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Recent aspects of chemical ecology: Natural toxins, coral communities, and symbiotic relationships*

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Abstract: The discovery of new ecologically active compounds often triggers the development of basic scientific concepts in the field of biological sciences, since such compounds have direct physiological and behavioral effects on other living organisms. We have focused on the identification of natural key compounds that control biologically and physiologically intriguing phenomena. We describe three recent aspects of chemical ecology that we have investigated: natural toxins, coral communities, and symbiotic relationships. Blarina toxin (BLTX) is a lethal mammalian venom that was isolated from the short-tailed shrew. Duck-billed platypus venom shows potent Ca^{2+} influx in neuroblastoma cells. The venom of the solitary wasp contains arginine kinase-like protein and is used to paralyze its prey to feed its larva. The ecological behaviors of corals are controlled by combinations of small molecules. The polyol compound symbiodinolide may serve as a defense substance for symbiotic dinoflagellates to prevent digestion of their host animals. These compounds reveal the wonder of nature, in both terrestrial and marine ecological systems.

Keywords: chemical ecology; natural toxins; marine natural products; coral larval metamorphosis; symbiosis; secondary metabolites.

INTRODUCTION

Many kinds of natural products with extraordinary structures and significant biological activities have been isolated from both marine and terrestrial organisms and characterized. The discovery of such new ecologically active compounds often triggers the development of basic scientific concepts in the field of biological sciences, since these compounds have direct physiological and behavioral effects on other living organisms. Chemical ecology is particularly important among social insects, such as ants, bees, and wasps, as a tool for communication. Defensive substances and deadly weapons, which can protect against predators or obtain more food for survival, have also been well studied. Meanwhile, researchers

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in marine chemical ecology have been attracted to reproductive systems as well as predator–prey and competitive interactions, settlement cues, and substances that defend against infection by microorganisms [1].

We have focused on the identification of natural key compounds that control biologically and physiologically intriguing phenomena [2–11]. The discovery of new bioactive molecules, facilitated by a deeper understanding of nature, will advance our knowledge of biological processes and lead to new strategies to treat disease. Recent technological advancements including spectroscopic analyses and genetic approaches have provided outstanding opportunities for new discoveries, even in the case of scarce, unstable, and composite compounds. We describe here three recent aspects of chemical ecology that we have investigated: natural toxins, coral communities, and symbiotic relationships.

NATURAL TOXINS

Among natural products, the toxic constituents produced by living organisms, commonly referred to as "venom", have attracted wide attention due to their potent activity and innovative modes of action. In addition to relatively small marine natural toxins such as tetrodotoxin, ciguatoxin, and palytoxin, many types of poisonous proteins have been identified in several species equipped with deadly weapons. Not only organisms such as pathogenic bacteria, plants, insects, and spiders, but also some vertebrates, including reptiles, fish, and a few mammals, produce toxins [12].

The toxic constituents produced by relatively lower animals have been well studied. Several birds endemic to New Guinea (genera *Pitohui* and *Ifrita*) have also been shown to use the steroidal alkaloid toxins homobatrachotoxin and batrachotoxin as a defense [13,14]. Meanwhile, venomous mammals are rare; only a few members of the order Insectivora (shrew and solenodon) and Monotremata (platypus) have been demonstrated to produce toxic venom. However, due 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.

Blarina toxin (BLTX): A mammalian lethal venom from the American short-tailed shrew *Blarina brevicauda*

Among Insectivora, only four species have been demonstrated to produce toxic compounds, including the Haitian solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*), the Mediterranean shrew (*N. anomalous*), and the American short-tailed shrew (*Blarina brevicauda*). In 1942, extracts of the salivary glands of *B. brevicauda* were first scientifically shown to be toxic to mice, rabbits, and cats [15]. Despite its relatively small body length (75–105 mm), the extract from the salivary glands of only one *Blarina* individual, when administered by intravenous (iv) injection, was shown to be capable of killing 200 mice weighing the same amount as the shrew itself [16,17]. Accounts of humans bitten by *Blarina* describe a local burning sensation around the tooth puncture marks and subsequent swelling.

Shrews have been characterized as having one of the highest metabolic rates among mammals [18]. For example, the common shrew *Sorex araneus* requires 80–90 % of its body weight (~8 g) in food daily, and thus it requires approximately 100 worms measuring 1 cm in body length per day. They feed on a wide variety of common invertebrates, particularly earthworms, spiders, and insect larvae. Meanwhile, venomous shrews are known to also eat vertebrates, even those larger than themselves. The short-tailed shrew *B. brevicauda* feeds on murid rodents and frogs. The semi-aquatic shrew *Neomys fodiens* feeds on frogs, newts, and small fish. Therefore, these shrew species may use venom to effectively catch and immobilize their relatively large prey to meet their own high metabolic demands [19,20].

Due to the interesting ecology of shrews, we have studied toxic substances from the shrew *B. brevicauda*. Recently, a novel lethal mammalian venom, BLTX, has been purified from its submaxillary

and sublingual glands (salivary glands) (Fig. 1) [21]. The LD_{50} value of BLTX against mice was approximately 1 mg/kg. Mice that were given BLTX intraperitoneally developed irregular respiration, paralysis, and convulsions before dying. BLTX was relatively unstable, and lost its proteolytic activity at room temperature or under basic conditions (pH >7). These properties indicate that the lethal toxicity of BLTX can be degraded by its own proteolytic activities.

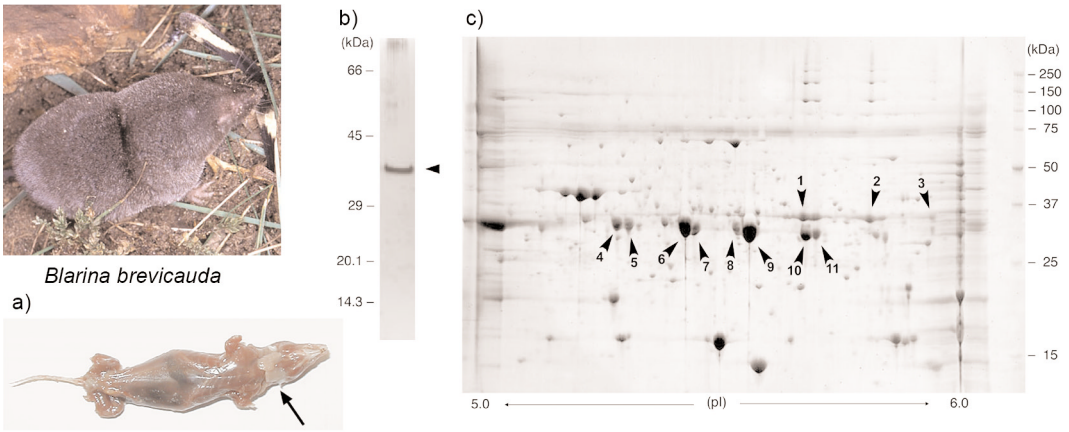

Fig. 1 American short-tailed shrew *B. brevicauda* and its lethal venom, BLTX. (a) Skinned *Blarina* specimen from the ventral point of view. The arrow indicates submaxillary glands. (b) SDS-PAGE analysis of purified BLTX (35 kDa). (c) 2D-PAGE analysis of the extracts of *Blarina* submaxillary glands. Arrows 1–3 and 4–11 indicate the spots for BLTX and blarinasin, respectively.

Purified BLTX yielded a single protein band with a molecular mass of approximately 35 kDa under reducing conditions on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (Fig. 1). 2D-PAGE analysis of purified BLTX showed three major spots with similar molecular sizes but different apparent pI values of between 5.6 and 5.9, suggesting the glycosylation of different complex oligosaccharides in each BLTX. The internal amino acid sequences of these three components obtained by trypsin digestion were identical, suggesting that the proteins contained in the analyzed spots possess common primary amino acid sequences.

Based on the amino acid sequence of the purified protein, BLTX cDNA was cloned. It consists of a pro-sequence and an active form of 253 aa with a presumed catalytic triad of serine proteases (His43, Asp109, and Ser206) (Fig. 2). Its amino acid sequence was most highly similar (53.4 and 45.1 % identity, respectively) to human tissue kallikrein 1 (hK1) and mouse tissue kallikrein 1 (mGK1). BLTX has highly conserved flanking residues at a catalytic triad and 10 highly conserved cysteine residues that may form disulfide bonds and stabilize the catalytic pocket. SDS/PAGE analysis of deglycosylated BLTX showed a single band with a molecular mass of 27–28 kDa, suggesting that BLTX is an N-linked microheterogeneous glycoprotein with a characteristic insertion of 10 residues, L^{94}TFFYKTFLG^{103}.

The enzymatic properties of BLTX (i.e., optimum pH, substrate, and inhibitor specificities) were similar to those of tissue kallikreins. BLTX also converted kininogens to kinins, which may be one of the toxic pathogens, and had dilatory effects on blood vessel walls. Furthermore, the acute toxicity and proteolytic activity of BLTX were strongly inhibited by aprotinin, a potent kallikrein inhibitor, suggesting that its toxicity is due to the kallikrein-like activity of the venom. The kallikrein-kinin system is important in blood pressure homeostasis. Kinins relax vascular smooth muscle, increase vasodilation, and enhance vascular permeability. Interstingly, however, kallikreins from other mammalian species are

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                                                    ◆
BLTX          IIGGWECDKHSQPWQALLTFTRKHNSVCGGVLVHSQWVLTAAHCIGDNYKVLLGLHDRSSEEST   064
Blarinasin    IVGGWECDKHSQPWQALLTFTNGLDGVCGGVLVHPQWVLTAAHCIGDNYKIKLGLHDRFSKDDP
hK1           IVGGWECEQHSQPWQAALYHF--STFQCGGILVHRQWVLTAAHCISDNYQLWLGRHNLFDDENT
mGK1          IVGGFKCEKNSQPWHVAVYRY--KEYICGGVLLDANWVLTAAHCYYEKNNVWLGKNNLYQDEPS
              * **  *   ****              *** *    ********        **
                                                          ◆
BLTX          VQEARVSARFPHPLYNMTLLNLLLSHKMNLTFFYKTFLGADFSHDLMLLRLDQPVQ-LTDAVQV   127
Blarinasin    FQEFQVSASFPHPSYNMRLLKLLLSDELNDTYYDEISLGADFSHDLMMMQLEKPVQ-LNDAVQV
hK1           AQFVHVSESFPHPGFNMSLLENHTRQAD-----------EDYSHDLMLLRLTEPADTITDAVKV
mGK1          AQHRLVSKSFLHPCYNMSLHRNRIQNPQ-----------DDYSYDLMLLRLSKPAD-ITDVVKP
               *   **  * **  ** *                       * * ***   *  *     * *

BLTX          LDLPTQEPQVGSTCHVSGWGRTSQNYENSFVLPEKLQCVEFTLLSNNECSHAHMFKVTEAMLCA   191
Blarinasin    LDLPTQEPQVGSKCHASGWG-SMDPYSRNFPRTGKLQCVDLTLMSNNECSRSHIFKITDDMLCA
hK1           VELPTQEPEVGSTCLASGWG---SIEPENFSFPDDLQCVDLKILPNDECEKAHVQKVTDFMLCV
mGK1          IALPTEEPKLGSTCLASGWG---SIIPVKFQYAKDLQCVNLKLLPNEDCDKAYVQKVTDVMLCA
                *** **  ** *  ****         *     ****   *  *       * *  *** **
                        ◆
BLTX          GHMEGGKDSCVGDSGGPLICDGVFQGIASWGSSPCGQQGRPGIYVKVFLYISWIQETIKAHS     253
Blarinasin    GHIKGRKDTCGGDSGGPLICDGVFQGTTSWGSYPCGKPRTPGVYVKIFSHVDWIREIIATHS
hK1           GHLEGGKDTCVGDSGGPLMCDGVLQGVTSWGYVPCGTPNKPSVAVRVLSYVKWIEDTIAENS
mGK1          GVKGGGKDTCKGDSGGPLICDGVLQGLTSWGYNPCGEPKKPGVYTKLIKFTSWIKDTLAQNP
              *   * ** * ******* **** **  ***  ***    *          **
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Fig. 2 Structure-based sequence alignment of BLTX and mammalian tissue kallikreins. ◆ indicates putative active site residues. An asterisk indicates an amino acid residue that is identical among all four sequences. The putative N-glycosylation sites are highlighted in black. hK1, human tissue kallikrein 1; mGK1, mouse tissue kallikrein 1.

not lethal. Thus, a type of kallikrein activity-linked vasodilatation, together with another undefined toxicity exerted by BLTX, may contribute to its lethality in pharmacological doses.

Several serine proteases have been identified in reptilian venom. Snake venoms are well known to contain thrombin-like enzymes due to their fibrinolytic activity and their interference with blood coagulation [22–24]. Various kallikrein-type proteases have been identified in snake venom, e.g., ancrod (*Calloselasma rhodostoma*), batroxobin (*Bothrops atrox*), and crotalase (*Crotalus adamanteus*), but they are not lethal. With regard to lizard venoms, gila toxin (GTX) and horridum toxin (HTX) resemble mammalian kallikreins with regard to their amino acid sequences and protease activities [25,26]. The LD_{50} values of GTX and HTX injected iv into mice are 2.5 mg/kg body weight [27], i.e., nearly equivalent to, or slightly higher than, that of BLTX. Both of these toxins have hypotensive effects when injected into rats, and are also hemorrhagic, yet only HTX causes exophthalmia.

Based on the crystallographic structure of hK1, homology modeling studies of BLTX have been carried out (Fig. 3) [28]. By comparison of the main elements of their secondary structures, it was found that most of the mature amino acid residues in BLTX highly overlapped those of hK1, including presumed active site residues. Meanwhile, the characteristic insertion of 10 residues in BLTX, L^{94}TFFYKTFLG^{103}, extended outward and was located around the active site residues (His43 and Asp109). These differences in the primary and secondary structures may contribute to the different enzymatic properties of BLTX and related mammalian tissue kallikreins.

Furthermore, blarinasin, a tissue kallikrein-like protease related to BLTX, has been purified from extracts of *B. brevicauda* salivary glands [29]. Blarinasin is a very abundant kallikrein-like protease, and represents 70–75 % of kallikrein-like enzymes in the salivary gland of *B. brevicauda*. Interestingly, however, blarinasin was not toxic in mice. Analysis of blarinasin and BLTX by SDS/PAGE revealed bands of different molecular sizes (32 and 35 kDa), and after deglycosylation these bands were shifted to a single band with a molecular mass of 27–28 kDa. Based on the typical N-glycosylation motif (Asn-Xaa-Ser/Thr), blarinasin has a putative N-glycosylation site at Asn93 (Fig. 2), while BLTX possesses two. This difference in glycosylation may play a role in the different toxicity and enzymatic properties of blarinasin and BLTX.

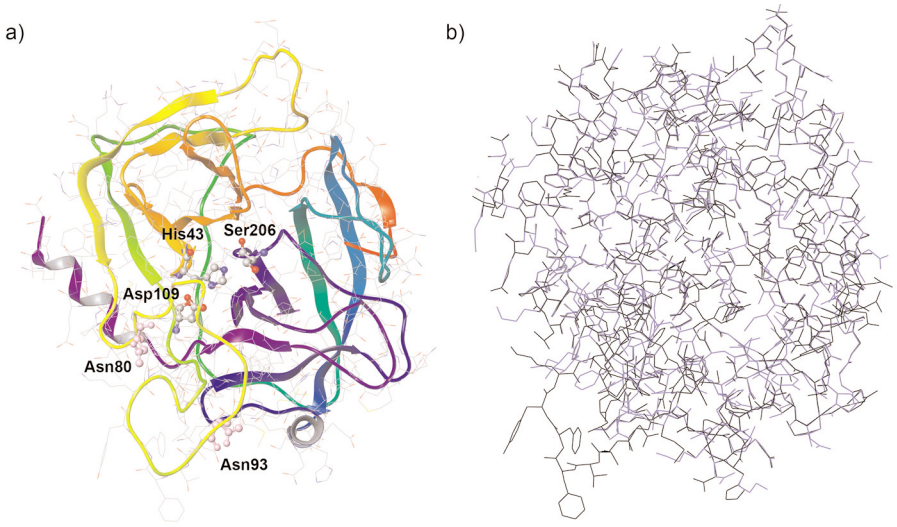

Fig. 3 Putative stereostructure of BLTX obtained by homology modeling (SWISS-MODEL program [30–32]). (a) Ribbon representation of BLTX illustrating the main elements of the secondary structure. Putative active site residues and N-glycosylated amino acids are shown in the ball-and-stick model. (b) Superimposed structures of BLTX (black) and hK1 (blue, PDB: 1spj).

Further structural studies of BLTX, including analyses of the expression of recombinant proteins, posttranslationally modified oligosaccharides, and crystal structure, are in progress. BLTX is the first lethal venom purified from mammalian origin to be fully characterized. It will be of great importance to clarify the true role of shrew venom in the ecosystem; i.e., whether it is used for defense, for food-capturing or -hoarding, for use in territorial disputes. The findings of the present study may lead to the development of valuable vasoactive agents and to a deeper understanding of the biological evolution of vertebrates.

Duck-billed platypus *Ornithorhynchus anatinus* venom

The duck-billed platypus *Ornithorhynchus anatinus*, a uniquely Australian Monotremata (egg-laying mammals) species, is one of the few living venomous mammals [33]. The adult male platypus carries a thorn on each hind leg, and fighting males are known to use this device to inject their competitors with poison. Envenoming by a platypus causes immediate excruciating pain, which evolves toward a long-lasting hyperalgesia (hypersensitivity to pain). Since this poison was shown to be toxic against rabbits at the end of the 19th century, the extracts of secreted venom or poison gland have been well investigated [34–36]. Several biologically active peptides and proteins have been identified from this venom, i.e., defensin-like peptides [37,38], C-type natriuretic peptides (ovCNPs) [39–41], nerve growth factor, and hyaluronidase. OvCNP-39 causes the relaxation of rat uterine smooth muscle, promotes histamine release from mast cells, and forms fast cation channels in lipid bilayers [42–44]. However, the precise mechanism of action leading to the excruciating pain caused by platypus venom in humans remains unclear. In 2008, the platypus genome project was completed. It revealed that the platypus genome consists of 18 500 protein-coding genes and 26 pairs of chromosomes, and that reptile and platypus defensin-like peptides have been co-opted independently from the same gene families [45,46]. To elucidate the structure and function of the actual active compounds in the platypus venom, however, continual investigations of the secreted venom fluid are essential.

Recently, in collaboration with the Taronga Zoo, Sydney, Australia, we successfully collected the fresh venom from a male platypus under anesthesia. The corrected venom was a clear viscous fluid, which was immediately frozen under liquid nitrogen and stored at –70 °C until use.

Despite the small amount (~15 μl) of platypus venom, several biological assays have been carried out. Intravenous or intracerebral administration (1–2 μl) in mice did not produce any significant symptoms, and no hemolytic activity was observed against rabbit or sheep red blood cells. Meanwhile, the crude platypus venom (0.5 μl) showed potent Ca^{2+} influx in neuroblastoma cells (Fig. 4) [47]. Notably, unlike the fast and temporal increase in $[Ca^{2+}]_i$ caused by KCl as a control, the Ca^{2+} uptake caused by the platypus venom was relatively slow and continuous. Furthermore, preliminary separation of the crude platypus venom by gel-filtration high-performance liquid chromatography (HPLC) suggested that the active components were relatively small-size compounds. Differentiated human IMR-32 neuroblastoma cells typically express L- and N-type voltage-dependent Ca^{2+} ion channels. It has been demonstrated that several marine polyether and polyol compounds, such as pinnatoxins, maitotoxin, and symbiodinolide, also cause a significant increase in $[Ca^{2+}]_i$ at pico- or nanomole concentrations [2,4]. Although it has not been confirmed that such a gradual increase in $[Ca^{2+}]_i$ caused by platypus venom is due to the activation of Ca^{2+} ion channels or specific binding to other receptor channels, the crude venom may contain some physiologically important neurotoxic substances.

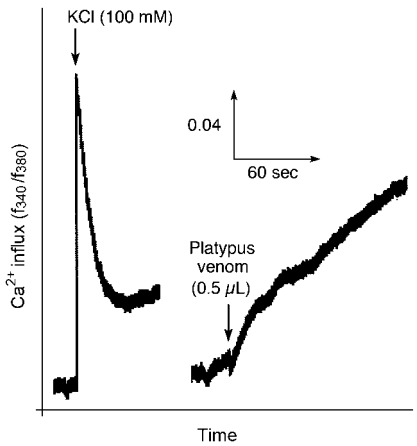

Fig. 4 Effects of platypus venom on Ca^{2+} uptake in neuroblastoma cells. Human IMR-32 cells were differentiated by treatment with 1 mM Db-cAMP for seven days, and then loaded with Fura 2-AM. The increase in the f_{340}/f_{380} ratio was monitored as the intracellular Ca^{2+} uptake rate.

In parallel with bioassays, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOFMS) analysis of the platypus venom was also carried out. After desalting using a Zip-Tip®C_{18} pipette tip column, more than 15 ion peaks were observed between *m/z* 800–4000. By the MS/MS analysis of one of the major ion peaks, the primary structure of a nonapeptide **1** has been established (Fig. 5). The amino acid sequence of **1** coincided with the nine N-terminal residues of ovCNP (Fig. 6). The amino acid sequences of mammalian CNPs are highly conserved, and a disulfide bond between two cysteine residues has been suggested to be essential for their activity [39]. Meanwhile, the N-terminal fragments of CNPs such as **1** have not been identified or characterized in any mammals. Thus, such unique small-size peptides liberated by enzymatic degradation can exhibit unique and hitherto unknown biological activities. Further chemical and biological studies on platypus venoms are currently underway.

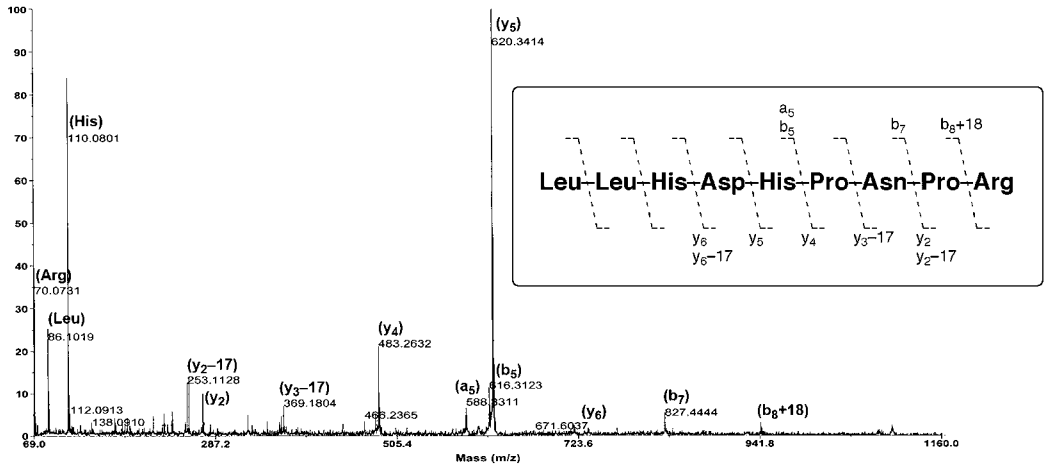

Fig. 5 MALDI-TOF/TOF MS analysis of a nonapeptide **1** identified in platypus venom [precursor ion: 1098.6 ($M+H^+$)].

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ovCNP-39        --------------LLHDHPNPRKYKPANKKGLSKGCFGLKLDRIGSTSGLGC
Peptide 1       --------------LLHDHPNPR-----------------------------
Human CNP-53    DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC
Mouse CNP       DLRVDTKSRAAWARLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC
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Fig. 6 Sequence alignment of the platypus and mammalian C-type natriuretic peptides. An asterisk indicates an amino acid residue that is identical among all three CNP sequences. Conserved cysteine residues are boxed.

The solitary spider wasp *Cyphononyx dorsalis*

Wasps of the family Pompilidae are well known for their unique oviposition behavior. Female wasps land on spiders and sting to instantaneously paralyze their prey. The paralyzed spider is then carried or dragged to a nest which, in some cases, the female wasps build from scratch or create by substantially modifying an existing cavity. The female then lays an egg on the paralyzed spider, and the larva devours the prey [48]. This interesting function of solitary wasp venom has attracted a great deal of interest. Several neurotoxic compounds with low molecular weight have been characterized from solitary wasp venom; e.g., philanthotoxins from *Philanthus triangulum* [49], megascoliakinins from *Megascolia flavifrons* [50–52], and pompilidotoxins from *Anoplius samariensis* and *Batozonellus maculifrons* [53,54].

The solitary spider wasp *Cyphononyx dorsalis* (Fig. 7) is well known to hunt spiders, such as the wandering spiders *Heteropoda venatoria* and *H. forcipata*. It uses its stinger to paralyze its prey to feed its larva. We collected both wasps and spiders in the field, and studied their paralytic toxins [55]. The venom reservoirs were manually squeezed, and venom droplets were collected from stingers (1–2 µl/specimen). When the crude venom fluid was injected into a spider, immediate paralysis was observed. These paralysis symptoms were reproducible, and the induced long-term paralysis lasted for up to 40 days (Table 1). In contrast, the venom did not cause paralysis in adult cricket (*Achera domestica*) by the same bioassay.

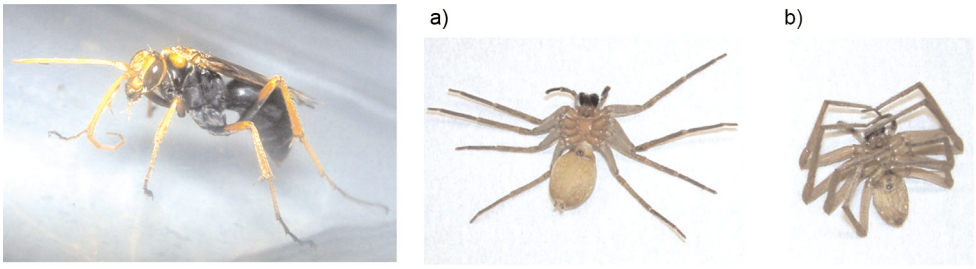

Fig. 7 The solitary spider wasp *C. dorsalis*. (a) Paralyzed spider taking the *Cyphononyx* venom. (b) Naturally dead spider.

Table 1 Paralytic activity of *C. dorsalis* venom.

	Number of paralyzed specimens/total specimens	
Dosed animal (body weight/mg)	Venom fluid (2 µl)	Control (water 2 µl)
Spider		
Heteropoda venatoria (300–800)	2/2	0/2
Heteropoda forcipata (200–800)	5/5	0/4
Nephila clavata (500–700)	4/4	1/5
Cricket		
Acheta domestica (300–500)	0/5	0/5

Guided by this bioassay, the crude wasp venom was fractionated by gel permeation and cation-exchange chromatography. Cation-exchange chromatography indicated that the pI value of the active principle was >6.5. 2D-PAGE analysis of the active fraction obtained by gel permeation chromatography showed three major spots of proteins. Two of them (25 kDa, apparent pI 8.5; 24 kDa, apparent pI 7.5) were analyzed by in-gel digestion and protein sequencing, and three proteins were identified: an arginine kinase (AK)-like protein, an elastase like-protein, and an unknown protein that was not homologous to any protein in the database. All the three proteins were cloned from mRNA of *C. dorsalis*.

Based on the molecular size and C-terminal catalytic domain of authentic AKs [56], Met109 was tentatively chosen as the start residue of recombinant truncated AK-like protein (AK109-355) (Fig. 8). As a result, recombinant AK109-355 expressed in *E. coli* showed significant paralytic activity against their natural prey, *H. venatoria* and *H. forcipata*. Injection of 1–3 µg recombinant protein per 100 mg of spider induced reproducible paralysis, which was similar to that seen with crude venom. Interestingly, the recombinant full-length AK-like protein (AK1-355) also showed paralytic activity, but was weaker than the truncated compound. Furthermore, a recombinant sea cucumber *Stichopus japonicus* AK also showed weak paralytic activity and its amino acid sequence showed 45.1 % identity to *C. dorsalis* AK-like protein. These results may imply that truncated *C. dorsalis* AK-like protein is one of the paralytic substances used against spiders.

```
  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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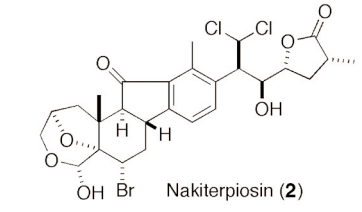
Fig. 8 Amino acid sequence of *C. dorsalis* arginine kinase-like protein. The sequences of the tryptic fragments determined by MS analysis are shown with solid underlines. Met109 is boxed.

CORAL COMMUNITIES

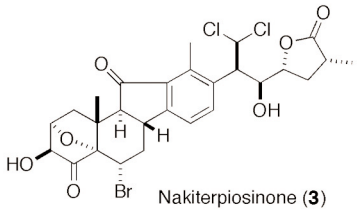
Coral reefs are rich resources as primary producers in tropical and subtropical areas. However, coral is being destroyed by many different external factors [57]. One of the extreme causes of such destruction is coral bleaching, in which all of the dinoflagellates, which usually live inside the coral, flee. Other causes of coral destruction include the overgrowth of organisms covering coral and feeding by coral predators.

Recently, we encountered a catastrophic change in the Nakijin coral reef in Okinawa Prefecture: the coral's surface was covered with a black sponge *Terpios hoshinota*. This sponge emits some compounds that kill corals and cover the dead bodies, which suggested that the sponge might be injecting a toxin into the corals. Guided by cytotoxicity, nakiterpiosin (**2**) [IC_{50} = 0.01 μg/ml], nakiterpiosinone (**3**) [IC_{50} = 0.01 μg/ml], and terpiodiene (**4**) were isolated from the sponge *T. hoshinota* (Fig. 9) [58,59]. Notably, nakiterpiosin (**2**) and its analog have a unique highly oxidized ring that corresponds to the steroid A-ring, and contain bromine and chlorine atoms. Its steroidal skeleton, consisting of C-nor and D-homo, can be found on land, but this is the first example to be found in the marine environment. Recently, nakiterpiosin (**2**) was synthesized and its stereostructure has been revised [60]. Besides the sponge *Terpios* species, many kinds of sponges, soft corals, and algae cover corals and compete for survival. Further studies on compounds from such overgrowth organisms are currently underway.

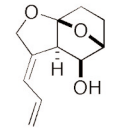
Terpios hoshinota



Nakiterpiosin (**2**)



Nakiterpiosinone (**3**)



Terpiodiene (**4**)

Fig. 9 Cytotoxic compounds from the Okinawan sponge *T. hoshinota*.

Feeding attractants for coral predators

The sea star *Acanthastar planci* (crown of thorns) is a well-known coral predator. We have been interested in determining what attracts *A. planci* to coral, why these organisms feed only on coral, and what we can do to get rid of these invertebrates. The fishermen of Okinawa found that *A. planci* gather around

the internal organs of Toxopneustidae. The shell of this sea urchin is an important Okinawan souvenir. The fishermen collect hundreds of Toxopneustidae but let their internal organs rot on the sandy beach. It was found that a battalion of *A. planci* gathers to feed on the organs. From extracts of Toxopneustidae internal organs, arachidonic acid (**5**) and α-linolenic acid (**6**) have been identified as feeding attractants (Fig. 10) [61]. Furthermore, when we used a trap containing compound **5** as an agar component, 10 sea star individuals were successfully trapped overnight. Corals are known to contain abundant triglycerides. Meanwhile, sea stars contain large amounts of phospholipase A_2 (PLA_2). Thus, it is expected that once a sea star begins to eat a coral, arachidonic acid (**5**) is liberated from the coral and spreads in the water, which invites more sea stars to the coral reef, until a whole congregation of them arrives to eat the coral.

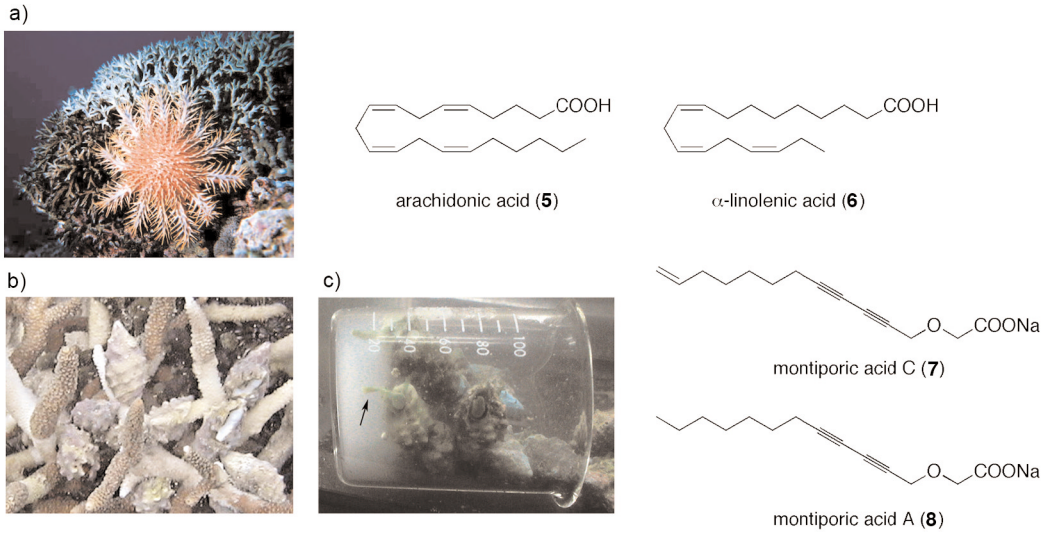


Fig. 10 Coral predators and their feeding attractants. (a) The sea star *A. planci* (crown of thorns); (b) the muricid gastropod *D. cornus*; (c) bioassay in an aquarium. *D. cornus* were attracted to and fed on agar that contained extracts of the coral *Montipora* sp. The arrow indicates the proboscis of *D. cornus*.

The muricid gastropods genus *Drupella* is another voracious coral predator, and is currently a major problem especially in Kochi Prefecture and at Amami Oshima Island, Japan. Outbreaks of them have accelerated the significant destruction of coral reefs, but the precise mechanism is poorly understood. In addition, due to the small size of *D. cornus* (2–3 cm in length), removal from a coral requires that divers must pick them up one by one with a pair of tweezers, which is an enormous amount of work. Two fatty acids, montiporic acids C (**7**) and A (**8**), have been identified as potent feeding-attractants from sea water extracts of the coral *Montipora* sp. [62] Meanwhile, none of the known feeding attractants for *A. planci*, i.e., arachidonic acid, α-linolenic acid, and betaine [63], were effective for *Drupella* sp. As described above, trapping of coral predators using feeding attractants could contribute to the prevention of coral damage caused by outbreaks of harmful coral predators.

Natural inducer of coral larval metamorphosis

Corals engage in simultaneous mass spawning at around the time of the full moon. These gametes become planktonic larvae after fertilization. Microscopic larvae are widely dispersed and transported by currents. After days to months, they begin to swim toward the bottom and search for suitable substrates for settlement and metamorphosis. It has been suggested that chemical signals of crustose coralline red

algae (CCA) or bacterial biofilms induce larval settlement and the metamorphosis of several scleractinian corals [64–67]. CCA are plants that deposit a particularly hard and geologically resistant form of calcium carbonate. These algae cement together large quantities of sand, dead coral, and debris to create a stable substrate.

We have tried to identify what compounds enable coral larvae to settle and metamorphose into the adult form. First, the larvae of the scleractinian coral *Pseudosiderastrea tayamai*, collected at Okinawa, Japan (Fig. 11), were incubated with a fragment of coral rubble with CCA. 92 % of the larvae metamorphosed with mesenteries and a central mouth on coral rubble and on the surface of the glass dish, but not on the surface of CCA. These results suggest that two types of chemical signals are released from coral rubble with CCA: defensive chemical substances and substances that induce larval metamorphosis.

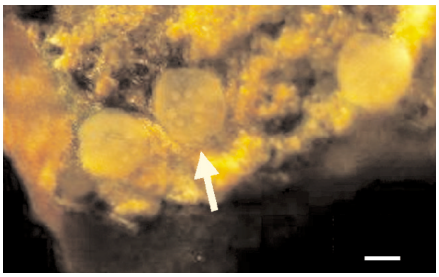


Fig. 11 Juvenile polyps of the coral *P. tayamai*. Scale bar = 1 mm.

Recently, defense substances emitted by CCA against *P. tayamai* larvae were identified: toxic brominated aromatic compounds, called corallinafuran (**9**) and corallinaether (**10**) (Fig. 12). Both compounds exhibited toxic activity against *P. tayamai* larvae, with LD_{50} values of 1.9 and 0.14 μg/ml, respectively [68].

Meanwhile, 11-deoxyfistularin-3 (**11**) has been identified as a larval metamorphosis-inducing substance, which is active at concentrations of 10^{-8} and 10^{-7} M, but the highest percentage of induced metamorphosis was not satisfactory compared to the results with living CCA. Interestingly, however, when either fucoxanthinol (**12**, 10^{-9} M) or fucoxanthin (**13**, 10^{-9} M) is present with **11** (10^{-7} M), the activity is significantly enhanced, even though neither of the carotenoids alone shows any activity. Furthermore, β-carotene (10^{-9} M) and lycopene (10^{-9} M) also have enhancing effects against *P. tayamai* larvae. These results suggest that a bromotyrosine derivative and carotenoids have a synergistic effect in the metamorphosis of *P. tayamai* larvae [69]. These synergistic effects indicate that larval metamorphosis might be rigidly regulated by multiple natural cues. The synergistic effect may provide a higher selectivity of recruitment than a single-component natural inducer for the selection of a surface to settle on, which may confer a survival advantage for benthic invertebrates in the marine environment.

Furthermore, larvae of the corals *Acropora digitifera*, *A. nobilis*, *A. surculosa*, and *Leptastrea purpurea* were incubated with **11** (10^{-7} M) and β-carotene (10^{-9} M) to determine whether the active compounds for *P. tayamai* larvae are also active for the larvae of other coral species. Although metamorphosis was induced in all four species by living CCA *Hydrolithion* sp., the combination of compounds **11** and β-carotene did not induce larval metamorphosis. These results suggest that such a natural inducer and enhancers for coral larvae are strongly species-specific.

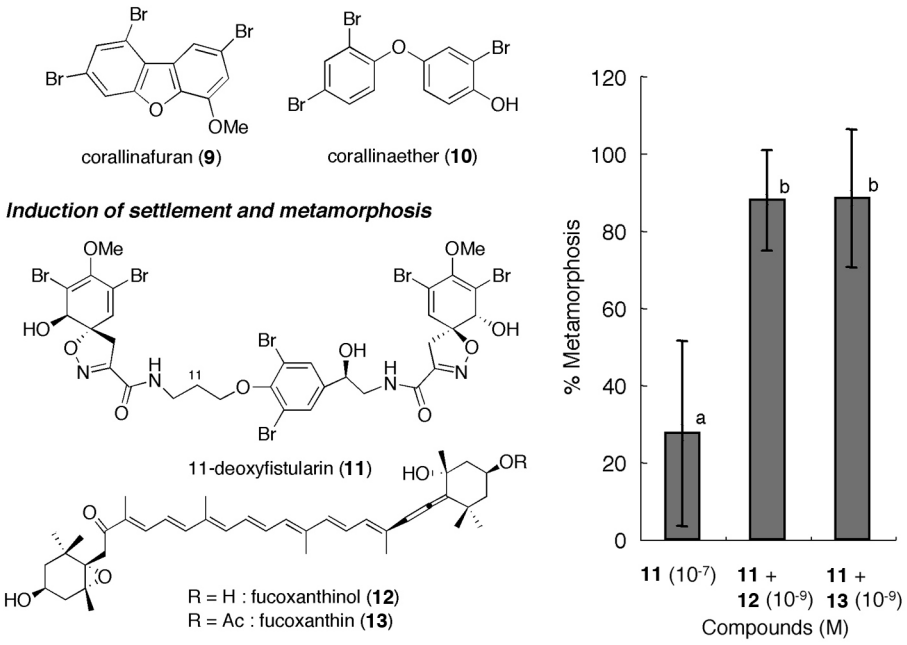


Fig. 12 Chemical inducers and defense substances isolated from CCA. Tested larvae were spawned on 1 August 2005 in FT and on 11 July 2005 in BT ($n = 6$). Larvae were incubated with compound **11** (10^{-7} M) and carotenoids **12** or **13** (10^{-9} M). Values (error bars) represent means (± SD). Values with different letters were significantly different from each other ($P < 0.05$; Tukey's test).

We have also investigated the chemical constituents of the CCA *Hydrolithion* sp. collected in Guam. We found that several coral larvae, *A. digitifera*, *A. nobilis*, *A. surculosa*, and *L. purpurea*, were induced to settle and metamorphose by extracts of this CCA species. Recently, the novel macrolide **14**, called luminaolide, has been isolated (Fig. 13) [70]. While compound **14** alone was not active, upon the addition of other fractions of the CCA extract it showed potent larval metamorphosis-inducing activity

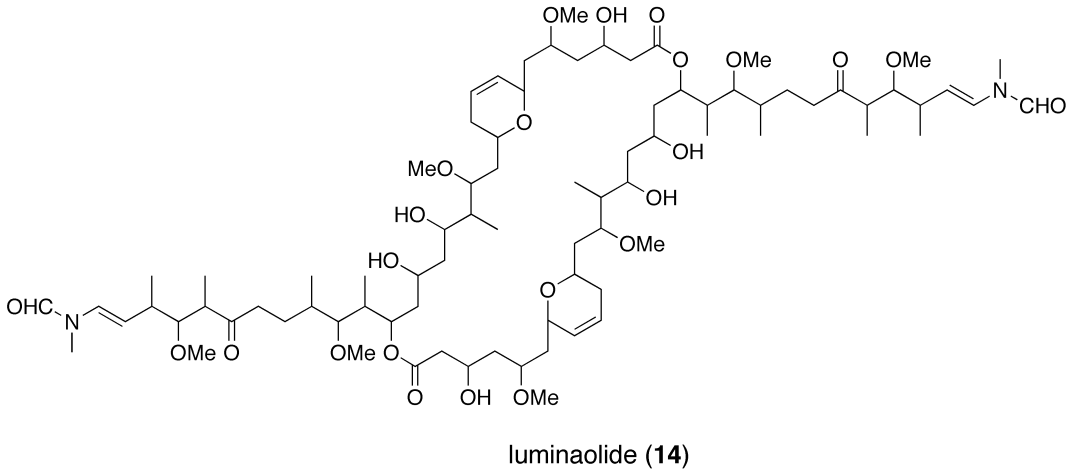
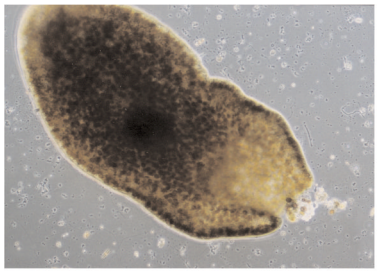


Fig. 13 Substance that induced the metamorphosis of *A. digitifera*, *A. nobilis*, and *L. purpurea* larvae isolated from Guam's CCA *Hydrolithion* sp.

against the corals *A. digitifera*, *A. nobilis*, and *L. purpurea*. Further purification and structural determinations of the chemical inducers are ongoing.

SYMBIOTIC RELATIONSHIPS BETWEEN MARINE DINOFLAGELLATES AND THEIR HOST ANIMALS

There is great potential for future studies with respect to the material level of symbiosis. For example, the Papuan jellyfish *Mastigas papua* (Fig. 14) has some algae living inside and around its legs, which may be related to coral bleaching. Notably, this jellyfish can survive >10 days in a plastic bottle, as long as it is irradiated with light. Under light, the dinoflagellates (zooxanthellae) within the jellyfish may produce oxygen and some nutrients to enable the Papuan jellyfish to survive. We have been especially interested in an ecological system in which the algae are removed from the host and a different alga is transplanted onto the host. This would enable us to identify the substances that are essential for symbiosis as well as the substances that allow for the removal and transplantation of the algae. Many questions must still be answered, such as how the symbiotic algae are taken into the host and whether the algae emit any substances that prevent the host from eating them. The answers to these questions should help us better understand a presently unknown, but very interesting, ecological system.



An Okinawan flatworm *Amphiscolops* sp.

A jellyfish *Mastigas papua*

A sea slug *Chelidonura fulvipunctata*

Fig. 14 Host animals of symbiotic marine dinoflagellates.

Isolation of bioactive secondary metabolites from symbiotic dinoflagellates

Marine dinoflagellates have been considered to be rich sources of bioactive compounds, and various unique secondary metabolites have been isolated from cultured symbiotic specimens. To identify physiologically important compounds between the host animals and their symbiont, we have investigated such unique secondary metabolites. A polyol macrolide, symbiodinolide (**15**) [71], and amphoteric iminium alkaloids, symbioimines (**16**, **17**) [72,73], have been isolated from the extracts of symbiotic dinoflagellates *Symbiodinium* sp., which were collected from the Okinawan flatworm *Amphiscolops* sp. (Fig. 15) Symbiodinolide (**15**) caused a significant increase in the intracellular free Ca^{2+} concentration at 7 nM against differentiated IMR-32 neuroblastoma cells in the presence of niphedipine (L-type Ca^{2+} channel blocker). This result revealed that symbiodinolide (**15**) possessed significant voltage-dependent N-type Ca^{2+} channel-opening activity. Symbioimine (**16**) suppresses differentiation into osteoclasts, and thus would be a candidate for treating osteoporosis. In addition, several unique polyol compounds have been isolated, such as durinskiols (**18**) [74–76] from *Durinskia* sp. and karatungiols (**19**) [77] from *Amphidinium* sp.

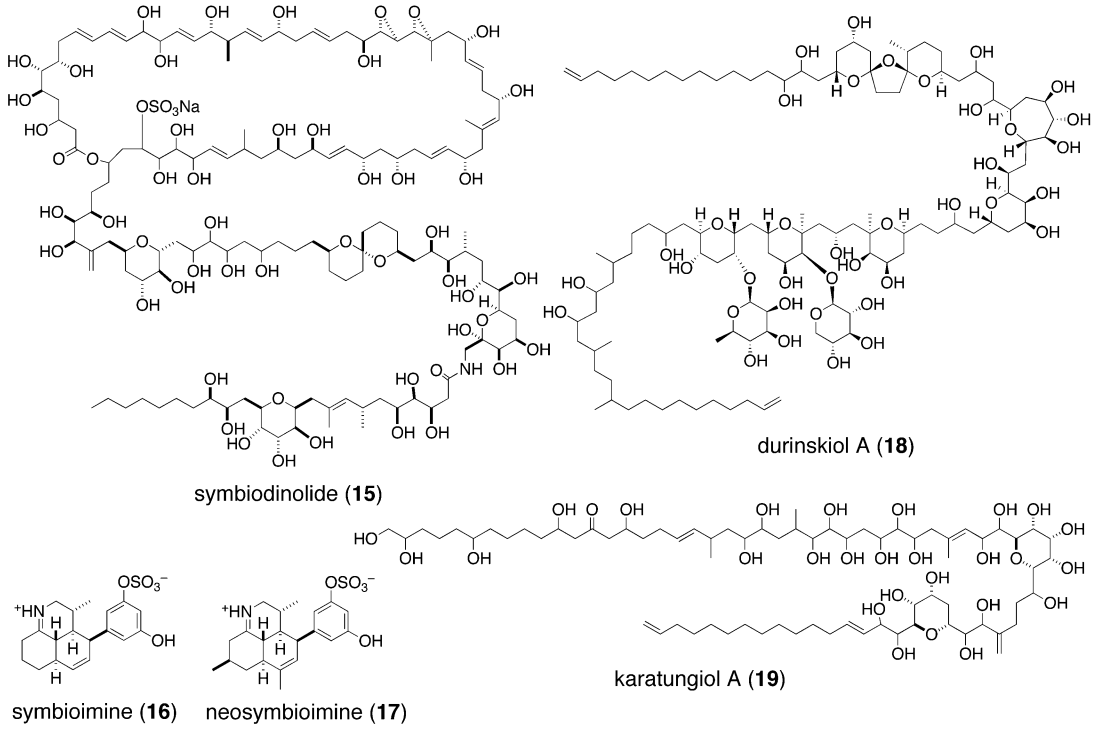


Fig. 15 Bioactive metabolites from symbiotic marine dinoflagellates.

Recently, lingshuiol B, which was previously isolated from *Amphidinium* sp. [78], was found in the cultured dinoflagellate derived from the jellyfish *M. papua* (see above). Notably, lingshuiol B significantly inhibited the expression of vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs) at a concentration of 10 μg/mL.

Degradation reactions of symbiodinolide and its effects on the host animal

The entire planar structure of symbiodinolide (**15**) has been confirmed by detailed analyses of the degradation products obtained by alkaline hydrolysis and ethenolysis reactions [71]. Symbiodinolide has a 62-membered mono-sulfated macrolactone moiety, bis-epoxide moiety, 6,6-spiroacetal and hemiacetal rings. With the use of an excess amount of the second-generation Hoveyda–Grubbs' catalyst (**25**), symbiodinolide (**15**) has been successfully degraded to afford three fragments: C14–C23, C24–33, and C34–41 (**20**–**22**) (Fig. 16) [79]. Meanwhile, the lactone ring in **15** has been opened by methanolysis, and subsequent ethenolysis using the second-generation Grubbs' catalyst (**26**) gave the C1–C13 fragment **23** and the C14–C25' fragment **24**. Unexpectedly, the allylic position of the 1,2-diol moiety at C13–C14 was specifically cleaved into α,β-unsaturated aldehydes. This degradation proceeded even under an air atmosphere, and none of the remaining polyol moieties and sulfate groups reacted under these conditions. We also found that the (*E*)-allyl *vic*-diol cleavage reaction proceeds catalytically in the presence of oxidant such as NMO or NaOCl. Therefore, this degradation may be applicable to various complex natural products which possess allylic diol moieties. To date, we have confirmed the relative configurations of C5–C7, C44–C51, and C64–C66 as well as the absolute configurations of C17–C40, C69–73, C83–C103, and C3'–C18' in symbiodinolide (**15**) by a detailed spectroscopic analysis and synthetic studies [80–84].

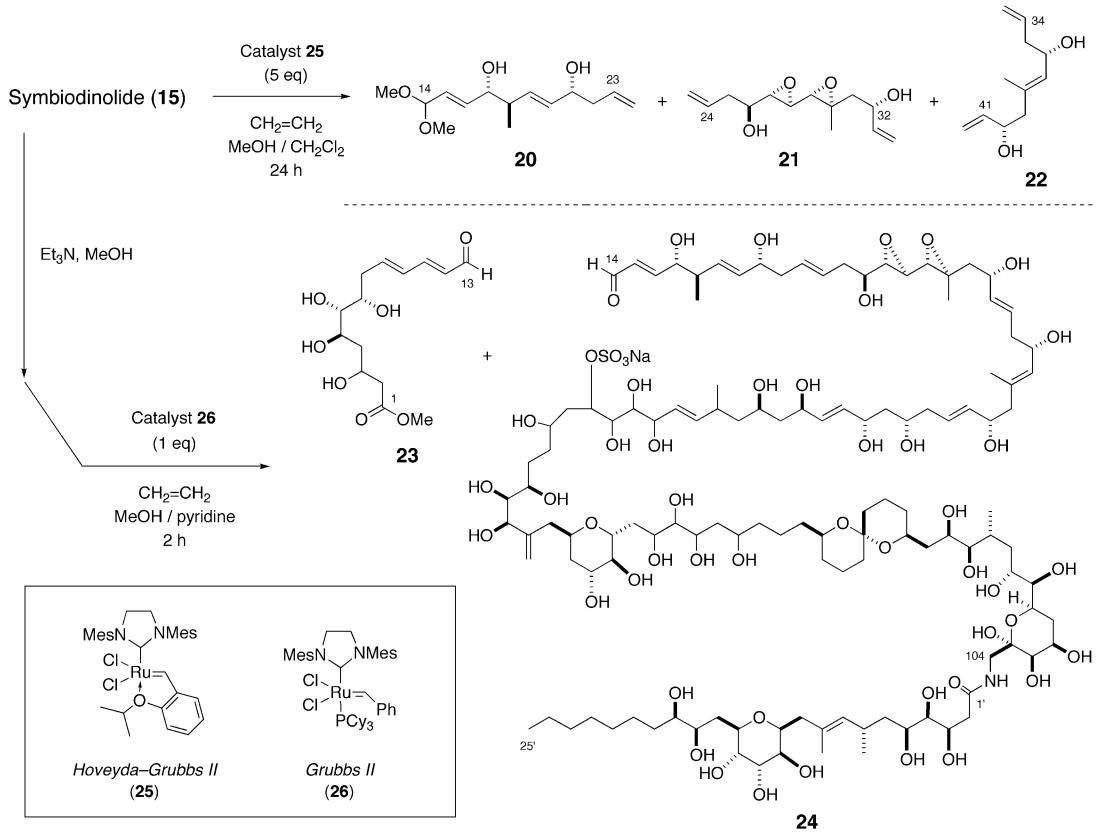


Fig. 16 Degradation reactions of symbiodinolide (**15**) using olefin metathesis catalysts.

Furthermore, to consider the role of long-carbon-chain polyol compounds in symbiotic relationships, these compounds were added to the host animals. Notably, symbiodinolide (**15**) caused immediate rupture of the tissue surface of the host animal (acoel flatworm *Amphiscolops* sp.) at 2.5 μM (Fig. 17) [71]. It is largely unknown how much polyol compounds, such as **15**, are accumulated in a flatworm. Still, our preliminary results suggest that symbiodinolide may act as a defense substance that prevents digestion of the host animal.

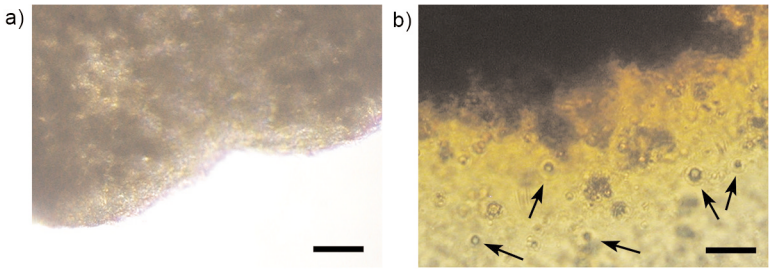


Fig. 17 Bioassay using symbiodinolide (**15**) against host animals (acoel flatworm *Amphiscolops* sp.). (a) Control, scale bar = 20 μm. (b) Treatment with **15** (2.5 μM) after 10 min. Arrows indicate dinoflagellates liberated from host animals.

There are many questions regarding long-carbon-chain polyol compounds, such as whether this type of compound has any limitations; why such a tiny dinoflagellate produces such a huge molecule; the biological role of the molecule; whether this product is made by accident, since it is present in such a small amount, but has a singular structure that does not seem to be a product of chance; and the need for this substance. These questions have intensified the interest in these compounds.

CONCLUSION

The discovery of new biologically active molecules has contributed to a better understanding of life dynamism. To preserve precious natural resources, it is important to have a broad knowledge of systems biology and chemical ecology at the level of the microenvironment. Needless to say, clarification of the function and role of chemical cues that significantly influence biological and physiological phenomena in living creatures is becoming increasingly essential. A wide diversity of natural products with specific bioactivities has been identified. These compounds provide a rich source of chemical diversity that could be used to design and develop new potentially useful therapeutic agents and pharmacological tools, such as anticancer, antimicrobial, or antiviral drugs. Newly discovered bioactive substances provide numerous opportunities for the creation of new scientific fields.

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