of tiaprofenic acid (TPA), which induce various inflammatory chemical signals such as TNF-α or IL-1α.



Scheme 1 Total synthesis of complanine. Keys: (a) 1. BH_3·SMe_2 (71 %); 2. cat. TsOH, Et_2CO (59 %); 3. TsCl, pyridine (80 %); (b) lithium acetylide ethylenediamine complex (1.2 equiv), DMSO, rt, 3 h (51 %); (c) 1-iodopent-2-yne (2.0 equiv), EtMgBr (1.6 equiv), CuI (cat.), THF, 0 °C to rt, 12 h (43 %); (d) H_2, Lindlar catalyst, EtOH, rt, 30 min; (e) AcOH, H_2O, rt, 12 h (43 % in 2 steps); (f) MsCl (1.1 equiv), pyridine, CH_2Cl_2, 0 °C, 2 h; (g) NaN_3 (4.0 equiv), DMF, 80 °C, 11 h (79 % in 2 steps); (h) PPh_3 (1.0 equiv), THF, H_2O, rt, 12 h (78 %); (i) *N*-[4-(trimethylammonio)butyryloxy]succinimide iodide (2.0 equiv), MeOH, rt, 18 h (44 %).

MARINE SUNSCREEN

The bodies of marine organisms are constantly exposed to strong UV radiation. How they avoid getting sunburned is of great interest. Since about 1974, we have been interested in biologically active substances in marine organisms and have continued to work in this area. We pursued novel and unexpected phenomena found in environments that are very different from those found in land organisms, particularly those that are harsh in that they have a high water pressure, UV radiation, and are carbon-poor. This research trend started at the beginning of the 1970s when focus shifted from mass chemicals to fine chemicals owing to the oil crisis. Large amounts of prostanoids could be obtained from coral, so most chemical companies became involved, and this area has remained a topic of research ever since.

UV light in the range of 280 to 400 nm is the most dangerous, and this is the range that causes sunburn or suntan. More specifically, it is known that this UV light causes considerable damage to genes, etc., and therefore it is not merely a cause of inflammation, but actually poses a major problem for living creatures. This phenomenon affects not only marine organisms, but also molds and related organisms. The Italian pigment researcher Prof. Giuseppe Prota (Napoli) was interested in substances with a maximum absorbance at 310 nm, and although he actively conducted research on those substances, he did not arrive at a conclusion. At that time, we were performing our research on palytoxin, the venom produced by *Palythoa tuberculosa*, and because we were seeking substances with a maximum absorbance of 310 nm, we began research on suitable structures.

As a result, we first determined the structure of Mycosporine-Gly (**7**), a substance that absorbs UV light at 310 nm [45]. This was identified by Dr. S. Ito, who had worked for two years under Prota as post-doctorate researcher. Subsequently, a series of UV-absorbing substances named palithine (**8**), palythinol (**9**), and polythene (**11**) were isolated (Fig. 7) [46–48].

The 360 nm-absorbing substance polythene forms pearl-yellow crystals, and its structure was determined by X-ray crystallography. In addition, Porphyra-334 (**10**) was isolated from the red alga *Porphyra tenera*, an edible seaweed that is also known as Asakusa laver [49]. These findings were published from 1977 to 1979. Recently, the presence of palythine was identified in the Papuan jellyfish *Mastigias papua*, and interestingly this pigment is present in the body of the jellyfish, rather than in its symbiotic dinoflagellate algae [50].

A considerable amount of research has been conducted on the significance of the existence of this group of molecules in the bodies of organisms. In living organisms, the biosynthesis of these molecules

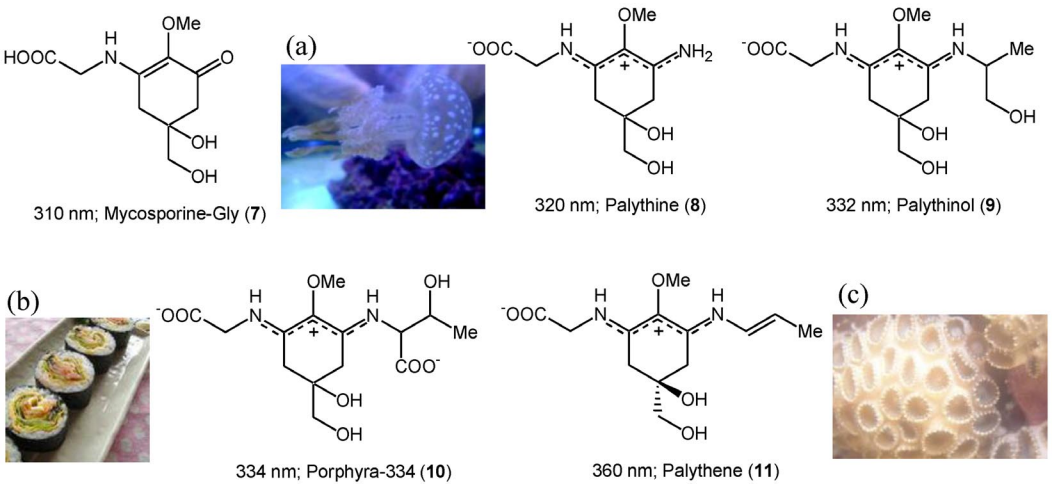


Fig. 7 Compounds that exhibit characteristic UV absorption at 310–360 nm and pictures of marine organisms that produce sunscreen: (a) a jellyfish *Mastigas papua*; (b) dry *Porphyra tenera* ("Asakusa" Laver); (c) *Palythoa tuberculosa*.

appears to proceed dependent on the amount of light, and thus these molecules can be called sunscreen molecules [51].

The biosynthetic genes for these molecules have been identified and their pathways reported. A group at Harvard University led by C. T. Walsh found that the biosynthetic pathway of mycosporine-glycine (**7**) led from sedoheptulose 7-phosphate (**12**) to 4-deoxygadusol (**13**), which is followed by the addition of glycine (**14**) (Fig. 8) [52]. In addition, a pathway was identified in which serine (**15**) is added

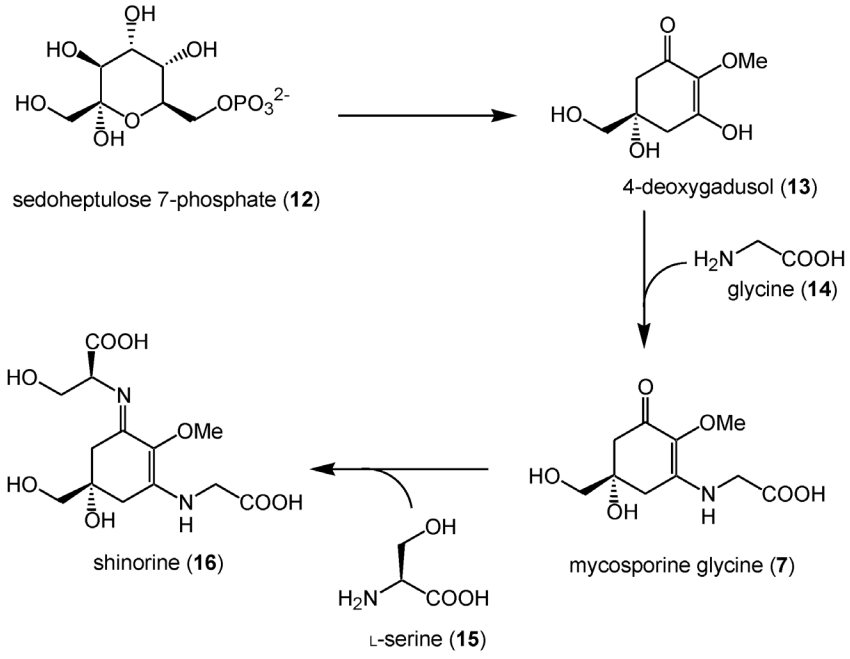


Fig. 8 Biosynthetic pathway for the assembly of shinorine (**16**) from sedoheptulose-7-phosphate (**12**).

to mycosporine-glycine (**7**) to produce the *O*-acyl form, and after spirocyclization it undergoes transformation to an amino group to arrive at the shinorine (**16**) molecule, for example.

It should be noted that this system is also present in the recently identified genes of coral, and it can be hypothesized that corals also synthesize UV-absorbing molecules [53].

MARINE HUGE MOLECULES

In marine ecology, we must consider symbiotic microorganisms such as dinoflagellates and blue–green algae (cyanobacteria), since they are suspected to be the real producers of marine toxins. Here we will describe dinoflagellate metabolites and blue–green algae-originated cyclic peptides.

Symbiotic dinoflagellates

Marine huge polyol and polyether compounds are remarkable molecules owing to their extraordinary structures and significant biological activities. Palytoxin (**1**) (2680 Da) and maitotoxin (3422 Da) [54] are currently believed to have the longest carbon chains in nature (more than 100 Å in length), except for biopolymers. Generally, marine huge molecules can be categorized as a polyketide metabolites without a repeating unit, which makes it difficult to elucidate their structures. During our quest to identify super-carbon-chain compounds (SCCs) from marine dinoflagellates, we found a group of huge molecules detected by matrix-assisted laser desorption/ionization with time-of-flight (MALDI-TOF) mass spectrometry (Table 2). After several extensive attempts, huge molecules were generally detected by linear positive with a matrix α-cyano-4-hydroxycinnamic acid (α-CHCA) as the best conditions thus far for showing very unstable and low-intensity huge masses.

Table 2 Huge molecules from symbiotic dinoflagellates and their hosts.

No.	Host	Estimated molecular weight (*m*/*z*) > 2000 daltons (in Dalton)*
1	Flatworm	2465, 2898, 3249, 3487, 4977, 5202
2	Nudibranch	2250, 2465, 2928, 3208, 4076, 4317
3	Zoanthid, *Palythoa* sp.	2200, 2642, 2973, 4000, 4304, 4330, 5389, 8245
4	Zoanthid, *Palythoa* sp.	Cluster 2000–2750, 3250, 3442, 4327, 5215, 5796
5	Zoanthid, *Palythoa* sp.	3101, 3292, 3631, 3957, 4246, 4346, 5198, 5383, 6026
6	Flatworm	2353, 2458, 4370, 4815, 5815, 6176

*Molecular weight was measured by MALDI-TOF using α-CHCA as a matrix.

As shown in Table 2, a marine dinoflagellate obtained from the zoanthid *Palythoa* sp. was shown to contain the largest molecule (8245 Da) so far. Purification of 8245 as a minor compound was hampered by instability of the molecule during separation, and further advances in separation methods are needed to isolate this unusual molecule. Huge polyol and polyether compounds larger than 2000 Da are considered to be mid-size molecules that fall between small (drug-like) natural products and biopolymers, and further studies on their 3D structures and dynamics should contribute to the creation of the new scientific fields.

New degradation reaction of marine huge molecules with Grubbs catalyst

Very recently, we reported the Grubbs II complex-mediated specific oxidation of allyl *vic*-diols to the corresponding aldehydes [55,56]. This oxidative *vic*-diol cleavage protocol is highly useful because only *vic*-diols with an allyl-alcohol moiety are oxidized, while dialkyl *vic*-diols are not consumed during the reaction. We have demonstrated that Grubbs II complex (**19**) is a suitable catalyst for the stoichiometric oxidation of diallyl *vic*-diol **17** to the corresponding aldehyde **18** in higher activity (94 %

yield) (eq. 1) than other Ru complexes such as $RuCl_3$, RuO_2, $RuCl_2(PPh_3)_3$, and Grubbs I complexes [57]. The major drawback of this reaction is that a stoichiometric amount of expensive Grubbs II complex must be used to achieve high efficiency (Fig. 9).

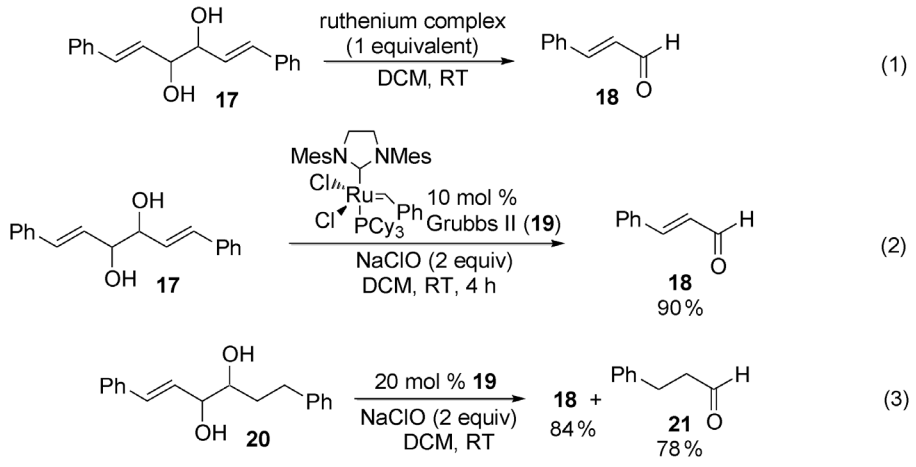


Fig. 9 Degradation reactions using Ru complex.

As a working hypothesis for oxidative cleavage of the C–C bond at an allyl *vic*-moiety in a catalytic manner, we considered the reaction pathway. The initial step involves ligand-exchange between the Grubbs II complex and the allyl *vic*-alcohol. In the second step, the diol is oxidized to the corresponding aldehydes and the Ru complex is reduced to Ru(II) species. Oxidation of Ru(II) species with an oxidant regenerates Ru(IV) species.

We chose **17** as a test substrate for the screening of co-oxidants in the Grubbs II complex-catalyzed oxidation. The reaction of **17** with a co-oxidant was carried out in the presence of 10 mol % Grubbs II catalyst (**19**) in dichloromethane at room temperature. The results showed that NaClO functioned as a good co-oxidant and led to **18** in high yield (90 %) compared to other oxidants (eq. 2). Under similar reaction conditions, except for the catalyst loading (20 mol %), mono-allyl *vic*-diol **20** was also oxidized to give the corresponding aldehydes **18** and **21** in respective yields of 84 and 78 % (eq. 3). On the other hand, dialkyl *vic*-diol was completely inactive under these reaction conditions. These results indicate that at least one allyl *vic*-diol substructure is essential for this Grubbs II complex-catalyzed oxidative cleavage reaction.

Two huge marine molecules such as SCCs [58], symbiodinolide, and *N*-*p*-BrBz-substituted palytoxin, contain allyl *vic*-diol and multiple *vic*-diol moieties and have been used as substrates to validate the practicability of the Grubbs II complex-catalyzed specific oxidation of allyl *vic*-diols using NaClO. A seco ester of symbiodinolide was first obtained by methanolysis, and the catalytic cleavage of seco-symbiodinolide (**22**) was then performed in the presence of **19** (10 mol %) with NaClO (7 equiv) in MeOH at room temperature for 4 h. The corresponding α,β,γ,δ-unsaturated aldehyde **23** (C1–C13 fragment) and α,β-unsaturated aldehyde **24** (C14–C25' fragment) were obtained in respective yields of 86 and 76 % owing to the selective cleavage of (*E*)-diallyl *vic*-diol (Fig. 10). The relative stereochemistries at C5, C6, and C7 of the C1–C13 fragment were assigned to be 5*R**, 6*R**, and 7*S** on the basis of the Universal NMR Database approach [59].

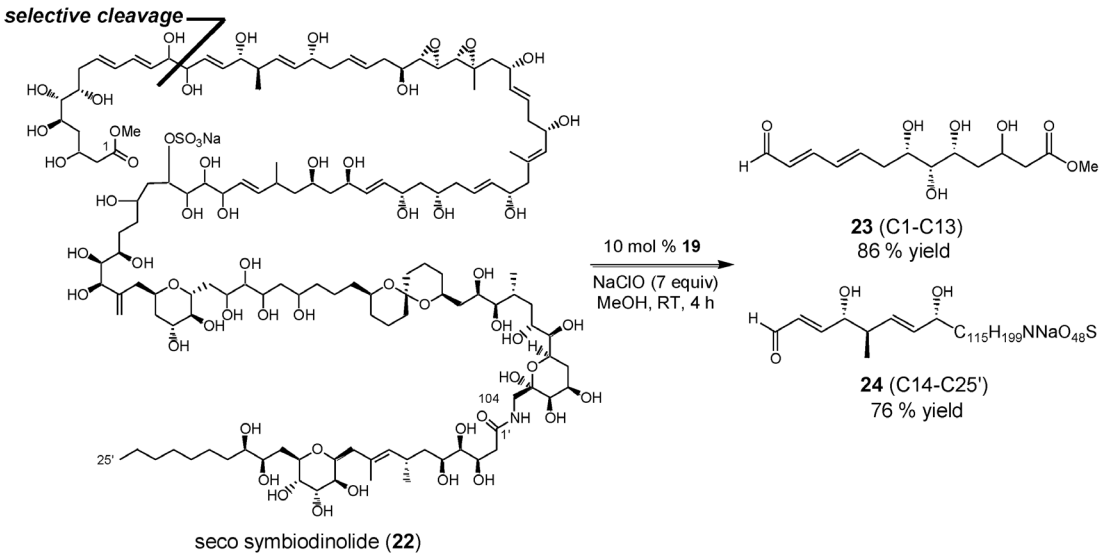


Fig. 10 Selective cleavage of seco-symbiodinolide (**22**).

In addition, a (*Z*)-monoallyl *anti*-diol substructure in *N*-*p*-BrBz palytoxin (**25**) was selectively cleaved in the presence of **19** (50 mol %) and NaClO (6 equiv) in MeOH at room temperature, and two degradation products, *N*-*p*-bromobenzoyl aldehyde **26** [61,62] and α,β-unsaturated aldehyde **27** (Cf–C100 fragment), were obtained (Fig. 11). These results indicate that Grubbs II complex-catalyzed selective cleavage using NaClO might be suitable for the conversion of both *E*- and *Z*-allyl *vic*-diols to aldehydes in natural products, especially large polyol compounds for stereostructural analysis, since multiple *vic*-diol moieties could not be cleaved during the reaction.

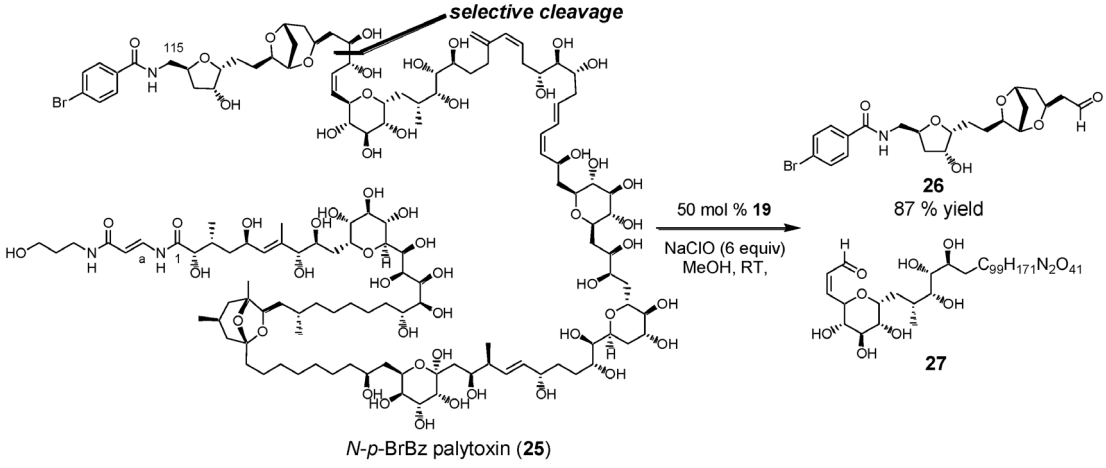


Fig. 11 Structures of *N*-*p*-BrBz palytoxin (**25**) and degradation products of *N*-*p*-BrBz palytoxin.

Another example of a diol cleavage reaction involves pseurotin A (**28**), which is a fungal metabolite with a diallyl-diol moiety [63,64], and gave the expected aldehyde **29** in >90 % yield without any side products (Fig. 12).

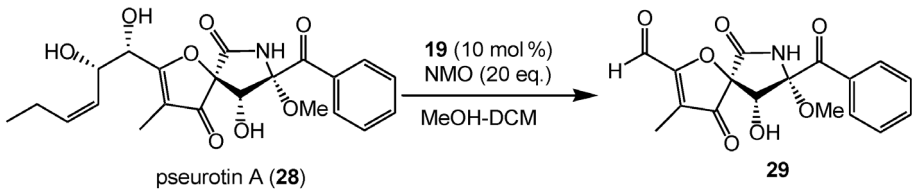


Fig. 12 Selective cleavage of pseurotin A.

For the present catalytic cleavage of allyl *vic*-diols using NaClO, we propose the reaction pathway shown in Fig. 13. Ru(II) species **C** was oxidized by NaClO (or some other co-oxidant) to give the oxoruthenium Ru(IV) species **A**. Ligand-exchange occurs between **A** and allyl *vic*-diol **B**, and the diol is then oxidized to the corresponding aldehydes and water. The Ru complex is reduced to Ru(II) species **C**, thereby completing the catalytic cycle.

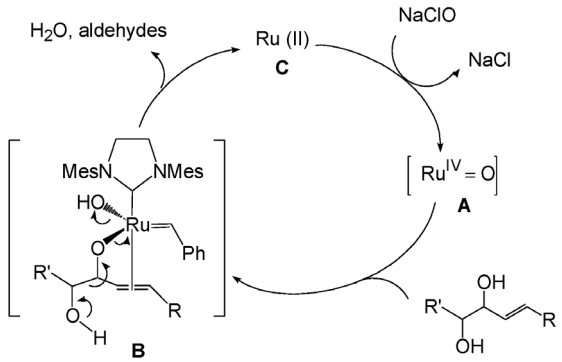


Fig. 13 Proposed reaction pathway for the catalytic cleavage of allyl *vic*-diols using NaClO.

In summary, we have developed a new efficient method for the Grubbs II complex-catalyzed oxidative cleavage of *E*- and *Z*-allyl *vic*-diol using NaClO or some other co-oxidant under very mild reaction conditions. We demonstrated the utility of this catalytic cleavage for the first time using two huge marine molecules, symbiodinolide and *N*-*p*-BrBz palytoxin. This reaction is highly useful for stereostructural analysis in natural products chemistry, especially in polyene alcohol compounds, since this cleavage reaction gives only simple products; i.e., while *vic*-diols with an allyl-alcohol moiety are oxidized, dialkyl *vic*-diol (e.g., triol or tetraol) in the same molecule is not [57].

Structure of new marine huge molecule, amdigenol A

Many species of microalgae adhere to the surface of the marine red alga *Digenea simplex*. We isolated and cultured some species of the dinoflagellates from the Okinawan *Digenea simplex*, and one of them, *Amphidinium* sp., was found to produce some huge compounds of more than 2000 mass units by MALDI or electrospray ionization-mass spectrometry (ESI-MS) measurements in seawater medium after culture of the dinoflagellate. Accordingly, we separated the medium, and a novel polyol compound with a molecular weight of 2169 mass units, amdigenol A (**30**) (Fig. 14) [65], was isolated. The structural elucidation of amdigenol A was performed by a combination of 2D NMR spectroscopic analyses, chemical degradation with a Grubbs catalyst and ESI-MS/MS analyses.

The dinoflagellate *Amphidinium* sp. was cultured in seawater medium. After two months of culture, the dinoflagellate was removed from the seawater medium by filtration. The seawater medium

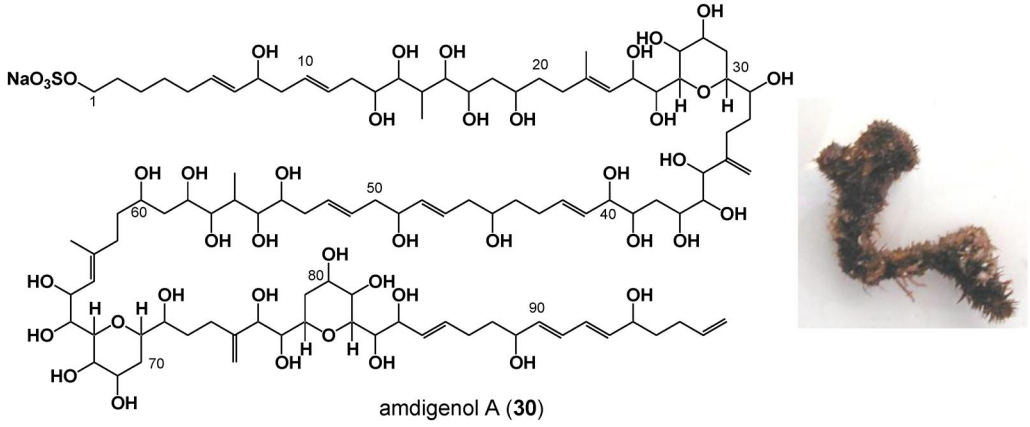


Fig. 14 The planar structure of amdigenol A (**30**) and the red alga *Digenea simplex*.

(120 L) was separated by column chromatography with TSK G-3000S polystyrene gel, DEAE-Sephadex, and Sephadex LH-20. Finally, purification by continuous reversed-phase high-performance liquid chromatography (HPLC) gave amdigenol A (**30**) (5.0 mg). The same procedure gave only 0.4 mg of amdigenol A (**30**) from the ethanol extract of the dinoflagellate.

The molecular formula of amdigenol A (**30**) was found to be $C_{104}H_{177}NaO_{43}S$ (*m/z* 2192.1187 for $[M+Na]^+$, *m/z* 2146.1389 for $[M–Na]^–$) by ESI-MS. From the $[M–SO_3Na+Na]^+$ (*m/z* 2189) ion peak in MALDI-TOF-MS, the structure was believed to contain one sulfate ester group. ^{13}C NMR spectra showed only 78 carbon signals, and some signals had a relatively high intensity. Therefore, we estimated that some of the same partial structures were present in this compound. 2D NMR spectra gave the three partial structures, C1–C13, C14–C54, and C55–C98. ^1H NMR integration and ^{13}C NMR signal intensity allowed us to surmise that the partial structures C12–C13, C15–C21, and C23–C31 were also present as C53–C54, C56–C62, and C64–C72, respectively. However, the linkages between the three partial structures were not clarified.

The linkages between the three partial structures were determined by the segment structure obtained by the degradation of amdigenol A (**30**). Olefin cross-metathesis is an effective method for the degradation of natural products [66–69]. In addition, degradation with a Grubbs catalyst occurs in parallel with the cleavage of allyl *vic*-diols [70,71]. The degradation reaction of amdigenol A (**30**) with the second-generation Hoveyda–Grubbs catalyst [72] gave many segments and one of them, segment **31**, was purified (Fig. 15). From the carbon skeleton and the oxidized positions, the C11'–C24' or

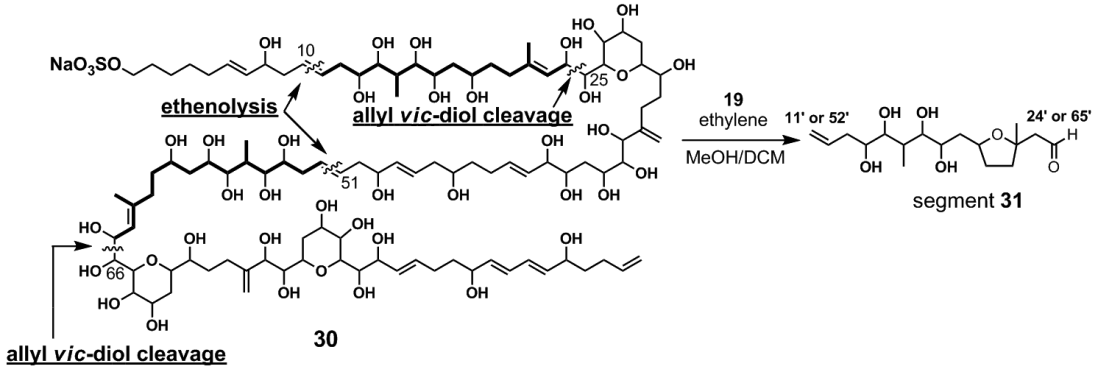


Fig. 15. Degradation of amdigenol A (**30**) with the second-generation Hoveyda–Grubbs catalyst.

C52'–C65' part of segment **31** was estimated to correspond with the C11–C24 or C52–C65 part of amdigenol A (**30**). The tetrahydrofuran of segment **31** was formed by cyclization of the hydroxyl group at the C19 or C60 position and the α,β-unsaturated aldehyde generated from cleavage of the C24–C25 or C65–C66 bond. Therefore, the partial structures C11–C14 or C52–C55 of amdigenol A (**30**) were determined the same as C11'–C14' or C52'–C55' of segment **31**. Based on this result, the C_{98}-linear carbon skeleton of amdigenol A (**30**) was determined to be as shown in Fig. 14.

The position of the sulfated oxygen was estimated and structure was confirmed by a tandem mass spectrometric (MS/MS) analysis. Fragmentation patterns could be interpreted based on the characteristic fragmentation for the sulfate ester group at C1 (Fig. 16). Accordingly, the remaining oxymethine carbons must contain hydroxyl groups. Based on these results, the planar structure of amdigenol A (**30**) is shown in Fig. 14.

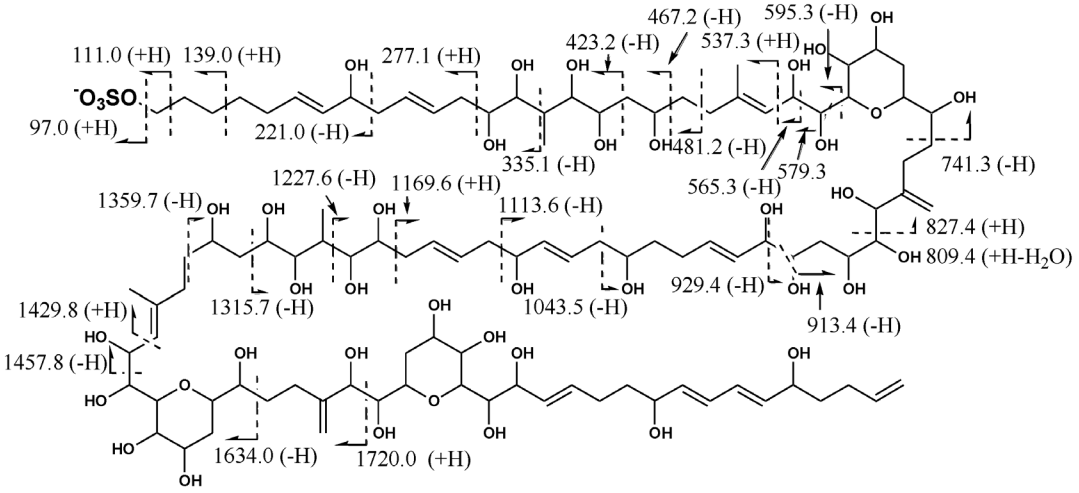


Fig. 16 The fragmentation patterns in MS/MS analyses of amdigenol A (**30**). Cleavage positions estimated only from the intense fragment ion peaks are illustrated.

Amdigenol A (**30**) has partial structures similar to those seen in amphidinol [73,74] analogs. Amphidinol analogs consist of a core part that includes a trisubstituted olefin, an *exo*-olefin and bis-tetrahydropyrans, and two long linear side chains. In amdigenol A (**30**), the C63–C82 part corresponds to a core part and C22–C41 would also correspond to the situation if the C37 hydroxyl group attacks the C41 carbon to produce a ring ether. Therefore, amdigenol A (**30**) is likely formed linearly by two amphidinol analogs. The sulfate ester terminal side chain of amdigenol A (**30**) is the same as that of lingshuiol B [75], luteophanol A [76], and symbiopolyol [77], while the olefin terminal side chain of amdigenol A (**30**) is the same as that of luteophanol D [78].

Amdigenol A (**30**) showed weak cytotoxicity against 3T3-L1 murine adipocytes (IC_{50} = 59 μg/mL). The antimicrobial activity was not examined. Further studies on the stereochemical analyses, the biosynthetic pathway and the mechanism of biological activities, including the relevance to those of the amphidinol analogs, are underway.

Cyclic peptides from cyanobacteria

Cyclic peptides are a major group of natural products isolated from marine and land micro-organisms. These metabolites are produced by the non-ribosomal peptide synthetase (NRPS) process and frequently include various nonproteinogenic amino acids in their structures. These cyclic peptides

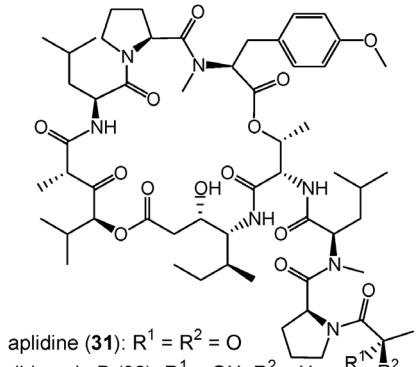
sometimes show strong biological activities, and are used as lead compounds for the development of antitumor drugs [79]. One recent example is aplidine (**31**).

Aplidine (**31**), which was isolated from the sea squirt *Aplidium albicans*, is a member of a class of cyclic depsipeptides, the didemnins (ex. **32**), from *Tridemnum solidum* [80,81]. Aplidine (**31**) showed potent cytotoxicity against tumor cells through a mechanism of activation of apoptosis and interruption of the cell cycle at G1-G2. Based on this powerful biological activity, aplidine (**31**) is under clinical investigation for the treatment of multiple myeloma in Europe and the United States.

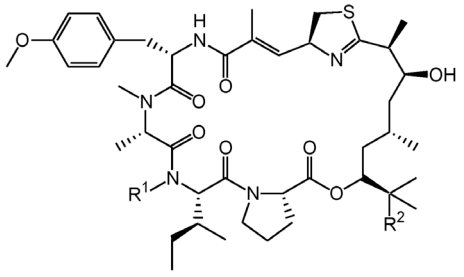
Cyanobacteria are photosynthetic prokaryotes that are widely distributed throughout marine and terrestrial environments (Fig. 17). Members of the marine cyanobacteria genus *Lyngbya* are known to produce structurally interesting and biologically active secondary metabolites. Typically, these metabolites can be divided into linear/cyclic peptides and depsipeptides which can exhibit potent cytotoxicity.

The cyanobacteria *Lyngbya* sp. was found to overgrow corals at Ishigaki Island. Apratoxin A (**33**) [82] was isolated as the main toxic compound from this alga. Apratoxins (**33–35**) were originally isolated from *Lyngbya majuscula* in Guam and Palau, which is known to cause swimmers' itch [83]. Apratoxin A has a macrocyclic structure with parts of amino acids and a polyketide unit, and shows potent cytotoxicity against P388 cells an IC_{50} value of 0.3 nM.

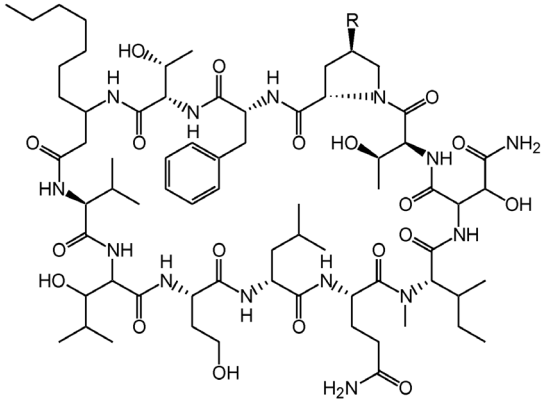
Another *Lyngbya* sp. was also collected at Ishigaki Island, and the aqueous ethanol extract of this alga showed strong cytotoxicity against tumor cells. Two new peptides, lyngbyacyclamides A (**36**) and B (**37**), were isolated via a guided bioassay [84]. The structures of these cyclic peptides were determined by spectroscopic analyses and degradation reactions. The amino acid residues of lyngbyacyclamides A (**36**) were assigned to proteinogenic L-amino acids, such as a Val, two Thr, a Pro, a Gln, and unusual amino acids including a D-Phe, a D-Leu, an *N*-methyl Ile, β-hydroxy leucine and asparagine, a homoserine and a β-amino decanoic acid. The structure of lyngbyacyclamides B (**37**) differs from that of lyngbyacyclamides A (**36**) only at the 4-hydroxyl proline. The biological activities of lyngbyacyclamides were examined with regard to cytotoxicity against B16 cells and toxicity against brine shrimp. After incubation, lyngbyacyclamides A (**36**) and B (**37**) showed an IC_{50} of 0.7 μM against B16 cells. However, they did not show definite toxicity against brine shrimp even at 70 μM. This result suggests that lyngbyacyclamides may have a unique mode of action and could contribute to the discovery of new drugs. The structures of lyngbyacyclamides resemble those of the natural products laxaphycins (**38**, **40**, **41**) [85,86] and lobocyclamides (**39**, **42**, **43**) [87]. Laxaphycins were originally isolated as major products from an antifungal extract of the cyanobacterium *Anabaena laxa* and *Lynbgya majuscule*. While the isolated laxaphycins did not show antifungal effect, laxaphycin B, in particular, showed strong cytotoxicity against three cell lines, lymphoblast cell line CCRF-CEM (IC_{50} = 1.1 μM), the vinblastine-resistant subline CEM/VLB100 (IC_{50} = 1.0 μM), and the subline CEM/VM-1 (IC_{50} = 1.4 μM). Interestingly, laxaphycin A showed weak cytotoxicity against these cell lines with IC_{50} values of over 20 μM. This difference may suggest a need for studies on the structure–activity relationships or the mode of action.



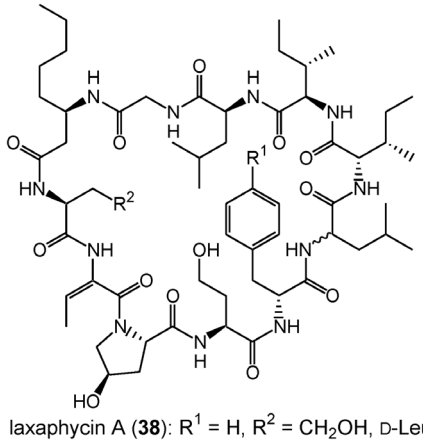
aplidine (**31**): $R^1 = R^2 = O$
didemnin B (**32**): $R^1 = OH$, $R^2 = H$



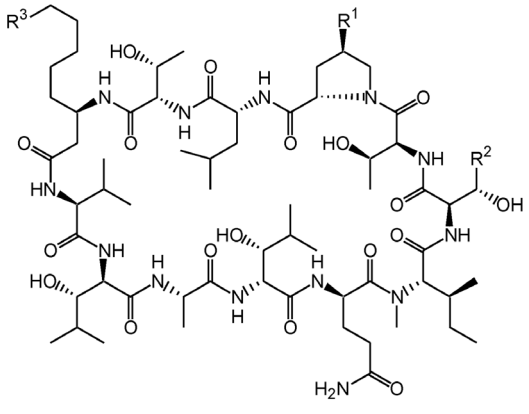
apratoxin A (**33**): $R^1 = CH_3$, $R^2 = CH_3$
apratoxin B (**34**): $R^1 = H$, $R^2 = CH_3$
apratoxin C (**35**): $R^1 = CH_3$, $R^2 = H$



lyngbyacyclamide A (**36**): R = H
lyngbyacyclamide B (**37**): R = OH



laxaphycin A (**38**): $R^1 = H$, $R^2 = CH_2OH$, D-Leu
lobocyclamide A (**39**): $R^1 = OH$, $R^2 = OH$, L-Leu



laxaphycin B (**40**): $R^1 = H$, $R^2 = CONH_2$, $R^3 = CH_2CH_3$
laxaphycin D (**41**): $R^1 = H$, $R^2 = CONH_2$, $R^3 = H$
lobocyclamide B (**42**): $R^1 = OH$, $R^2 = CH_2OH$, $R^3 = CH_2CH_3$
lobocyclamide C (**43**): $R^1 = OH$, $R^2 = CH_2OH$, $R^3 = H$

Fig. 17 Cytotoxic compounds from marine cyanobacteria.

SUMMARY

As exemplified by the compounds described here, there are still many intriguing organic molecules in nature. By identifying their specific structures and various functions, we can expect to contribute greatly to the emergence of a novel and astonishing new field of science. Following the compass of postgenome research, we must be prepared for new conceptual directions in the chemistry of natural substances, and we must extend our research consistently along with the field of drug discovery. Research in this field is also related to progress in chemical biology, and even greater developments in that field can be expected.

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