Chemical and biological aspects of water soluble heterocyclic marine natural products

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Abstract
Water soluble marine natural products are interesting as they generally do not penetrate into the cells but exhibit biological activities through cell surface receptors, ion channels, and glycans. Some molecules, however, interact with cell membrane and disrupt it. In this review, discovery, structures, and biological activities of water soluble marine natural heterocyclic molecules are summarized with special emphasis on their biological activity and functions.

Key words.
marine natural products, sponge, tunicate, zoanthid, giant clam, glutamate receptor, neuronal receptor, membrane, excitatory amino acid, peptide, polycation, polyamine, mycosporine

1
Introduction

According to marine natural products (MNP) database MarineLit, more than 35,000 compounds are known from the sea to date. Most reported marine-derived compounds, especially bioactive compounds are extracted by organic solvents. The reason for this is that organic soluble compounds often exert potent biological activity including cytotoxicity and antimicrobial activity by interacting with intracellular targets upon crossing lipid bilayer membrane. Contrary, hydrophilic or ionic compounds such as sugars and amino acids cannot cross the cell membrane thus require specific transporters to enter the cell. Thus, natural products with such physico-chemical properties likely fail to show 'bioactivity' in cell-based screening assays such as cytotoxicity and antimicrobial assays. Moreover, most of conventional separation techniques such as silica gel chromatography or solvent partition used to separate organic molecule may not be applicable for highly polar or ionic molecules [1]. For these reasons, water-soluble compounds are left relatively untapped. However, they are potentially interesting source for new biologically active molecules, because they can interact with cellular targets on the cell surface, including ion channels, receptors, glycans and membrane itself. Many interesting examples such as tetrodotoxin/saxitoxins that target voltage gated sodium channel; kainic acid/domoic acid that target ion channel glutamate receptors (iGluR); The *Conus* peptide toxins such as ω-conotoxin MVIII that target various neuronal ion channels and receptors including voltage gated calcium channel; lectins that target sugar chains, and polyalkylpyridiniums/saponins that target lipid bilayer [2]. The existence of these classical examples suggested that water soluble marine metabolites are interesting source to explore molecules that potentially target cell surface machineries that often govern intercellular communications and tissue integration in multi-cellular animals and plants. Moreover, biosynthesis and origin of water-soluble molecules are often quite interesting but elusive. Because most biosynthetically well-known class of molecules such as polyketides, lipids, steroids, terpenes, non-ribosomal peptides are organic soluble in general but those of some water-soluble small molecules such as tetrodotoxin are puzzling. This suggests to us that water soluble molecules are intriguing source to explore newer chemical space and biological activities in the science of marine natural products. In this review, I summarize works regarding water-soluble MNPs mainly conducted in my laboratory with special emphasis on discovery, structure and biological functions of heterocyclic molecules.

2
Discovery of water-soluble marine heterocycles

To screen bioactive water-soluble MNP, we first employed *in vivo* assay in mice. Because central nervous system is a showcase of receptors and ion channels expressed in cell surface. An intracerebroventricular (i.c.v.) injection of water-soluble compounds in mice can deliver them directly

to synaptic machineries. In the early stage in conotoxin research, mouse behavioral assay was used to discover potentially interesting molecule and it turn out to discover many important classes of *Conus* peptides [3]. Inspired by this finding, the *in vivo*-based behavioral assay was extended to aqueous extracts of various marine organisms. We screened more than 1000 marine aqueous extracts to date and found various small to middle secondary metabolites as well as bioactive proteins.

3
Excitatory amino acids

3.1. Kainic acid. Excitatory amino acid (EAA) is an acidic amino acid that triggers excitatory neuronal transmission by binding with ionotropic glutamate receptors (iGluRs). iGluRs are categorized structurally and functionally into NMDA (*N*-methyl-D-aspartate) and non-NMDA types, and later are further divided into kainite and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate)-type iGluRs. As the names suggest, the classification was based on the specific ligands that activate each subtype. Kainic acid (kainite), a classical marine heterocyclic compound, has played a key role in earlier studies of iGluR research and became a standard tool to investigate kainite-type iGluRs. Kainic acid (**1**) is a product of red algae *Digenea simplex* that occurs in temperate to subtropical shallow water of Indo-Pacific and Atlantic seas. The alga has been traditionally used as anthelmintic medicine in human, and kainic acid was isolated as an active principal. Later, domoic acid (**2**), a higher congener of kainic acid was found from another red algae *Chondria armata* which has also been used as anthelmintic medicine in Japan. Domoic acid is produced not only by the red algae, but also by diatoms [4]. Thereby bloom of domoic acid-producing diatoms, a genus of *Pseudo-nitzchia* species, causes damage in health of marine animals as shellfish or fishes may accumulate domoic acid through food web [4]. Ingestions of contaminated foods by marine animals as well as human result in abnormal behaviors and death in the worst cases. Both **1** and **2**, and in addition mushroom toxin acromelic acids share the substructure of kainic acid and thus they are collectively called kainoids. Kainoids generally possess agonist activity for iGluR selective for KA/AMPA types. Because **1** is indispensable tool in neurophysiological researches, and has attractive structural feature, more than 40 stereoselective syntheses of this molecule have been reported [5,6].

We are interested in this molecule as to biological aspect in the producing algae *D. simplex* because no direct relevance between the known physiological action of kainite and algal physiology seemingly exist. However, the fact that some glutamate related signaling are identified in plants may suggest some importance of kainic acid in the producing algae [7]. Towards further understanding of this aspect we choose to determine cellular localization of kainic acid using immunohistochemical (IHC) techniques [8]. A polyclonal antibody that specifically recognizes kainic acid was successfully raised in rabbit. The immuno-staining of the section of algae clearly showed that kainic acid is localized most densely in the outer epithelial layer of fine cylindrical thallus. The cortical cells, but

not the inner layers of the main axis, and cells of the rhizoid were also stained with this antibody. These observations suggested some defensive roles of kainic acid in the algae. Interestingly subcellular localization revealed by transparent electron microscopy (TEM) suggested that kainic acid is localized in nucleus, granule body (unannotated electron-dense compartment), and pit plug (red algae specific cell connection), however no immunoreactivity was observed in the chloroplasts.

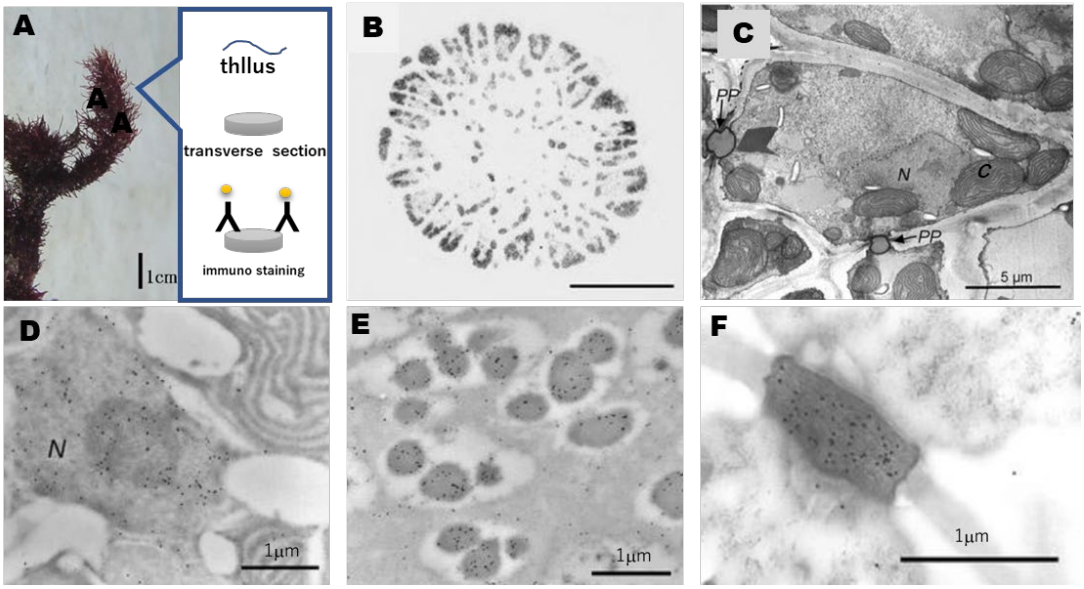
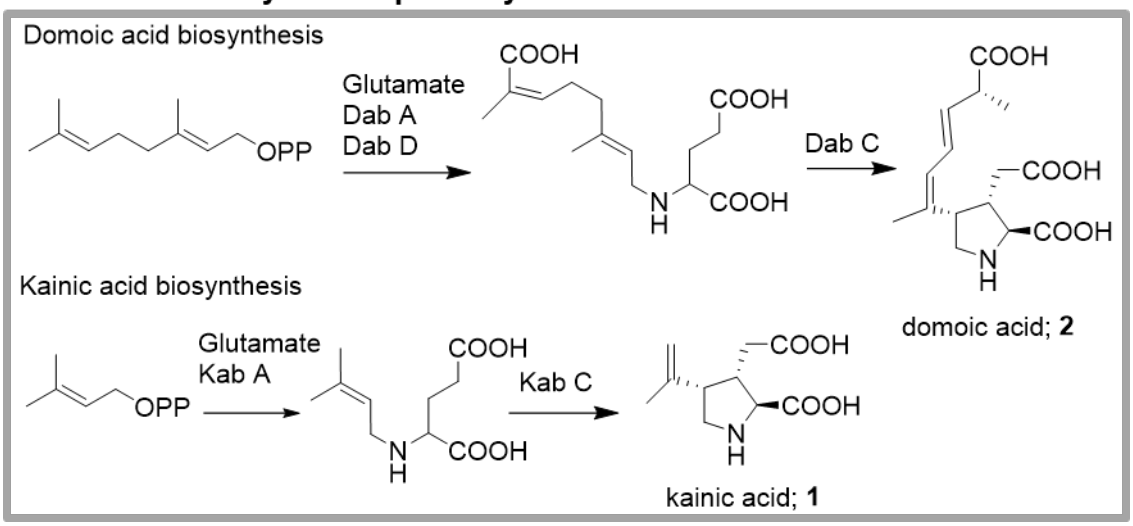


Fig.1. Cellular and subcellular localization of **1** revealed by IHC experiments. (A) *Digenea simplex*. A hair-like thallus was cut and subjected to immuno-staining by anti-kainate antisera. (B) Light micrograph of kainic acid immunoreactivity in a transverse section of thallus labeled by gold nano particle. Dark parts are kainic acid immunoreactivity. (C) TEM of non-labeled *D. simplex* cell showing nucleus, N, and pit plugs, pp. TEM of (C) chloroplast, (D) nucleus, (E) granule body, (F) pit plug. Kainic acid immunoreactivity was labeled as black dots.

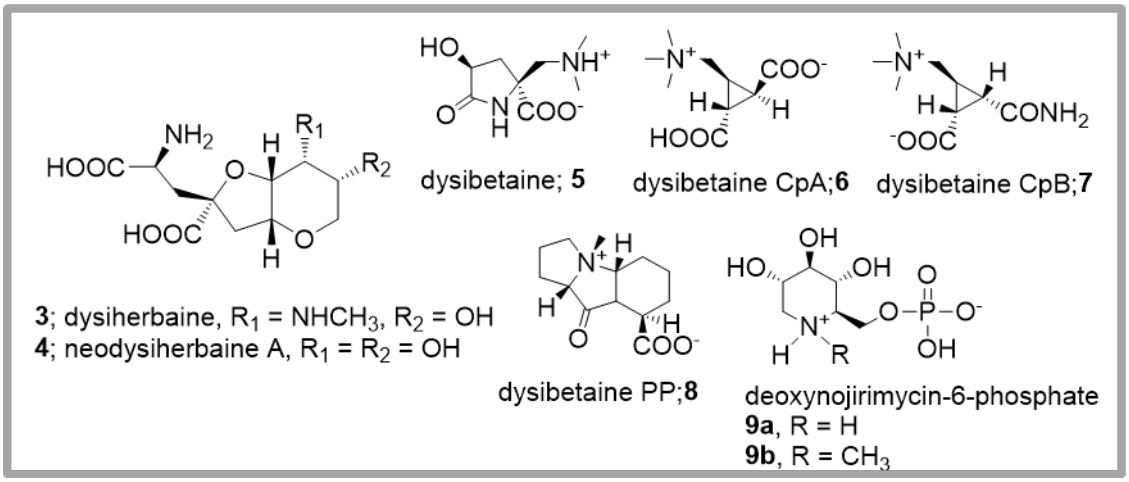
Recently, biosynthetic pathway of kainic acid [9] was uncovered along with that of domoic acid [10] by genomic approaches. As proposed earlier, condensation of geranyl diphosphate and L-glutamic acid is a key step in domoic acid biosynthesis in *Pseudo-nitzschia*, followed by oxidation to form carboxylic acid and then oxidative cyclization to construct the pyrrolidine ring. In *C. armata*, isolation of putative biosynthetic intermediates allowed to propose DA biosynthesis in the red alga where *N*-geranylated *β*-hydroxy glutamate was found as key intermediate for pyrrolidine ring formation [11]. This general enzymatic conversion was conserved both in the diatom and in *D. simplex* [9]. Because plant terpene synthesis generally takes place in the chloroplast through MEP pathway [12], our finding that chloroplast in *D. simplex* was devoid of kainic acid immunoreactivity suggests that biosynthesis of kainite takes place in cytosol rather than chloroplast using chloroplast-derived precursor.

Scheme 1. Biosynthetic pathway of domoic acid and kainic acid



2.2. *Dysiherbaines and related compounds.* We found a Micronesian sponge initially identified as *Dysidea herbacea* [13] but revised later as *Lendenfeldia chondrodes*, exhibited potent pro-convulsant in mice in our *in vivo* screening. We isolated dysiherbaine (**3**) and neodysiherbaine-A (**4**) as the responsible compounds for excitatory activity in the extract. We also found several other compounds without neuronal activity in the same sponge. These compounds include dysibetaine (**5**), dysibetaine CPa (**6**) and CPb (**7**), PP (**8**) [14], and deoxynojirimycin-6-phosphate (**9a**) and its *N*-methyl derivative (**9b**) [15]. Absolute stereochemistry for **6** and **8** were later confirmed by total syntheses [16,17], and that for **7** was supported by synthesis of desmethyl analogue [18]. The structural diversity of small heterocyclic compounds found in the sponge demonstrated its rich biosynthetic capability. Interestingly, sponges collected only in Yap state Micronesia contained dysiherbaines, but not in other areas in central Pacific including Guam and Palau, thereby production of dysiherbaines not by sponge itself, but associated microorganisms are suspected. It has long been known that certain species of Pacific Dictyoceratid sponges, including *D. herbacea* (*Lammelodysidea herbacea*) and *L. chondrodes* heavily harbor symbiotic cyanobacteria *Oscillatoria spongeliae* up to 30-50% of sponge volume. Interestingly, however, *L. chondrodes* not only harbors *O. spongeliae* but also contains another cyanobacterial symbiont *Synechocystis* sp [19].

Scheme 2. Dysiherbaines and other water-soluble metabolites form *Lendenfeldia chondrodes*



We observed that both of these symbiotic cyanobacteria in mesohyl of the sponge. An immunohistochemical study combined with microscopic observations using dysiherbaine-specific antibody raised in rabbit revealed that immunoreactivity of dysiherbaine was localized specifically in the cells of *Synechocystis* sp., but not in *O. spongeliae*, eubacteria or sponge cells. This result suggested that *Synechocystis* sp. plays important roles in production or storage of dysiherbaine [20]. *Synechocystis* sp. identified in the sponge is closely related to obligate symbionts *Prochloron didemni* and *S. trididemni* of tunicates. *Prochloron* is known to produce secondary metabolites represented by patellamides in the tunicate *Lissoclinum patella* [21], however, metabolic potential of symbiotic *Synechocystis* is left to be investigated.

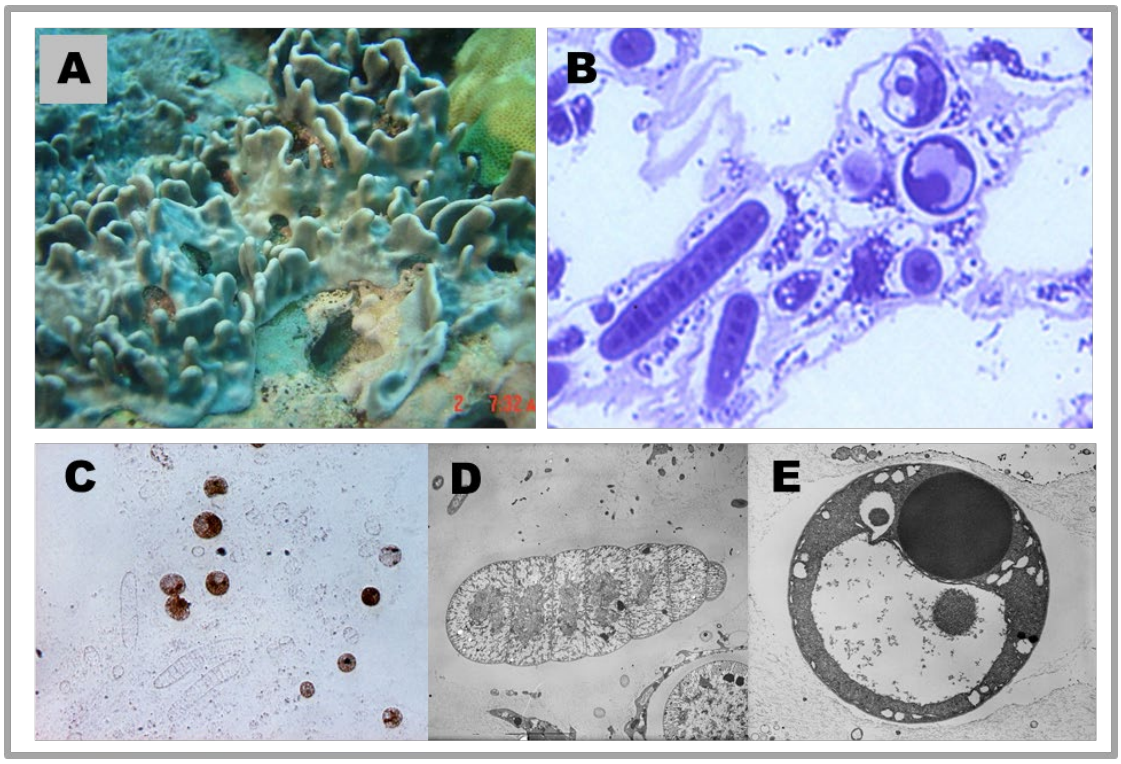


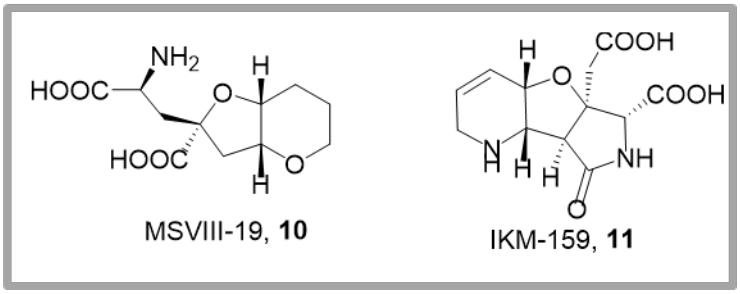
Fig.2. Cellular and subcellular localization of dysiherbaine (**3**). (A) Underwater photography of *L. chondrodes*. (B) A Light micrograph of toluidine blue-stained section. (C) A section stained by anti-dysiherbaine antisera. TEM for (D) *Oscillatoria spongeliae* and (E) *Synechocystis* sp.

Dysiherbaine (**3**) exhibited very potent excitotoxicity in mice, inducing long lasting convulsion with IC_{50} value of 6 pM/mouse, six times as potent as that of domoic acid, thus regarded as the most potent naturally occurring excitatory amino acid known to date [22]. Dysiherbaine (**3**) bind to GluK1 and GluK2 subtypes of kainite receptors very potently at *K*i values of 0.74 and 1.2 nM, respectively. Because of this potency, **3** selectively activates GluK1 site in the hetero tetrameric complex of GluK1/K4 receptor, showing new insight into behavior of heteromeric GluRs [23]. To gain structural insight into the potency and subtype selectivity, we performed structure-activity relationship study of dysiherbaines by synthesizing 11 analogues. We found that, besides importance of glutamate substructure of **3**, substituent of the perhydrofuro-pyrane ring plays significant roles in the activity. Further, crystal structures of ligand binding domain (LBD) of GulRs complexed with **3** indicated that binding of the ligand lead to conformational change in the receptor stabilizing 'closed' conformation, typical of those observed in glutamate and other agonists. Interestingly, a truncated analogue, MSVIII-19 (**10**), synthesized by Sasaki's group that exhibited *in vivo* a transient coma-like sleep also bound to the LBD in the same fashion as **3**, however, it stabilized desensitized state of the receptor and inactivate it by functioning as antagonist [24]. This is an unexpected outcome because in AMPA receptors, closure of the LBD lobe has been thought to correlate to its channel opening [25,26]. Thus the structure

and functional observations with dysiherbaine and congeners posed complexity in GluR biophysics [27]. Because **10** was shown to be a functional antagonist for GluK1 and this receptor has been thought to be involved in pain transmission, it antinociceptive activities was tested in rat pain models revealed that it selectively reduced pain-related behaviors in neuropathic pain model while no effect was seen in the peripheral model [28]. This result showed potential of selective GluR1 antagonists as analgesic drugs.

Inspired by the structure and activity of naturally occurring EAAs including kainic acid and dysiherbaine, Ikoma and Oikawa designed and synthesized a new molecule IKM-159 as racemic mixture (rac-**11**) using three component Ugi reaction coupled with olefin metathesis to construct tri hetero cyclic amino acids (Scheme 3) [29]. These compounds especially rac-**11** induced behavioral changes in mice in that the mice became totally flaccid after intracerebroventricular injection [30,31]. Electrophysiological characterization indicated that these compounds attenuate excitatory neurotransmission through AMPA-type glutamate receptors [32]. Crystal structure of LBD of AMPA receptor complexed with IKM-159 indicated that the 2*R* congener from rac-**11** binds to the ligand binding cavity and change the conformation to 'open state' which is typical of the conformation bound to GluR antagonist. These observations suggested that (2*R*)-IKM159 functions as antagonist for GluRA2 receptor. Stereospecific synthesis of both (2*R*)- and (2*S*)-IKM159 followed by *in vivo* bioassay in mice showing the activity in only 2*R* isomer **11**, confirmed the above observation [33].

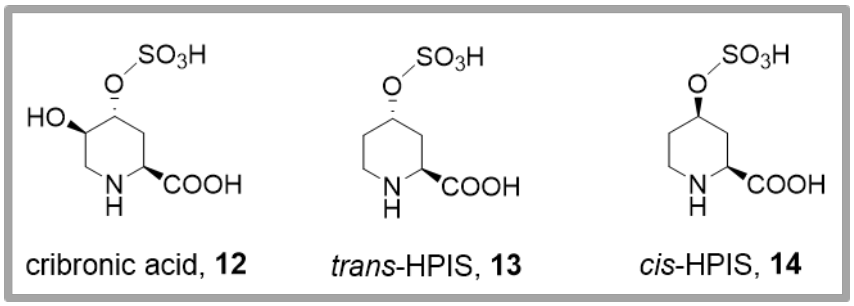
Scheme 3. Synthetic antagonists



2.3. *Sponge-derived 4-sulfooxypiperidine-2-carboxylic acids.* During further investigation for neuroactive compounds we found that the aqueous sponge extracts induced convulsant behaviors characterized by violent but transient running and jumping behaviors. These behaviors are also observed after injection of NMDA agonists in our previous study. From two distinct sponges, *Stylotella aurantium* and *Axinella carteri* collected in Yap State, Micronesia, a known (2*S*,4*S*)-4-sulfooxypiperidine-2-carboxylic acid (*trans*-HPIS, **13**) was isolated as an active principal (Scheme 4). On the other hand, separation of a Palauan sponge *Cribrochalina olemda* afforded an isolation of cribronic acid [34], (2*S*,4*R*,5*R*)-5-hydroxy-4-sulfooxypiperidine-2-carboxylic acid (**12**) (Scheme 4). They both bind to NMDA site in the rat synaptic preparation at IC_{50} values of 214 and 83 nM, but not to AMPA or KA sites. *trans*-HPIS (**13**) was previously reported from the seed of the legume

Pletophorum africanum and identified as an NMDA agonist [35,36]. Of note, synthetic *cis*-HPIS (**14**) [36] did not show any activity in mice. Several other NMDA ligands are found from marine organisms and plants indicated that they are potential source of novel NMDA receptor-targeting drugs [2].

Scheme 4. NMDA agonists from sponges



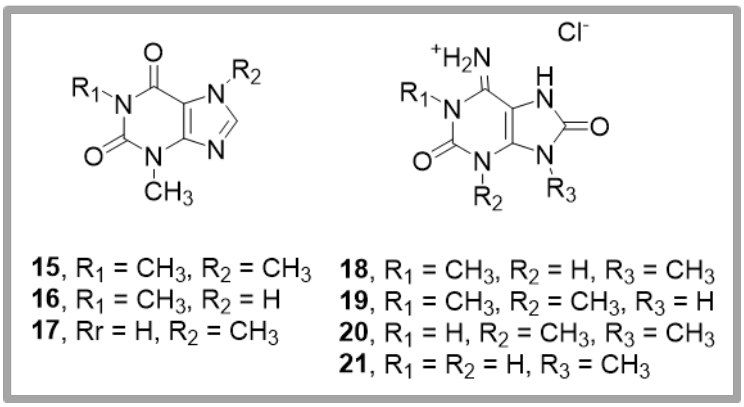
3. Neuroactive purine derivatives from marine sponges.

Purines are one of the most frequently encountered class of molecules during the separation of aqueous extracts. Plant methyl xanthines, such as caffeine (**15**) theophylline (**16**), and theobromine (**17**) are the most important class of bioactive purines. Needless to say, caffeine is widely used in recreational and medicinal purposes, and theophylline is clinically used as antiasthma drug. Marine organisms are rich source of bioactive purines exhibiting cytotoxicity, antimicrobial activity, enzyme inhibition, antiangiogenic activity, and neuronal activity [37].

We found that aqueous extracts of Palauan Haplosclerida sponges, *Cribrochalina olemda*, *Haliclona* sp. and *Amphimedon* sp. induce transient running-jumping convulsion in mice via i.c.v. route. Bioactivity-guided separation of *C. olemda* resulted in isolation of 1,9-dimethyl-8-oxoisoguanine (**18**) as active principal [38]. Compound **18** and related purines **19-21** were also found in *Haliclona* sp. and *Amphimedon* sp. collected in the same region. All these purines induced convulsion in mice, and the behavioral phenotypes including transient running-jumping convulsion resembled to that of NMDA agonists, however, **18** did not interact directly with GluRs in the radioligand binding assay. Note that caffeine did not induce such convulsant activity in the mouse assay. It was shown however, that **18** clearly showed hyperexcitability in cultured rat hippocampal neuron in an electrophysiological assay. Further physiological experiments indicated that **18** modify both inhibitory and excitatory neurotransmissions with higher impact on the inhibitory one. In a target screening panel, provided by Psychoactive Drug Screening Program (PDSP) showed moderate affinity for α-adrenoceptors (*K*i values of 885, 758, and 497 nM for each α1A, α1B, and α1D, respectively) and weak affinity for the serotonin receptor 5-HT_{1E} (2.9 μM), nicotinic receptor α3β2 (4.2 μM), and

κ- and μ-opioid receptors (1.8 μM and 8.9 μM, respectively). The action of **18**, however, was not affected by inhibitors of α-adrenoceptors or $GABA_B$ receptors which regulate inhibitory neurotransmission [38]. These results together indicated that **18** modulates inhibitory neurotransmission to turn neuron into excitatory phase, however, actual molecular targets of the purine are remained to be identified. This study illustrated interesting and complex nature of neuronal modification by purines. As represented by caffeinee, the multiple actions of the drug may be ascribed to its wide target selectivity [39]. Further investigation on this class of neuroactive molecules would result in next generation of purine-based drug discovery [37].

Scheme 5. Neuroactive purines



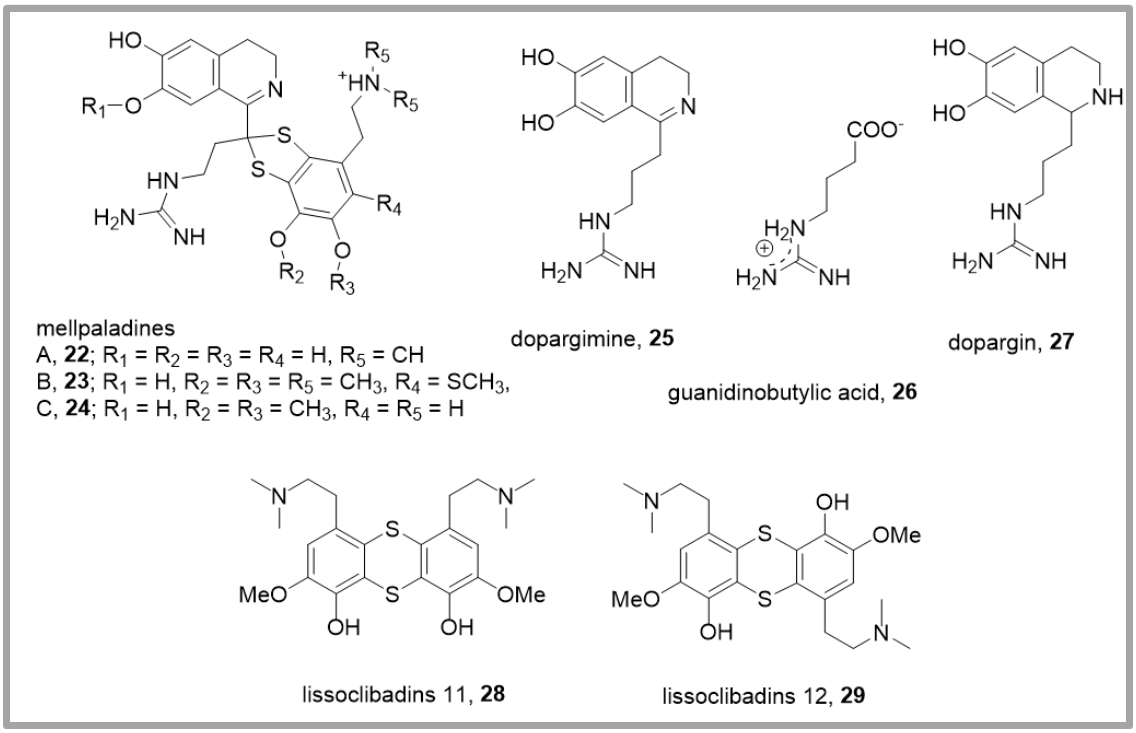
4. **Tunicate-derived heterocyclic molecules**

Tunicates are marine invertebrate belongs to the phylum Urochordata, close to vertebrates. It is, however, known to contain variety of bioactive secondary molecules including ecteinascidins [40] which has been developed as first marine-derived anticancer drug. Because of this, tunicates have received intense attentions from marine natural product community and a number of biologically interesting compound are reported. Most of which, however, are lipophilic metabolites and biosynthesized likely by symbiotic microorganisms [41]. Aqueous extracts of tunicate are studied mainly in a context of physiological and ecological interests but are relatively untapped [42]. Here, we show our recent work looking into aqueous extract of tunicates using our assay systems.

4.1. *Mellpaladines*; A crude aqueous extract of Palauan Didemnidae tunicate suppressed motor activity of mice. This activity suggested a presence of compounds that interact with neuronal receptors. Separation of the extract guided by the mouse assay resulted in isolation of mellpaladines A-C (**22-24**), dopargimine (**25**), as active principals, along with known compounds guanidinobutyric acid (**26**), and dimeric polysulfur dopamines lissoclibadins 11 (**28**) and 12 (**29**) [43]. Dopargimine and mellpaladine A-C induced behavioral changes in mice. They induced tremor or convulsion at high

dose (<50 nmol/mouse), but at lower dose (14 nmol/mouse) the motor activity of mice was largely suppressed by administration of these compounds. Compound **26** induced moderate behavioral change, but **28** and **29** did not show noticeable behavioral changes at 50 μg/mouse. Note that **26** is known to be a physiological substance in the brain and intracisternal injection of **26** in rabbit was convulsive at 5 mg/kg [44]. The molecular targets of the active compounds were screened in PDSP using radio ligand binding assay showing that **25** displace radioligands for δ-opioid receptor (DOR Ki = 485 nM) and α2B adrenergic receptor (5.9 μM). Mellpaladines A and B were also shown to bind to wide range of synaptic receptors including serotonin, α-adrenalgic, dopamine, histamine, opioid, and Sigma receptors, and transporters such as dopamine and noradrenaline transporters. The structure of **25** is closely related to plant guanidine alkaloid dopargine (**27**) which is known as a key ingredient of black cohosh, a herbal medicine used to treat menopausal symptoms [45].

Scheme 6. Mellpaladines and related compounds



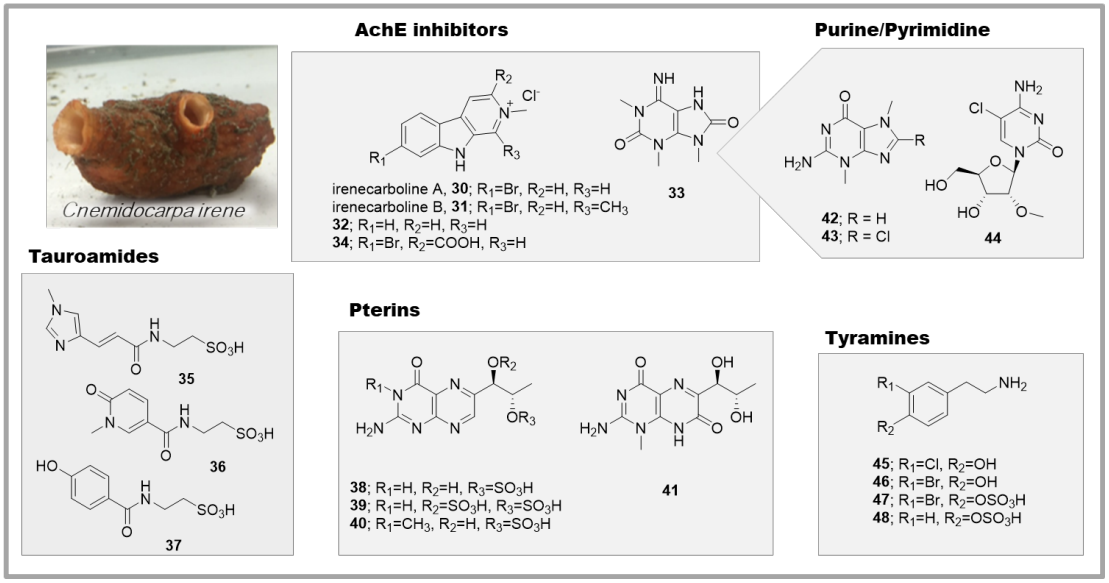
Other related guanidine alkaloids trypargine derivatives were found for various organisms including tunicates [46], a sponge [47], a flog [48], and a spider [49]. All these alkaloids are likely formed by Pictet-Spengler reaction between monoamines and an aldehyde derived from arginine. Isolation of putative precursor, **26** from this tunicate supported the above hypothesis. Mellpaladines are the first natural products that possess 1,2-dithoketal structure, although examples of dithioacetal compounds were reported in tunicates. Isolation of polysulfur dopamines **28** and **29** suggested that the tunicate is

in a chemotype that produce polysulfur dopamines [50], thereby mellpaladines are speculated to be biosynthesized by condensation of dithiol or relevant derivatives of dopamine and dopargimine derivative. Biosynthesis especially origin of sulfur atoms in mellpaladines and polysulfur dopamines are of particular interest in further investigations [51].

4.2. Aromatics from *Cnemidocarpa irene*

Aqueous extract of the solitary tunicate *Cnemidocarpa irene* collected in Kojima inlet, off-Hakodate, Hokkaido was shown to be rich in small water-soluble aromatics. Because the extract inhibited acetylcholinesterase activity *in vitro*, further separation was carried out.

Scheme 7. Aromatic heterocycles from *Cnemidocarpa irene*



As a result, new *β*-carboline derivatives irenecarboline A (**30**) and B (**31**) along with *N*-methyl-*β*-carbolinium chloride (**32**) and 1,3,9-trimethyl-8-oxoisoguanine (**33**) were isolated as active compounds [52]. *N*-Methyl-*β*-carbolinium-3-carboxylate (**34**) was also isolated but was devoid of the activity. Irenecarboline B (**31**) showed the most potent inhibition with IC_{50} value of 0.47 μM which is comparable to galantamine a clinically used acetylcholinesterase inhibitor for treatment of Alzheimer's disease. Compound **33** is the first example of purines that exhibited this activity. Of note, **33** is closely related to a series of neuroactive purines isolated from sponge (See section 3).

Further isolation work resulted in identification of fifteen more aromatics with various structures [53]. These compounds can be categorized into tauroamides **35-37**, pterin derivatives **38-42**, guanine derivative (**43**) and nucleoside (**44**), and tyramines **45-48**. These compounds failed to inhibit growth of tumor cell line and to inhibit activity of acetylcholinesterase, however,

biopterin disulfate **39** modulated behaviors of mice, in that, an i.c.v. injection resulted in flaccidness in mice. At higher doses (5–25 nmol/mouse), mice became completely flaccid and died 6–40 min after the administration. A lower dose (2.5 nmol/mouse) induced the loss of lighting reflex and ataxia motion. These behavioral profiles resembled to that induced by AMPA-type glutamate receptor antagonists, including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), GYKI52466 and IKM-159. Radioligand binding assay showed moderate affinity for NMDA, AMPA, and KA receptors at IC_{50} values of 1.9, 0.91, and 0.68 μM, respectively. All other pterin derivatives isolated here did not show behavioral activity in mice. This is the first report that biopterin derivative possessing neuronal activity. Tyramine sulphates **47** and **48** caused violent convulsions upon i.c.v. administration, leading to death at the highest doses (17 and 92 nmol/mouse, respectively). At lower doses (3.4 and 23 nmol/mouse), **47** and **48** induced motor suppression. Target molecules of these compounds are not identified. Halogenated tyramines **45** and **46** induced minor behavioral changes, including loss of balance and frequent grooming, suggesting they modulate synaptic transmission. Notably, **45** is known as a ligand for the dopamine D2 receptor [54]. Structural and biological diversity of these aromatic metabolites suggested some relevance to the ecology and physiology of the tunicate. Interestingly it was found that the blood of the tunicate was fluorescent [52]. HPLC analysis showed that the *β*-carboliniums **30** and **32** were present in the blood serum while pterin **42** in the blood cells. LC-MS analysis for the juvenile tunicates found near the adult animal, and larvae found in aquarium-grown tunicate lacked these aromatic metabolites. These observations suggested that the above aromatic secondary metabolites were accumulated with growth after the metamorphosis of this animal.

In conclusion, water soluble metabolites from tunicates provides us with insight into very unusual metabolism of tunicates. The isolates we show here may largely be of metabolites of tunicate rather than symbiotic bacterium, although origin of polysulfur compounds is debatable. The water-soluble metabolites are structurally close to primary metabolites, and some of them should even be categorized as primary metabolites. For instance, biopterin disulfate **39** isolated as neuroactive molecule here could exist in brain given that biopterin is known as a key cofactor in monoamine synthesis [55]. Presence of many neuroactive molecule suggests some physiological function of these compounds in tunicates.

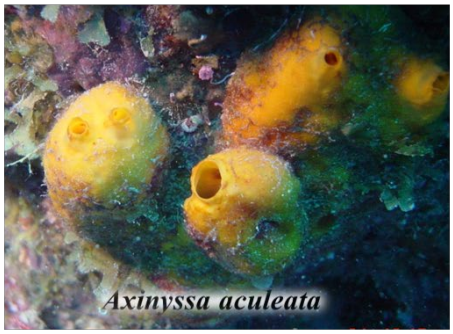
5
Marine polycations

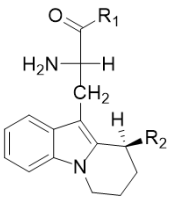
Marine sponges often contain polycationinc molecules, presumably for defense against predators or fouling organisms. The most prominent example is alkyl pyridinium salts (APS) found in several different taxon of sponges on mainly in the order Haplosclerida [56,57,58].

Common structural unit of APS is *N*-alkylated pyridinium but the *N*-alkyl chain is interconnected to the 3-position of the other pyridinium unit to form cyclic dimers, oligomers to polymers [57]. An example of monomeric molecule and liner APS were recently reported [57,59]. Length of the alkyl chain varies and double bonds or methyl branches are located in the alkyl chain in some APS [57]. Generally small APS are soluble in organic solvents, but polymers are water soluble. The molecular size of poly-APS from *Reniera sarai* was determined to be 5.5 and 19 kDa (corresponds to 26 and 100-mers, respectively) based on MALDI-TOFMS data, however behaviors in gel filtration and dynamic light scattering experiment suggested that the molecule exist as aggregate with the size of 3 x 10^6 Da [60]. The poly-APS displays discrete biological activities from small APS as represented by potent hemolytic and acetylcholinesterase inhibition activity [61,62,63]. The membrane interaction of PAP resulted in the unique property to translocate certain molecules into the cells [64]. Poly-APS thus translocated GFP-plasmid and the gene was successfully expressed [63]. We often observe in the mouse assay, aqueous extract containing polymeric Poly-APS induces convulsion thus can be a nuisance constituent when one is seeking for novel neuroactive compounds. However, unique membrane activity, enzyme inhibition and other ecologically-related functions of poly-APS, as well as its distribution in the sponge and biosynthetic relation to other marine polycations are interesting, and it is worthy endeavor to explore more chemical and biological aspects in poly-APS [58].

We found another polycationic middle-sized molecule aculeines from the marine sponge *Axinyssa aculeata* [64,65,66]. The aqueous extract of this sponge induced convulsion in mice. Three aculeines (Acu) A-C were isolated, but further structure determination was carried out only for Acu A and B because Acu C was obtained as complex mixture of homologues [69]. Aculeins A (**49**) and B (**50**) were characterized as a peptide with 44-amino acid residues (AcuPep) modified by long chain polyamine (LCPA) in combination of spectrometric, spectroscopic, and *c*-DNA cloning [64]. The AcuPep is a ribosomal peptide, and the gene encodes the peptide originally containing a codon for tryptophan at the *N*-terminal of the mature peptide, however, in Edman degradation the first amino acid was undetected instead, the sequencing started from aspartic acid the second amino acid residue in the *c*-DNA [64]. The structure of the *N*-terminal amino acid has long been elusive as no direct spectral interpretation on NMR was realistic due to broad spectral data. Isolation of free protoaculein B (pAcu-B, **51**) from the same extract, however, overcame this problem [65]. The spectral analysis of free pAcu-B lead to a proposal of *N*-terminal residue of Acu-B. The structure of **51** proposed here is unique as LCPA was attached to the unusual tricyclic tryptophan derivative. Detailed mass spectral analysis for both **49** and **50** indicated that both of those molecules contain AcuPep as a common substructure but two LCPAs are attached to the *N*-terminal tryptophan-derived amino acid in the case of **49** instead of one in **50**, although detailed structure in the LCPA moiety of **49** is still unknown (Scheme 8) [65].

Scheme 8. Aromatic heterocycles From *Axinissa aculeata*

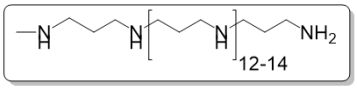




AcuPep

-NH-YDTVACFREDEICSGVPIPLPNSDVCC
STGGLSYRGAIPTCNEC-OH

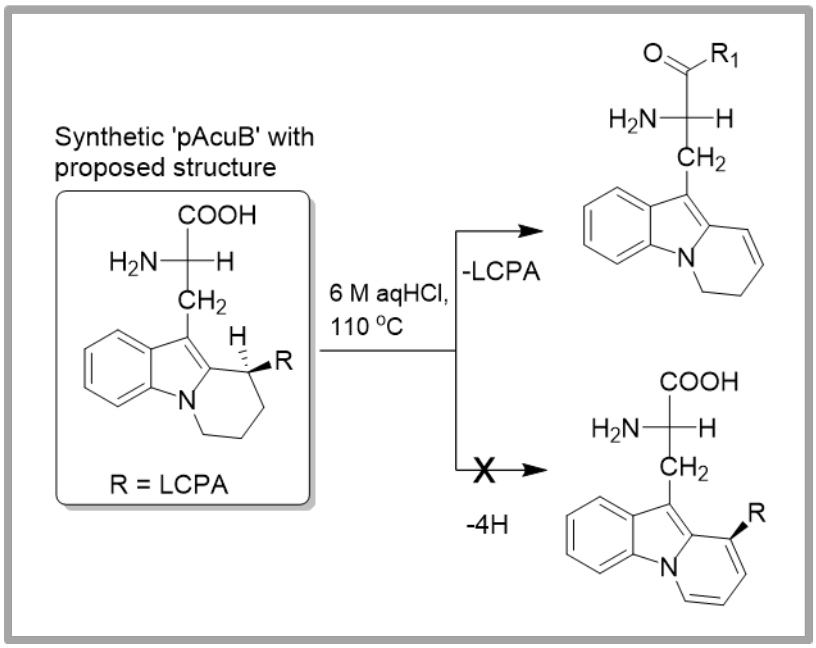
LCPA



Acu A; **49**, R_1 = AcuPep, R_2 = LCPA + $C_{46}H_{105}N_{15}$
AcuB; **50**, R_1 = AcuPep, R_2 = LCPA
pAcu B; **51**, Proposed structure, R_1 = OH, R_2 = LCPA

This structural characteristic, LCPA-modified polypeptide, is unique as no other examples besides diatom derived silica mineralization protein silaffin [67], has been found in nature. Moreover, six cysteine residues in AcuPep was shown to form three disulfide bonds [65]. Although the pattern of disulfide bond formation is not determined, the interval of the cystatin residues is in a typical pattern found in cysteine knot peptide toxins including conotoxins [66]. Together the gross structures of **49** and **50** were proposed as novel LCPA modified peptide. Recently Oikawa's group attempted to synthesize **51**, they however encountered difficulty due to poor solubility of nitrobenzenesulfonyl (Ns)-protected LCPA [70]. This problem was overcome by using both *t*-butoxycarbonyl (Boc) and Ns as protecting groups for secondary amine and photoremovable 1-(2-nitrophenyl)ethoxycarbonyl (NPEC) group at the terminal primary amine. The fully protected pAcu-B was thus synthesized [69]. The stepwise deprotection of the fully protected pAcu-B was further elaborated and finally **51** with the proposed structure was synthesized [69]. The NMR data for the synthetic and natural **51**, however, were found to differ significantly [69]. Therefore the chemical reactivity of them was compared. Natural **51** and a synthetic model compound was reported to undergo dehydrogenation/aromatization upon acid treatment in 6 M aqHCl. This hallmark reactivity in the natural product was not observed for the synthetic compound: namely, the same treatment resulted in de-amination first and then aromatization. These observation lead to the conclusion that the structure proposed for **51** was incorrect. The structural revision of **51** in now in progress.

Scheme 9. Reactivity of proposed pAcu B



Aculeines are discovered as 'neuroactive' compound because it induced convulsion in mice after i.c.v. administration, but they also exhibited cytotoxicity and hemolytic activity [64]. No antibacterial activity, however, was found. It was proposed that all the above bioactivities of aculeines is due to their action to disrupt cell membrane [64]. The deferential hemolytic activity of **49** was observed in the erythrocyte of several different animal species [64]. This observation suggested that the activity of **49** depends on phospholipid in the cell surface. Interestingly, however, **49** failed to disrupt artificial membrane in POPC liposome, suggesting that **49** targets other phospholipids or some other cell surface molecules [64].

In conclusion, middle-sized water soluble marine polycations such as poly-APS and aculeines both display interesting membrane activities. This action leads to wide variety of biological activities that have been reported especially for poly-APS. Membrane targeting is a common and widely used chemical defense strategy in nature as represented by antimicrobial peptides (AMP) that present in wide variety of organisms including mammals. Since chemical and biological research for AMPs have led to development of unique cell penetrating peptides that can translocate membrane-impermeable molecules into the cells. Thus, deeper insight into marine water-soluble polycations may lead to development of some useful drug delivery systems.

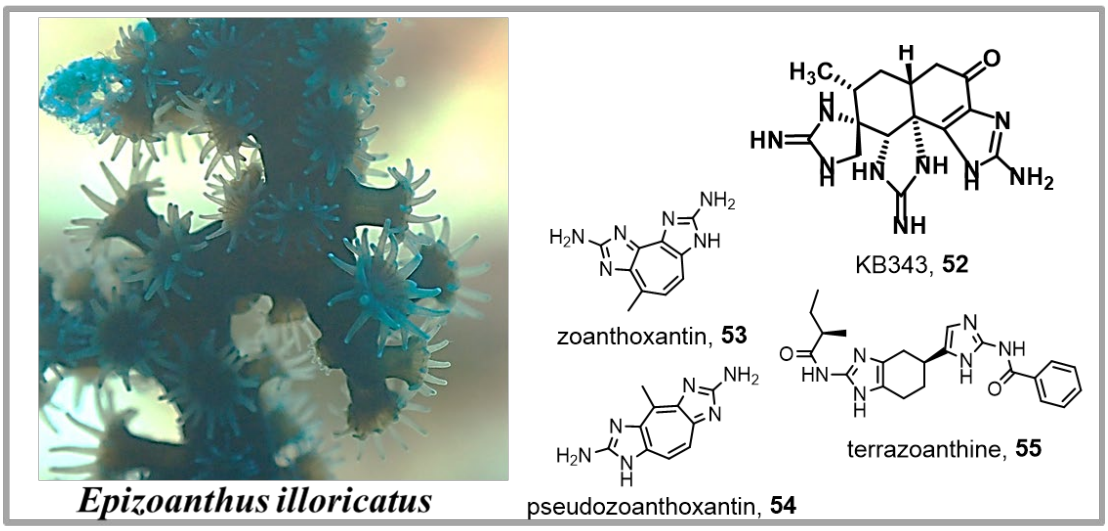
6

Polyguanidine alkaloids from zoantharians

Zoantharians, often referred to as zoanthids, belongs to hexacorallia, have been recognized

as rich sources of bioactive molecules [70]. The most prominent example, palytoxins are complex polyols with potent toxicity in animals. In our screening study, we found the aqueous extract of *Epizoanthus illoricatus* collected in Palau induced convulsion in mice. Separation of the extract afforded a fraction that induce the convulsant activity, and a fraction that shows cytotoxicity. Further separation of the cytotoxic fraction resulted in isolation of a KB343 (**52**) [71]. The structure of **52**, determined by extensive analysis of NMR and mass spectral data to be a novel tris-guanidine alkaloid where three guanidines are condensed in the southern hemisphere of the molecule. Although several polyguanidine alkaloids, such as Palau'amine [72] are known in sponges, the compound that have three guanidino group in one carbon skeleton is not precedented.

Scheme 10 Guanidine alkaloids from zoanthalian

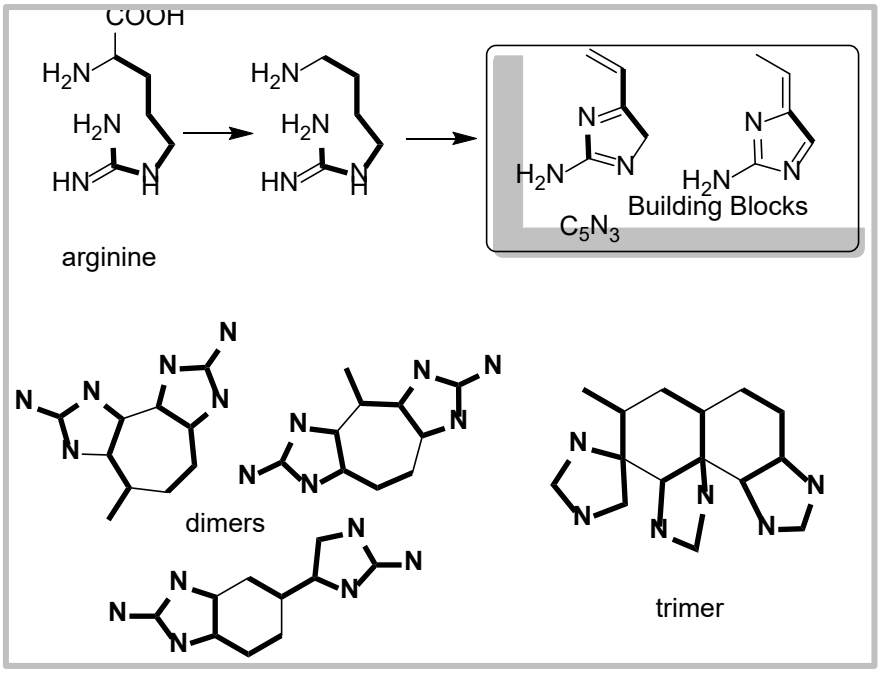


KB 343 was toxic to both mice and to cultured cells. An i.c.v. injection of **52** in mice resulted in death without any noticeable neuronal symptoms with slow onset (1 to few days). Cytotoxicities of **52** against murine leukemia L1210, human tumor Hela and model neuronal cell SH-SY5Y were moderate with IC_{50} value of 1.96, 4.93, and 3.90 μM, respectively. Compound **52** also suppressed growth of baker's yeast. These observations collectively suggested that **52** permeate the cell membrane slowly and interact to putative cellular target to evoke biological activity. Given that the high-water solubility and *C*LogP value (-4.75), it is reasonable to assume that the molecule cannot permeate cell membrane by simple diffusion. Interestingly, however, guanidino group is known to play a key role in polar molecules that can penetrate cell membrane [73]. For example, cell penetrating peptides (CPP) generally contain several arginine residues and the guanidino group interact negatively charges species in the cell surface resulting in internalization through endocytosis or other undefined mechanisms [73]. Thus, a unique structural feature, three

guanidines arranged in the bottom of the molecule, may play a key role in cell penetration of this molecule.

Several structurally related guanidino derivatives are reported in *Epizoanthus*, *Parazoanthus*, *Terrazoanthus*, and *Palythoa* [70]. These compounds commonly contain 2-amino imidazole groups as found in KB343. Zoanthoxantin (**53**) and pseudozoanthoxantin (**54**) form 14π aromatic system with liner and angular arrangement of two aminoimidazole via 7-membred ring, respectively. Terrazoanthine (**55**) also has two aminoimidazole units without fused aromatic system [74]. These molecules are hypothesized to be composed of arginine derived C_5N_3 building blocks. Cyclic addition of the building block lead to the formation of the above compounds. KB343 (**52**) is also envisioned as the biosynthetic product of the C_5N_3 building block, as tandem Diels-Alder type cycloadditions between three building blocks can formulate the skeletal structure of **52**. Thus, zoanthalians have biosynthetic machinery to convert arginine to the C_5N_3 compounds and assemble them into structurally diverse polyguanidine alkaloids.

Scheme 11 The C5N3 building blocks



7

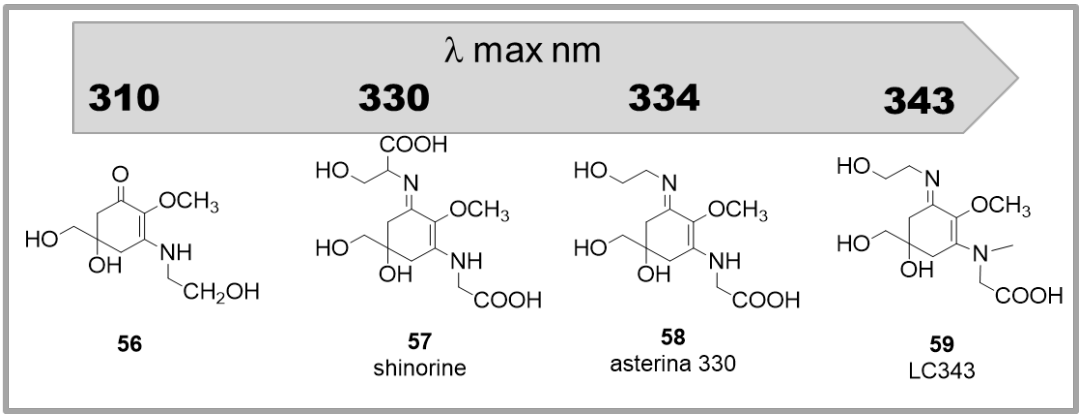
Mycosporines

Mycosporines (MA) are water soluble heterocyclic amino acid derivatives found widely in aquatic organisms [75,76, 77]. They commonly derived from 4-deoxygadusol where one or two amino acid sidechains are incorporated by forming aminocyclohexenone or aminocycloheximine ring, respectively. MAs absorb harmful UV-A light very efficiently thus thought to play roles as natural sunscreen. Other additional functions beneficial to aquatic

organisms including regulation of osmotic stress, heat or desiccating stress, antioxidation have also been found [77].

MAs are distributed widely in marine organisms, ranging from bacteria, microalgae, dinoflagellates, to red algae. They are also found both in invertebrates and vertebrates. This ubiquitous presence of MAs in aquatic organisms implicate its importance in marine ecosystem. Because MAs are found often in invertebrate species that associate symbiotic microorganisms, MA profiling, i.e. determining the presence of MA may help us to gain insight into the symbiotic relationship between the host animals and the symbionts.

Scheme 12. Mycosporines from *L. chondrodes*



This idea in mind, we isolated MAs in the aqueous extract of *L. chondrodes*, the sponge that produces dysiherbaine [75]. The major MA in the extract absorbed λmax of 343 nm was named LC343 (**59**). We also isolated asterina 330 (**58**) and shinorine (**57**) as known MA and mycosporine ethanolamine **56** as a new MA. The physical properties of LC 343 were particularly interesting; first, it gave only broad NMR signals in water at room temperature and were tend to be broadened even at higher temperature while closely related **58** gave sharp NMR signal. Because the methyl signal was particularly broadened suggesting that the methyl substituent at the imine nitrogen drastically affected the dynamics of the molecule. Furthermore, introduction of the methyl group markedly shifted absorption maxima by 14 nm. Although the mechanism of the bathochromic shift is yet to be investigated, it is interesting that a small change in the sidechain structure of MAs can fine tune the photochemical property of the molecule. Because *L. chondrodes* inhabits over shallow tropical coral reefs in Micronesia, it is constitutively exposed to strong sunlight. Therefore, the presence of MAs is the key to their survival.

Because in the depth where the sponge thrives (5-10 m), UV-A (315-400 nm) light can penetrate sea water to reach to the benthic organisms, covering wavelength with various MA is reasonable strategy to maximize the protection efficacy.

Tropical marine invertebrate largely relies their survival on symbiosis. The most well-

known example is symbiosis between coral and symbiotic symbiodiniacean cells (dinoflagellate family Symbiodiniaceae). It has been generally accepted that symbiodiniacean cells have ability to biosynthesize MA, and coral utilize these metabolites for protection. Recently, however, gene set for MA biosynthesis present in coral genome has suggested that coral itself can be a producer of MA [78]. These recent observations illustrated complex nature of MA dynamics in coral reef symbiotic ecosystem. Besides corals, giant clams are representative benthic invertebrates that harbor symbiodiniacean cells. We recently studied MA profiles in a species of giant clam, *Tridacna crocea* (*T. crocea*), commonly referred to as "boring clam". The clam, inhabiting in shallow coral reef, harbours symbiodiniacean cells in its mantel. The clam shell is open during the day and symbiodiniacean cells in the outer mantel were exposed to strong sunlight (Figure 3). This life style maximize production of photosynthetic substance by symbiodiniacean cells while risk of UV exposure needs to be managed. This problem is thought to be compromised by MAs, however, exact mechanisms of protection was not known. We used metabolomics approach as well as mass and UV imaging analysis to visualize localization of MAs in the mantel of the clam and identified eleven MAs with varying absorption properties [76]. The imaging experiment indicated that the localization of MA was 'selective' as the outmost layer of the mantel had high density of processed MAs which absorbs light with 330-360 nm, while the inner layer was rich in precursor MAs that can be transformed to more complex derivatives. These finding suggested the 'smart use' of precious sun screen in the clam where costly but most effective derivatives are transformed and accumulated at the site where the frontline defence from the UV light required the most.

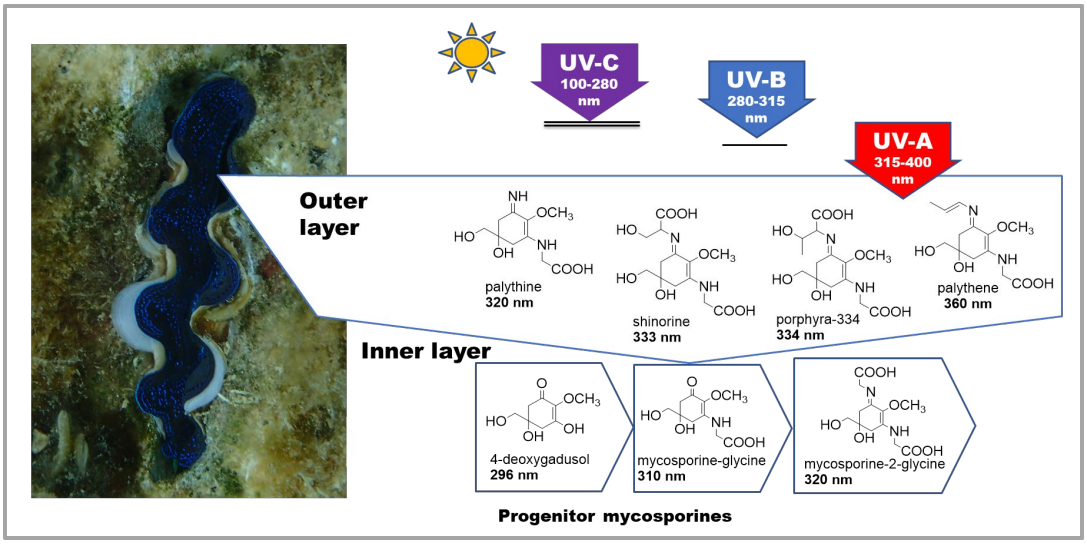


Fig. 3. "Smart use" of mycosporines in the giant clam. Mass-imaging experiments showed uneven distribution of MAs in the mantel tissue of *T. crocea*, where MA and putative precursors with UV-B

absorbing capability were localized all over the mantel. Biosynthetically 'processed' derivatives localized only in the outer layer of the mantel where is most severely suffered by UV-A penetration.

8
Conclusion

We have demonstrated that aqueous extracts of marine organisms are attractive sources of biologically active and functional molecules with chemical space distinguishable from the lipophilic compounds. Most of the above compound possess new-class chemical structure and biosynthesis. As represented by dysiherbaine, molecule with unique structure lead to discovery of yet undescribed physiological functions of living organisms and that may lead to drug discovery. Marine ecosystem differs largely from that of terrestrial as it is in the water milieu. Water soluble molecule like mycosporines can be useful markers to truck complex food web and symbiotic relationship of marine animals. Moreover, large production, either by chemical or biological means, mycosporines are of great commercial value in the area of skin care/cosmetic industries.

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