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Cellular Origin of Dysiherbaine, an Excitatory Amino Acid Derived from a Marine Sponge

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The cellular origin of dysiherbaine, a marine-sponge toxin, was investigated immunohistochemically by using an anti-dysiherbaine antibody. Dysiherbaine-like immunoreactivity was found to be localized in spherical cells harbored in the sponge mesohyl. A combination of ribosomal RNA gene (rDNA) analysis and cell-morphology analysis revealed that the spherical cells were Synechocystis *cyanobacteria. However, the sponge, identified as* Lendenfeldia chondrodes *on the basis of its rDNA sequence, appeared to contain two different chemotypes—dysiherbaine-producing (DH^+) and nondysiherbaine-producing (DH^-)—both of which inhabited the same region.* Synechocystis *cells in the DH^- sponge were not labeled with antibody, although the 16S rDNA gene profile of the cyanobacteria in the DH^- sponge was indistinguishable from that of the cyanobacteria in the DH^+ sponge. On the basis of these results, we hypothesize that dysiherbaine is a metabolite of certain varieties of endosymbiotic* Synechocystis *sp.*

Introduction

Although the chemical diversity of marine-derived secondary metabolites is attractive for drug discovery and development, difficulties in the sustainable production of the structurally complex compounds limit their industrial use. Mariculture and large-scale synthesis can only be applied to highly promising targets.[1] Thus, the large chemical and genetic diversity of marine natural products cannot be exploited effectively by the pharmaceutical industry. Because marine invertebrates that are rich in bioactive metabolites, such as sponges and tunicates, often harbor symbiotic microorganisms, such as bacteria, microalgae, or fungi, the secondary metabolites are thought to originate from the symbionts, especially when the biosynthetic pathways of the metabolites are related to those of known microorganism products.[2] However, the replication of the secondary metabolism in a laboratory culture by isolating symbiotic microorganisms is difficult. Perhaps the unique secondary metabolism takes place only in the symbiotic milieu and depends on unique host–simbiont interactions. In many cases, the true origin of a secondary metabolite is an unculturable symbiotic microorganism.[3]

Recently, metagenomic approaches enabled the identification of several sponge-derived polyketide genes on the basis of homology-based screening with known polyketide synthese (PKS) sequences. However, the culture-independent production of the bioactive molecules by heterologous expression techniques is still at an experimental stage.[3–7] To date, the only examples of successful heterologous production of marine secondary metabolites have been the production in *Escherichia coli* of patellamides,[8,9] cyclic peptides produced by tunicate-associated cyanobacteria. In these cases, knowledge of the putative producer of the peptide and genomic information seem to reduce the inherent complexity of gene collection.[10] Thus, information about the actual producer of secondary metabolites would be a great advantage for selecting target cells. Although the direct identification of the actual producer of certain metabolites in sponge tissue might be difficult, knowledge of the cellular localization of the metabolite would be a good starting point. Such information could be used to select the cell itself, although the site of biosynthesis might not always be the same as the site of storage.

In the present study we investigated the cellular localization of dysiherbaine (DH), a novel excitatory amino acid isolated from the marine sponge formerly identified as *Dysidea herbacea* and collected in Yap, Micronesia.[11,12] *D. herbacea* and related dictyoceratid sponges are common species found in shallow coral reefs in tropical Pacific waters and known to produce a variety of secondary metabolites, such as brominated diphenyl ethers, chlorinated peptides, and terpenoids.[13] As this species forms a consortium with endosymbiotic microorganisms, including the photosynthetic cyanobacterium *Oscillatoria spongeliae*,[14,15] there has been significant interest in determining the actual producers of the secondary metabolites in the sponges.[13,16,17] The cellular localization of some metabolites was assessed by cell-separation studies, the results of which indicated that the halogenated compounds had their origin in

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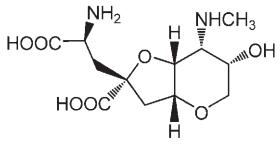
the cyanobacteria, whereas the terpenoids were produced by the sponge cells.[13,16,17] Moreover, the catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) technique revealed that the biosynthetic genes of chlorinated peptides were localized in the symbiotic cyanobacteria and confirmed the biosynthetic origin of the metabolites.[18] In the case of DH, the putative producer was difficult to predict from its chemical signature, as the structure of DH is not related to that of any known products from sponges or cyanobacteria, or to that of other known natural products.[11]

Herein, we report evidence that DH is localized within endosymbiotic cyanobacterial cells of the genus *Synechocystis* harbored in the host sponge *L. chondrodes*, which was formerly identified as *D. herbacea*. We employed an immunohistochemical approach to assess the cellular localization of the compound; this enabled the visualization of DH immunoreactivity at subcellular levels. The exclusive localization of DH in the cyanobacterial cells suggested its bacterial origin.



dysiherbaine

Results

rDNA sequences of the sponge and symbiotic cyanobacteria

The DH-containing sponge was formerly identified as *Dysidea herbacea* solely on the basis of its morphology; however, it has been shown previously that the identification of the order of a sponge is extremely difficult.[19] A recent study indicated that genetic analysis can aid in the conclusive identification of such sponges.[19] We therefore examined the 5.8S–internally transcribed spacer 2 (ITS-2)–28S rDNA sequences of selected sponge specimens, as well as the 16S rDNA sequences of the cyanobacteria associated with the sponge specimens collected in Yap (Table 1).

Phylogenic analysis of the rDNA of the four sponge specimens indicated that all specimens belonged to a single clade of *L. chondrodes*, and that they differ distinctly from other *O. spongeliae*-associated dictyoceratid sponges, including *L. herbacea*, with high maximum-likelihood (ML) and neighbor-joining (NJ) bootstrap values (Figure 1). These results indicate that the taxonomy of the specimen that was formerly identified as *D. herbacea* should be revised. We next compared the 16S rDNA sequences of the *O. spongeliae* associated with the above-mentioned four representative sponge specimens. It was evident that all associated *O. spongeliae* strains belonged to the same clade, that is, the clade detected in *L. chondrodes* (Figure 1).

Occurrence of DH in sponges associated with *O. spongeliae*

During our continuing search for unusual amino acid derivatives in *D. herbacea* collected in Yap,[20,21] we noted that the DH content varied significantly among individual sponge specimens. However, the factors responsible for this difference, including taxonomic, geographic, ecological, and physiological factors, have not been investigated. We therefore collected individual (single colony) sponge specimens from different sites in Yap ($n = 24$) and analyzed these specimens for their DH content in the aqueous extract. The DH content was assessed by using a mouse behavioral assay,[22] as well as by analysis by high performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–ESIMS/MS). With both methods it was possible to detect DH in solution at a concentration of approximately 1 ppm, and the results obtained with the two techniques were in good agreement with each other.

The results showed clearly that *L. chondrodes* specimens obtained from Yap could be divided into two groups, namely, the chemotypes DH^+ and DH^- (Table 1). Although the concentration of DH in the DH^+ specimens varied significantly, the DH^- specimens did not contain detectable amounts of DH. No distinguishable morphological feature between DH^+ or DH^- specimens was evident; on the contrary, geographic factors appeared more important for DH production. As shown in Table 1, the sponges collected from the north-eastern and eastern reefs, sites 4 and 5, respectively, differed with regard to the occurrence of the DH^+ chemotype. Interestingly, none of the eastern-reef collection contained detectable amounts of DH.

Table 1. DH content revealed by using a mouse assay, and LC–MS analysis of various *L. chondrodes* specimens collected from different sites in Yap, Micronesia.

Sample	Site[a]	Toxicity[b]	*c*(DH)[c] [ppm]	Selected samples
Yap 030801-2-1	1	6	0.5	
Yap 030801-2-2	1	6	12.1	
Yap 030801-2-3	1	3	0	DH^- 1
Yap 030801-2-4	1	6	8.2	
Yap 030801-3-1	2	2	0	
Yap 030801-3-2	2	6	15.7	DH^+ 1
Yap 030801-3-3	2	6	11.5	DH^+ 2
Yap 030802-2-2	3	6	12.6	
Yap 030802-2-3	3	3	0	
Yap 030802-2-4	3	3	0	
Yap 030731-1-1	4	7	27.1	
Yap 030731-1-2	4	3	0	
Yap 030801-4-1	4	2	0	
Yap 030801-4-2	4	2	0	
Yap 030731-2-1	5	3	0	
Yap 030801-5-1	5	3	0	
Yap 030801-5-2	5	3	0	
Yap 030801-5-3	5	3	0	DH^- 2

[a] Location of the collection sites: 1: Sunrise Reef, 9.64119° N, 138.2320° E; 2: Choul Reef, 9.61956° N, 138.2445° E; 3: Wanyan Reef, 9.561024° N, 138.2555° E; 4: Pelak Reef, 9.499054° N, 138.2311° E; 5: East Reef, 9.445839° N, 138.1417° E. [b] The behavioral toxicity of the extract was determined by using a mouse assay. The activity was graded by using scores ranging from 1 to 7 (see the Experimental Section for details). Note that a score of 3 might result from the action of other excitatory amino acids, such as glutamate. The absence of DH in all cases was confirmed by LC–MS. [c] Concentration of DH in the diluted extract (1 mg mL^{-1}).

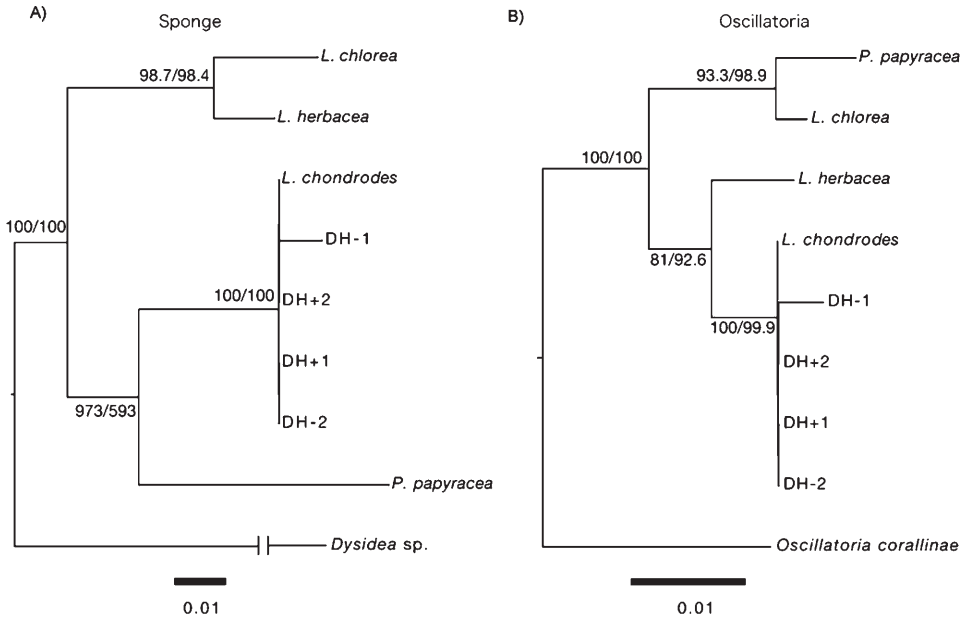


Figure 1. Maximum-likelihood phylogenetic trees constructed by using data on the rDNA of sponges and symbiotic cyanobacteria. Four different specimens (two DH^+ and two DH^- specimens, see Table 1) were used. The phylograms are derived from A) the sponge rDNA gene from *Dysidea* sp. (GenBank accession number: AY613969) as an outgroup, and B) the 16S rDNA gene of *O. spongeliae* associated with each of the named sponges, with *Oscillatoria* cf. corallinae (GenBank accession number: X84812) as an outgroup. The ML and NJ bootstrap values are given at the appropriate nodes (ML/NJ). The scale represents the expected number of nucleotide substitutions per site. The following sequences were used: *Lamellodysidea herbacea* (GenBank accession number: AY613962), *Lendenfeldia chondrodes* (AY613965), *Lamellodysidea chlorea* (AY613963), *Phyllospongia papyracea* (AY613968); *O. spongeliae* from *Lamellodysidea chlorea* (AY615504), from *Lamellodysidea herbacea* (AY615503), from *Lendenfeldia chondrodes* (AY615506), and from *Phyllospongia papyracea* (AY615509). The rDNA sequences from hosts and symbionts were deposited with the DNA Data Bank of Japan (DDBJ; AB364245–AB364252).

Morphological and histological overview

We next compared the morphological and histological characteristics between two representative specimens from each of DH^+ and DH^- Yap sponge populations. *L. chondrodes* from Yap share a common appearance: they have a thickness of 2–5 mm and encrust dead corals. We observed that the surface color of our specimens varied from green to grey; the surfaces were either smooth with a latticelike pattern or exhibited a projecting digitate outgrowth. However, there were no significant individual variations in the subdermal color (pale brownish violet). Light microscopy of the sections revealed very similar characteristics: filamentous cyanobacteria *O. spongeliae* were observed in the sponge mesohyl (Figure 2A, D, white arrows). Most notably, both types of specimens contained characteristic spherical cells of 10 μm in diameter (Figure 2A, D, black arrows). The spherical cells and *O. spongeliae* cells were separated from the fresh specimens, fixed, and then observed under light and epifluorescent microscopes. Spherical cells were distinguishable by their translucent appearance (Figure 2I, K < xfigr2). Both *O. spongeliae* cells and spherical cells emitted an orange fluorescence upon excitation at 460–490 nm; this suggests the presence of a phycobilin chromophore in both cell types (Figure 2G, H). DNA staining with 4′,6-diamino-2-phenylindole (DAPI) indicated the absence of a nucleus in the spherical cells (Figure 2J); however, fluorescent fragments found in some cells, which were probably in an early stage of cell division, showed the disperse localization of DNA (Figure 2L). These results suggest that at least two morphologically distinct endosymbiotic cyanobacteria reside in the sponge tissue. No distinguishable features between DH^+ and DH^- samples were revealed by histological observations.

Immunohistochemical studies

We next examined immunohistochemically the localization of DH in the sponge sections. Having recently demonstrated the cellular localization of kainic acid (KA) in the KA-producing red alga *Digenea simplex* at a high resolution by using immunohistochemical (IHC) techniques,[23] we applied similar protocols in the present study. We first prepared a DH-specific rabbit anti-DH antibody by using DH as a hapten. Rabbits were immunized, and the resulting serum was subjected to affinity purification with two columns: a protein A column to give immunoglobulin G (IgG), followed by an affinity column to which DH was conjugated (DH column). The fraction retained on the DH column was eluted to give the anti-DH antibody. The specificity of the antibody for DH was assessed by enzyme immunoassays. Concentration-dependent recognition was observed between 6 and 400 μM when DH was conjugated to bovine serum albumin (BSA) coated on the polystyrene plate, but not when a control amino acid was used in place of DH (Figure 3). Given that the concentration of DH in the sponge crude extract was about 1500 μM, this result indicates that the antibody can selectively label aldehyde-fixed DH in sponge tissue.

Localization of DH in sponge tissue

A freshly collected sponge (DH^+) was immersed in a paraformaldehyde–glutaraldehyde fixative. The sponge sections were treated with the anti-DH antibody or a rabbit-serum control and then labeled with a colloidal gold-conjugated antirabbit IgG antibody, followed by silver enhancement. Observation under a light microscope showed clearly a dense label on the spherical cells when the anti-DH antibody was used (Figure 2B, black arrows). However, the cells were not labeled when the antibody-treated section was washed with a DH-containing

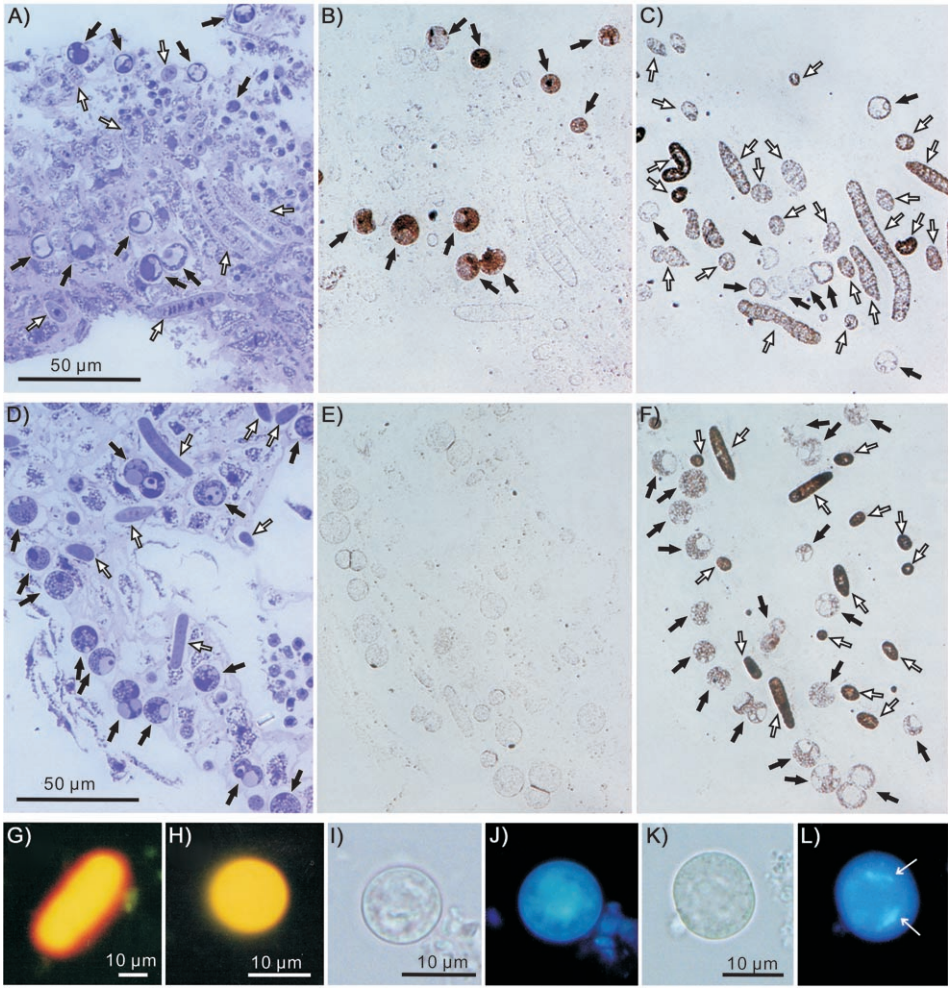


Figure 2. Light micrographs of the sponge-specimen sections: A) and D) stained with toluidine blue; B) and E) labeled with the anti-DH antibody; C) and F) labeled with an anti-phycoerythrin antibody. Sections A)–C) and D)–F) were prepared from the DH^+ and DH^- specimens, respectively. Both the *O. spongeliae* cells and the spherical cells (*Synechocystis* sp.) were labeled, as indicated by open and black arrows, respectively. G) Fluorescent image of an *O. spongeliae* cell; H) fluorescent image of a spherical cell; I) and K) light micrographs of spherical cells isolated from the sponge; J) and L) fluorescent images of DAPI-stained cells. The cell in K) and L) is presumed to be in an early stage of cell division.

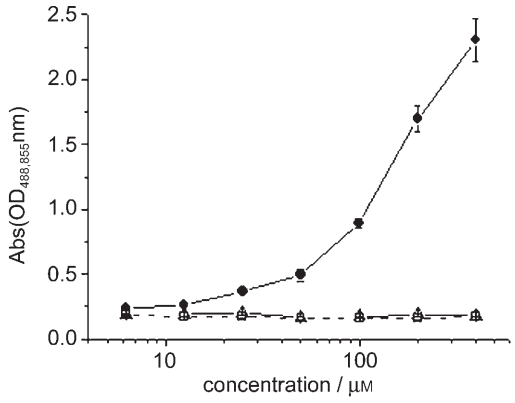


Figure 3. Recognition by the anti-DH antibody of DH (●), glutamic acid (△), and KA (□), each conjugated to a BSA-coated polystyrene plate with an aldehyde.

buffer (see Figure S1 in the Supporting Information). These results indicate that the labeling corresponds to DH immunoreactivity. The same IHC experiment with a DH^- specimen, however, did not result in labeling, although a large number of spherical cells were present (Figure 2D, E). The labeling appeared exclusively in the DH^+ specimen; that is, all spherical cells in the DH^+ section were labeled, but none in the DH^- section.

As epifluorescent microscopy revealed that the spherical cells contained phycoerythrobilin chromophore, the sponge section was treated with an anti-phycoerythrin antibody. As expected, both the *O. spongeliae* cells and the spherical cells were labeled (Figure 2C, F, arrows).

Cytological and immunocytochemical analysis of the spherical cells under a transmission electron microscope

As our results suggested that DH was present almost exclusively in the spherical cells, we observed the cells in detail under a transmission electron microscope (TEM). The cells had no apparent organelle; not even a nucleus was observed (Figure 4). A rigid thylakoid structure was absent, and the thylakoid suspended alone within the cytoplasmic area appeared somewhat dilated. Several other distinguishable inclusions were detected: 1) a large electron-dense body the diameter of which was almost half that of the cell (Figure 4, EDB), 2) a carboxysome (thin arrows), 3) a large vacuole (V) containing a large electron-dense cluster (*) or some fuzzy inclusions (open arrow), which are probably aggregates of the vacuole contents that result from fixation, and 4) smaller vacuoles distributed evenly in the cytoplasm. The cells could be grouped on the basis of their vacuolation pattern (Figure 4A, B). The outermost layer of the cell was the 30 nm-thick cell wall (Figure 4C), and dividing processes with cell-wall cleavage were observed (Figure 4D).

In contrast to the spherical cells, the *O. spongeliae* cells present in the same specimen formed a multicellular "hormogonium" protected by a surrounding sheath, as described previously (Figure 4E).[14] Each cell contained well-developed thylakoids (Figure 4F, Th) and had a thin cell wall (15 nm; Figure 4F, ar-

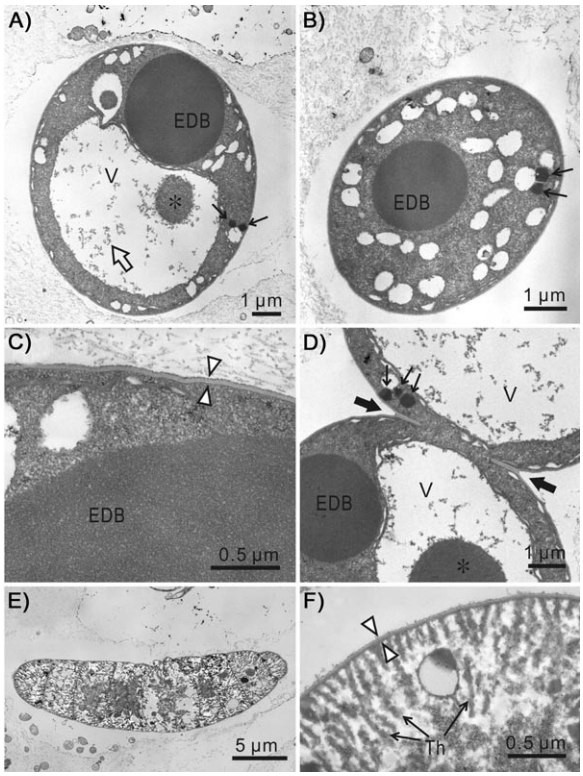


Figure 4. A)–D) TEM images of spherical cells showing a dilated thylakoid, large vacuoles (V) containing electron-dense clusters (asterisk), fuzzy inclusions (open arrow), an electron-dense body (EDB), and a carboxysome (thin arrow). B) Some cells contained smaller vacuoles distributed evenly in the cytoplasm. C) Magnified image of the spherical cell; the 30 nm-thick cell wall can be seen (arrowheads). D) A dividing cell; a cleft can be seen between the cells (black arrows). E) Image of an *O. spongeliae* cell. F) Magnified TEM image of an *O. spongeliae* cell showing a clear thylakoid structure (Th); the cell wall (15 nm thick) is shown between the arrowheads.

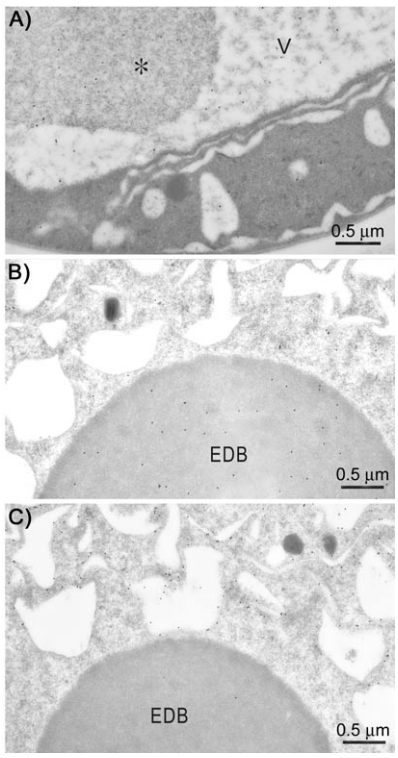


Figure 5. TEM images of immunolabeled sections of the spherical cells. The antigens are labeled with dots of colloidal gold: A) and B) Anti-DH antibody labeling showing DH immunoreactivity of the content of the large vacuole (V), an electron-dense cluster in the vacuole (*), and an electron-dense body (EDB). C) Section labeled with an anti-phycoerythrin antibody; the antigen is located mostly on the dilated membrane-like structure.

rowheads). Stellar bodies, which have been reported in some cases for this species,[14] were not observed in our specimens.

When the cell was treated with the anti-DH antibody followed by the colloidal gold-conjugated secondary antibody, the ultrastructures of the spherical cell were labeled distinctly. The contents of the large vacuoles were labeled most densely (Figure 5A). Gold particles were observed in the electron-dense cluster (*) as well as in the large EDB (Figure 5B). The dilated thylakoid-like cytoplasm was labeled to a lesser extent. Sponge cells (of any type) and the *O. spongeliae* cells were rarely labeled. The extent of labeling of the mesohyl, choanocyte, and *O. spongeliae* cells was negligible relative to that of the spherical cells. As the gold-particle density and antigen concentration have a linear relationship,[24] we compared the particle count in given areas of cells of the cyanobacteria and cells of the sponge. The average gold density in spherical cells, *O. spongeliae* cells, and sponge cells was 7.6 ($\pm$2.7), 0.25 ($\pm$0.45), and 0.25 ($\pm$0.45) counts μm^{-2}, respectively ($n = 10$ for all cell types). The two latter values are comparable with those obtained from a control experiment, in which the anti-DH antibody was "inactivated" prior to use by treatment with DH (see Figure S1 in the Supporting Information). Thus, we conclude that the labels in the sponge and *O. spongeliae* cells resulted from nonspecific labeling. As seen in the light microscope experiment, the treatment of the sponge section with an anti-phycoerythrin antibody led to clear labeling of both the *O. spongeliae* cells and the spherical cells; however, the localization pattern differed distinctly from that observed with the anti-DH antibody (Figure 5C). Prominently, the gold particles were most dense on the thylakoid-like inclusions, but were rare in other parts of the cell.

Identification of the spherical cell

Analysis of the 16S rDNA sequences of cyanobacteria revealed two representative genotypes besides *O. spongeliae*. On the basis of phylogenetic analysis, both sequences were found to be highly similar to that of *Synechocystis* sp.; one was nested within the lineage of the cyanobacterial clade reported to be associated with the *L. chondrodes* of Palau,[19] and the other was distantly separated from them at the node (ML bootstrap value: 100%, NJ bootstrap value: 99%; Figure 6). *Synechocystis* sp. has been known to associate with the sponges *Spirastrella* aff. *decumbens* and *Prianos* aff. *melanos*, and tunicates *Trididemnum tegulum* and *T. clinides*.[25] The morphological characteristics of the *Synechocystis* sp. associated with these organisms have been described in detail.[25,26] The electron micrograph of the *Synechocystis* sp. associated with Palauan

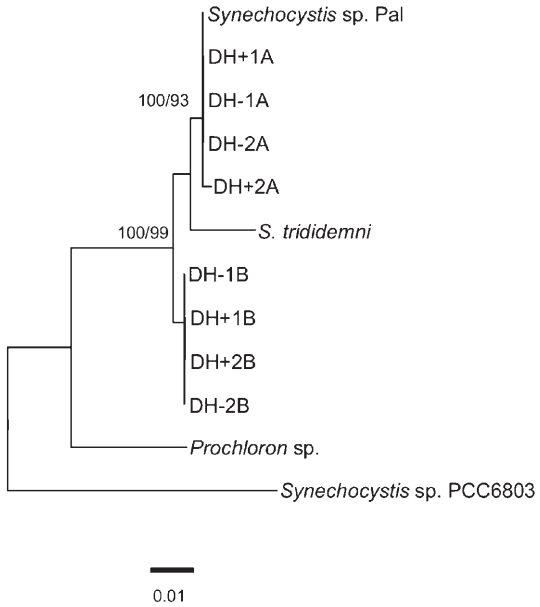


Figure 6. Maximum-likelihood phylogenetic trees constructed by using data from the 16S rDNA of symbiotic *Synechocystis* associated with *L. chondrodes* from Palau (*Synechocystis* sp. Pal; GenBank accession number: AY845229), *L. chondrodes* from Yap (two DH^+ and two DH^- specimens, see Table 1), *S. tridemni* (GenBank accession number: AB011380), and *Prochloron* sp. (GenBank accession number: X63141). *Synechocystis* sp. PCC6803 (GenBank accession number: BA000022) was used as the outgoup. Each of the Yap sponge specimens contained two distinguishable sequences of *Synechocystis*. The bootstrap probabilities of ML and NJ are given at the appropriate nodes (ML/NJ). The scale represents the expected number of nucleotide substitutions per site. The rDNA sequences of symbionts of *L. chondrodes* were deposited with the DDBJ (AB364253–AB364260).

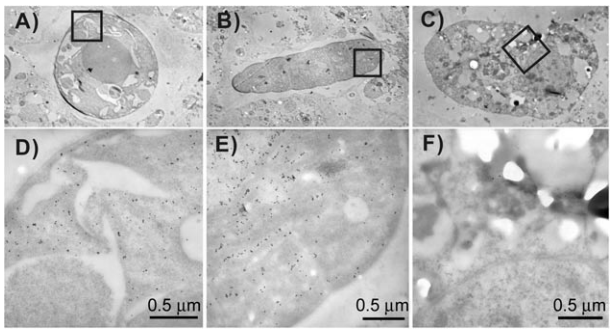


Figure 7. TEM images of the sections labeled with an RNA probe generated from full-length 16S rDNA of *O. spongeliae*: A) a spherical cell, B) an *O. spongeliae* cell, and C) a sponge cell. Gold particles were observed on both *O. spongeliae* cells and spherical cells. D)–F) Magnified images of the areas indicated in A)–C).

L. chondrodes has also been reported.[27] As the spherical cells found in the present study closely resemble those reported to have been found in the Palauan sponge, and as no other cells besides *O. spongeliae* that possess the characteristics of cyanobacteria were observed in our sponge specimens, the spherical cells are probably cyanobacteria.

This supposition was confirmed by in situ hybridization. When the section was treated with a DIG-labeled probe (DIG: digoxigenin) generated from full-length 16S rDNA of *O. spongeliae*, both the *O. spongeliae* cells and the spherical cells were labeled (Figure 7). This result shows clearly that the spherical cells are cyanobacteria. On the basis of the morphological, cytological, and genetic evidence presented herein, we conclude that the spherical cells are members of the genus *Synechocystis* sp., although we found two distinguishable sets of *Synechocystis* genes in one sponge specimen and different morphological characteristics (represented by different vacuolation patterns). The relationship between morphology and genetic differences has yet to be studied.

Discussion

By using the IHC approach, we have identified the cells of the symbiotic cyanobacterium *Synechocystis* sp. as the site inhabited by the excitatory amino acid dysiherbaine in the sponge *L. chondrodes*. Previously, the cellular localization of natural products derived from several sponges and tunicates has been determined by using various techniques, including cell separation,[13,16,17] cell culture,[28,29] and other visualization techniques.[30] In recent studies, the producers of some polyketides were tracked down by molecular biological strategies and identified as symbiotic bacteria.[4,8,10,18] Only two studies on the IHC localization of marine natural products have been described.[23,31] However, the IHC technique is advantageous because it enables the visualization of the in situ location of the antigen at a very high resolution. One of these studies, conducted by us, took advantage of this aspect of IHC and showed the localization of KA in the cells of the KA-producing alga *Digenea simplex*.[23] It revealed that KA is not only localized in the alga cells but also distributed densely within certain organelles, such as the nucleus.

The present study also demonstrated the localization of DH at a high resolution. The presence of DH exclusively in the cyanobacterial cells lends strong support to the idea that DH is biosynthesized within the cells; however, further evidence is required to conclude this issue. Observations on a subcellular level showed that DH is localized to a great extent in droplet-like inclusions of middle-to-high electron density and to a much lesser degree in the dilated thylakoid, which contains phycoerythrin. These observations suggest that the photosynthetic apparatus is not involved directly in DH biosynthesis and storage.

Although the symbiotic cyanobacterium *Synechocystis* sp. has been found in a few species of tunicates and sponges, including a specimen of *L. chondrodes* collected in Palau, its connection with the production or storage of secondary metabolites has not been discussed previously.[25,27,32] Interestingly, the symbiotic *Synechocystis* sp. bacteria described thus far share unusual characteristics in their morphology, which resembles closely the morphology of *Prochloron* sp.—a symbiotic cyanobacteria found in didemnid ascidians.[25,26,33] Judging from their morphological characteristics, *Synechocystis* sp. associated with sponges or tunicates are, as in the case of *Prochloron* sp., obligate symbionts that have presumably coevolved with the invertebrate hosts.

As didemnid ascidians are rich sources of bioactive peptidyl secondary metabolites, the relationship between the symbionts *Prochloron* sp. and the metabolites has been a matter of great interest. Recently, the production of patellamides, which are antitumor cyclic peptides from the didemnid ascidian *Lissoclinum patella*, was ascribed to the symbiotic cyanobacterium *Prochloron didemni*.[8,10] The gene that encodes the amino acid sequence of the putative patellamide precursor peptide was identified in the genomic sequence of *P. didemni*, and evidence was found for the ribosomal biosynthesis of patellamides. Notably, not all individuals of *L. patella* produce the peptide, but both peptide-producing and nonproducing specimens exist. Both types contain *Prochloron*, and no morphological difference between them is apparent. This observation is strikingly similar to the results of the present study with *L. chondrodes*. In the case of patellamide biosynthesis, the responsible gene clusters *pat* A–G were present in the patellamide-producing strains but not in the nonpatellamide-producing strains, although their 16S rDNA sequences were identical. These results suggest that in the case of the *L. chondrodes–Synechocystis* system, the cyanobacterial gene clusters responsible for DH production might be present only in the DH^+ specimens. The biosynthetic pathway of DH is not known, although there might be a polyketide pathway or a coupling of sugar and amino acid precursors; therefore the identification of such a gene cluster responsible for this process would be a significant challenge. Further genomic analysis of *Synechocystis* sp. of both DH^+ and DH^- specimens would provide a clue towards the identification of the genes responsible for DH biosynthesis.

Recent chemical and biological studies on marine secondary metabolites in relation to symbiosis have helped accumulate significant insight into the genetic origins of these metabolites. However, if the biological activity of a metabolite is not connected directly to any ecological event, then the role of that particular secondary metabolite in the producing organism and in the ecosystem might remain elusive. With regard to DH, whether its production affects the physiology of the sponge or that of the cyanobacteria is not clear. The survival of DH^+ and DH^- sponges together in the same environment and the fact that the population of the DH^+ sponge was not overwhelming indicate that there is no reproductive advantage related to DH production. The demonstration of positive DH immunoreactivity by all *Synechocystis* cells in DH^+ specimens suggests the vertical transmission of the cyanobacteria in the event of symbiosis; that is, certain types of *Synechocystis*, with or without the ability to produce DH, are probably acquired maternally. The pharmacological target of DH, an ionotropic glutamate receptor (iGluR) of the non-NMDA type (NMDA: *N*-methyl-D-aspartate), seems to have little significance in sponge physiology, in which no developed neuronal system exists. However, GluRs are highly diverse, and genes that encode GluRs and related molecules have been found not only in well-developed animals but also in sponge,[34] and even in the genomic sequences of cyanobacteria.[35] Therefore, DH might have functional roles in the sponge–cyanobacteria consortium. Further systematic study of both the sponge and symbiont is required to resolve this problem.

Experimental Section

Collection of sponge specimens: Specimens of *L. chondrodes* were collected in July 1998, August 2000, and August 2003 by scuba diving from various regions in the Yap State, Micronesia, after obtaining permission from the Department of Marine Resources, Yap State. Information on individual collections is listed in Table 1.

Fixation of the sponge: Each of the sponge specimens, Yap 030801-3-2 (DH^+) and Yap 030801-5-3 (DH^-), was cut into pieces (5 mm thick) and transferred to a tube that contained a fixative comprising glutaraldehyde (2.5%), sucrose (0.1 M), and sodium cacodylate (0.1 M) with 50% natural sea water for ultrastructural observation. For IHC and in situ hybridization, a fixative containing paraformaldehyde (4%), glutaraldehyde (0.05%), and sucrose (0.1 M) in 50% sea water was used. The fixed specimen was transferred to phosphate buffer (0.1 M) and maintained at 4 °C until further processing.

Extraction of the sponge: About 1 g of each sponge specimen was cut, homogenized with the same amount (*w/v*) of water, and centrifuged. Subsequently, the aqueous extract was lyophilized to afford the crude extract. About 50 mg of crude extract was obtained from 1 g of the sponge specimen. The crude extract was dissolved in water to a final concentration of 1 mg mL^{-1} for the bioassay and HPLC analysis.

DNA-sequence analysis: Total DNA was extracted from 10-mg samples of each specimen (Yap 030801-2-3, Yap 030 801-3-2, Yap 030801-3-3, and Yap 030801-5-3) by using the Puregene DNA Purification Kit (Gentra, Minneapolis, USA) according to the manufacturer's instructions. Touch-down PCR[36] was performed in order to amplify the 5.8S-ITS2–28S rDNA of the sponge by using the forward primer SP58bF (5′-AATCATCGAGTCTTTGAACG-3′) and the reverse primer SP28cR (5′-CTTTTCACCTTTCCCTCA-3′).[37] Sponge DNA was amplified by using KOD Dash DNA polymerase (Toyobo) according to the instructions of the manufacturer. The PCR was carried out under the following conditions: an initial denaturing step (5 min) at 95 °C; followed by 15 cycles at 98 °C for 10 s, 66 °C for 10 s (the lower temperature was decreased by 2 °C every fifth cycle until the temperature reached 62 °C), 72 °C for 25 s; followed by 15 cycles at 98 °C for 10 s, 60 °C for 10 s, 72 °C for 25 s; and a final elongation step at 72 °C for 3 min. The PCR products were purified by gel-permeation chromatography with Sephacryl S-400 HR gel (Amersham Biosciences, New Jersey, USA).

Cyanobacterial 16S rDNA was amplified by using the forward primer CYA359F (5′-GGG GAA TTT TCC GCA ATG GG-3′) and the reverse primer CYA1509R (5′-GGT TAC CTT GTT ACG ACT T-3′);[38] the total DNA isolated from each sponge specimen was used as template. The PCR was carried out under the following conditions: an initial denaturing step (5 min) at 95 °C; followed by 28 cycles at 98 °C for 10 s, 60 °C for 2 s, 72 °C for 20 s; and a final elongation step at 72 °C for 3 min.

The PCR product was purified with a QIAEX II gel extraction kit (Qiagen, Maryland, USA), according to the instructions of the manufacturer. The purified PCR product was cloned directly into the pCR4-TOPO plasmid (Invitrogen), and transformed into competent *E. coli* DH5α (Takara, Shiga, Japan). The sequencing reaction was carried out by using a DYEnamic ET terminator cycle sequencing kit (GE Healthcare, Buckinghamshire, UK) with the forward and reverse primers employed in the preceding PCR experiments. Sequencing was performed by using an ABI PRISM 3100-Avant Genetic Analyzer.

The nucleotide sequences obtained were aligned by using the program ClustalW 1.83[39] and checked manually. Phylogenetic trees were constructed by using the neighbor-joining method and the maximum-likelihood method without a molecular clock in PHYLIP 3.65.[40] For the neighbor-joining method, the distance matrix was computed by using the F84 model.[41] Ten replicate heuristic searches were performed in both methods with the random addition of sequences. To assess the reliability of the tree, bootstrap analysis was performed by using 1000 replicates.

In situ hybridization of cyanobacterial rRNA: In situ hybridization was performed according to the procedure of Waller and McFadden.[42] Each fixed and dehydrated sponge specimen was embedded in LR White acrylic resin (London Resin, London, UK). DIG–dUTP-labeled RNA probes were prepared by using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer. Sense and antisense probes were generated from one of the plasmid clones of the *O. spongeliae* 16S rDNA by using one of the two promoters of the pCR4-TOPO vector. The DIG-labeled RNA probes (5 ng μL^{-1}) were hybridized to ultrathin sponge sections at 65 °C for 3.5 h, and the bound probes were labeled with the anti-DIG antibody conjugated to colloidal gold (10 nm in diameter; British Biocell, London, UK). The sections were then observed as described below.

Behavioral assay: As DH displays characteristic epileptogenic activity in mice at very low doses (< 10 pmol per mouse) administered by an intracerebroventricular (i.c.v.) injection, the presence of DH was first surveyed by using a mouse assay.[22] The i.c.v. injection of the aqueous extract (1 mg mL^{-1}, 20 μL) into mice resulted in various behavioral toxicities when DH was present. The observed behaviors were scored as reported previously; the scores reflected the DH concentration in the extract. Animal experiments were carried out under the regulations of the Kitasato University Animal Experimentation and Ethics Committee. All efforts were made to minimize both suffering and the number of animals used.

HPLC–MS analysis: Each extract was analyzed by using an HPLC–ESIMS/MS system. HPLC was conducted with a Wako C30 NAVI (2× 150 mm) column (flow rate: 0.2 mL min^{-1}; 0.2% hexafluorobutyric acid/CH_3CN, 0–100% gradient over 8 min). N_2 was used as the collision gas for MS/MS analysis. The presence of characteristic product ions at *m/z* 287 and 186[12] was monitored. The DH concentration in each extract was estimated on the basis of the peak area of the selected ion chromatogram.

Preparation of DH-succinated carrier-protein conjugates: Succinated keyhole limpet hemocyanine (KLH–SU) was purchased from Sigma. BSA was succinated by treatment with succinic anhydride. DH was purified from *L. chondrodes* as described previously.[12] DH was conjugated to the succinated carrier protein by using the water-soluble-carbodiimide method.[43] Typical procedure: *N*-Hydroxysuccinimide (10 mg) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (10 mg) were added to KLH–SU (10 mg), and the resulting mixture was stirred for 10 min at 0 °C. DH (3.5 mg) and *N*-methylmorpholine (10 μL) were then added, and the reaction mixture was allowed to incubate for 12 h at room temperature. The mixture was then dialyzed against distilled water. To estimate the amount of DH conjugated per molecule of carrier protein, the product (KLH–SU–DH, 1 mg) was hydrolyzed with 6 N HCl at 115 °C for 12 h. The concentration of DH in the hydrolysate solution was estimated to be 1% by using the mouse assay. BSA-conjugated DH (BSA–SU–DH) was prepared in the same way with KLH–SU–DH.

Immunization and preparation of DH IgG: The antigen (KLH–SU–DH, 3.5 mg) was mixed with Freund's complete adjuvant (Rockland, 5 mL) and serine (5 mL). This mixture was injected subcutaneously into female albino rabbits. The antigen suspension was administrated at four different sites (150 μL at each site) along the back of the animals once every alternate week for 10 months. The production of anti-DH antisera was monitored by dot blotting with BSA–SU–DH as the positive control. The animals were sacrificed 10 months after immunization. The serum (35 mL) was subjected to affinity purification by using a HiTrap Protein A column (1 mL, Amersham Pharmacia Biosciences) according to the instructions of the manufacturer to yield the IgG fraction.

Preparation of anti-DH IgG: DH (1 mg) was conjugated to an *N*-hydroxysuccinimide-activated HiTrap column (1 mL, Amersham Biosciences) according to the instructions of the manufacturer. The IgG fraction obtained by the procedure described above was applied to this column, and the fraction bound to the column was eluted with an elution buffer to obtain the DH-binding fraction. This fraction was concentrated by centrifugal ultrafiltration to give anti-DH IgG.

Detection of antibody activity by ELISA: A polystyrene multiwell plate was coated with BSA or KLH (1 μg, 100 μL^{-1} in phosphate-buffered saline (PBS)) for 12 h at 4 °C. Serial dilutions of DH, glutamic acid, and kainic acid (50 μL each, starting with 400 μM) were added to the plates. The fixative (2% paraformaldehyde, 0.5% glutaraldehyde, and 0.2 M sucrose in phosphate buffer; 150 μL) was added to each well, and the plate was incubated at 37 °C for 2 h, then washed twice with washing buffer (2×200 μL), once with PBS (150 μL), and again with washing buffer (200 μL). The primary antibody (50 μL) was added to the plate, which was then incubated at 37 °C for 1 h. The plate was washed three times with a washing buffer, then a coloring buffer (0.1% *o*-phenylenediamine, 0.06% H_2O_2 in 0.05 M citrate buffer; 100 μL) was added and allowed to react for 10 min at room temperature. The reaction was quenched by the addition of 2 N H_2SO_4 (50 μL), and absorptions at 490/650 nm were measured. Mean triplicate ± SE values were plotted, and each data point was fitted to a sigmoid curve.

Transmission electron microscopy (TEM): The aldehyde-fixed specimen was processed for TEM as described previously.[23] In brief, the fixed sample was washed with the cacodylate buffer (0.1 M, pH 7.2), postfixed in 1% OsO_4 for 1 h at 4 °C, treated with hydrofluoric acid, and dehydrated. The sample was then embedded in Spurr's resin. For IHC preparation, LR White resin (London Resin) was used instead of Spurr's resin. The ultrathin sections were stained with uranyl acetate and lead citrate prior to observation under a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan) operating at 80 kV.

Immunohistochemistry: For IHC observation under a light microscope, a thin section (ca. 700 nm) was cut and blocked with Block Ace (Dai-Nihon Seiyaku, Osaka, Japan) for 30 min. The section was incubated with various concentrations of anti-DH IgG (starting concentration: 0.5 mg mL^{-1}), anti-phycoerythrin IgG (established from cryptophycean phycoerythrin, 0.015 mg mL^{-1}), or 2% rabbit normal serum, and then labeled with antirabbit IgG (H + L) goat polycolloidal gold (5 nm) conjugate (British Biocell, London, UK), followed by silver enhancement with a kit (British Biocell, London, UK). The sections were observed under a light microscope (BX-40, Olympus, Tokyo, Japan). The section without immunostaining was counterstained with toluidine blue for 5 min at 80 °C. For TEM, an ultrathin (70 nm) section was processed as above, labeled with the colloidal-

gold-conjugated secondary antibody (15 nm), and stained with uranyl acetate followed by lead acetate.

Fluorescent microscopic observation of cells separated from the sponge: A sponge sample was cut into pieces and filtered with a nylon mesh (50 μm). The filtrate was fixed with the same fixative used for the whole sponge and stored at 4 °C until use. This suspension contained sponge-cell debris, well-preserved *O. spongeliae* cells, and the spherical cells. The spherical cells were picked up manually by a thin grass capillary under a light microscope. These cells were observed under blue fluorescent light for the unstained phycoerythrin. DAPI (0.25 $\mu g\,mL^{-1}$) was then added to the cell preparation, and DNA-complexed DAPI (excitation at 330 nm) was observed.

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[1] J. Jimeno, J. A. Lopez-Martin, A. Ruiz-Casado, M. A. Izquierdo, P. J. Scheuer, K. Rinehart, *Anticancer Drugs* **2004**, *15*, 321–329.
[2] W. C. Dunlap, C. N. Battershill, C. H. Liptrot, R. E. Cobb, D. G. Bourne, M. Jaspars, P. F. Long, D. J. Newman, *Methods* **2007**, *42*, 358–376.
[3] J. Piel, *Nat. Prod. Rep.* **2004**, *21*, 519–538.
[4] J. Piel, D. Hui, G. Wen, D. Butzke, M. Platzer, N. Fusetani, S. Matsunaga, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16222–16227.
[5] J. Piel, *Curr. Med. Chem.* **2006**, *13*, 39–50.
[6] J. Kennedy, J. R. Marchesi, A. D. Dobson, *Appl. Microbiol. Biotechnol.* **2007**, *75*, 11–20.
[7] L. Grozdanov, U. Hentschel, *Curr. Opin. Microbiol.* **2007**, *10*, 215–220.
[8] E. W. Schmidt, J. T. Nelson, D. A. Rasko, S. Sudek, J. A. Eisen, M. G. Haygood, J. Ravel, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7315–7320.
[9] P. F. Long, W. C. Dunlap, C. N. Battershill, M. Jaspars, *ChemBioChem* **2005**, *6*, 1760–1765.
[10] E. W. Schmidt, S. Sudek, M. G. Haygood, *J. Nat. Prod.* **2004**, *67*, 1341–1345.
[11] R. Sakai, G. T. Swanson, M. Sasaki, K. Shimamoto, H. Kamiya, *Cent. Nerv. Syst. Agents Med. Chem.* **2006**, *6*, 83–108.
[12] R. Sakai, H. Kamiya, M. Murata, K. Shimamoto, *J. Am. Chem. Soc.* **1997**, *119*, 4112–4116.
[13] M. D. Unson, D. J. Faulkner, *Experientia* **1993**, *49*, 349–353.
[14] R. J. Berthold, M. A. Borowitzka, M. A. Mackay, *Phycologia* **1982**, *21*, 327–335.
[15] R. Hinde, F. Pironet, M. A. Borowitzka, *Mar. Biol.* **1994**, *119*, 99–104.
[16] M. D. Unson, N. D. Holland, D. J. Faulkner, *Mar. Biol.* **1994**, *119*, 1–11.
[17] A. E. Flowers, M. J. Garson, R. I. Webb, E. J. Dumdei, R. D. Charan, *Cell Tissue Res.* **1998**, *292*, 597–607.
[18] P. Flatt, J. Gautschi, R. Thacker, M. Musafija-Girt, P. Crews, W. Gerwick, *Mar. Biol.* **2005**, *147*, 761–774.
[19] C. P. Ridley, P. R. Bergquist, M. K. Harper, D. J. Faulkner, J. N. Hooper, M. G. Haygood, *Chem. Biol.* **2005**, *12*, 397–406.
[20] R. Sakai, C. Oiwa, K. Takaishi, H. Kamiya, M. Tagawa, *Tetrahedron Lett.* **1999**, *40*, 6941–6944.
[21] R. Sakai, K. Suzuki, K. Shimamoto, H. Kamiya, *J. Org. Chem.* **2004**, *69*, 1180–1185.
[22] R. Sakai, G. T. Swanson, K. Shimamoto, T. Green, A. Contractor, A. Ghetti, Y. Tamura-Horikawa, C. Oiwa, H. Kamiya, *J. Pharmacol. Exp. Ther.* **2001**, *296*, 650–658.
[23] R. Sakai, S. Minato, K. Koike, K. Koike, M. Jimbo, H. Kamiya, *Cell Tissue Res.* **2005**, *322*, 491–502.
[24] O. Waerhaug, O. P. Ottersen, *Anat. Embryol.* **1993**, *188*, 501–513.
[25] G. C. Cox, R. G. Hiller, A. W. D. Larkum, *Mar. Biol.* **1985**, *89*, 149–163.
[26] M. Hernandez-Marine, X. Turon, J. Catalan, *Phycologia* **1990**, *29*, 275–284.
[27] C. P. Ridley, D. J. Faulkner, M. G. Haygood, *Appl. Environ. Microbiol.* **2005**, *71*, 7366–7375.
[28] E. Richelle-Maurer, J. C. Braekman, M. J. De Kluijver, R. Gomez, G. Van de Vyver, R. W. Van Soest, C. Devijver, *Cell Tissue Res.* **2001**, *306*, 157–165.
[29] E. Richelle-Maurer, M. J. De Kluijver, S. Feio, S. Gaudencio, H. Gaspar, R. Gomez, R. Tavares, G. Van de Vyver, R. W. M. Van Soest, *Biochem. Syst. Ecol.* **2003**, *31*, 1073–1091.
[30] C. Salomon, T. Deerinck, M. Ellisman, D. Faulkner, *Mar. Biol.* **2001**, *139*, 313–319.
[31] O. Gillor, S. Carmeli, Y. Rahamim, Z. Fishelson, M. Ilan, *Mar. Biotechnol.* **2000**, *2*, 213–223.
[32] V. L. Webb, E. W. Maas, *FEMS Microbiol. Lett.* **2002**, *207*, 43–47.
[33] G. Cox, *New Phytol.* **1986**, *104*, 429–445.
[34] S. Perovic, A. Krasko, I. Prokic, I. M. Müller, W. E. Müller, *Cell Tissue Res.* **1999**, *296*, 395–404.
[35] G. Q. Chen, C. Cui, M. L. Mayer, E. Gouaux, *Nature* **1999**, *402*, 817–821.
[36] R. H. Don, P. T. Cox, B. J. Wainwright, K. Baker, J. S. Mattick, *Nucleic Acids Res.* **1991**, *19*, 4008.
[37] R. W. Thacker, S. Starnes, *Mar. Biol.* **2003**, *142*, 643–648.
[38] A. J. Martinez-Murcia, S. G. Acinas, F. Rodriguez-Valera, *FEMS Microbiol. Ecol.* **1995**, *17*, 247–255.
[39] J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* **1994**, *22*, 4673–4680.
[40] J. Felsenstein, University of Washington, Department of Genome Sciences, Seattle, **2005**.
[41] H. Kishino, M. Hasegawa, *J. Mol. Evol.* **1989**, *29*, 170–179.
[42] R. F. Waller, G. I. McFadden, *Methods Mol. Biol.* **2000**, *123*, 259–277.
[43] J. R. Moffett, M. A. Namboodiri, J. H. Neale, *J. Histochem. Cytochem.* **1993**, *41*, 559–570.

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