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Zooxanthellamide A, a novel polyhydroxy metabolite from a marine dinoflagellate of *Symbiodinium* sp.

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Abstract—A novel polyhydroxy metabolite named Zooxanthellamide A, $C_{128}H_{222}N_2O_{54}S_2$, was isolated from a cultured marine dinoflagellate, *Symbiodinium* sp., and the chemical structure was determined by spectroscopic methods. © 2003 Elsevier Science Ltd. All rights reserved.

1. Introduction

Marine dinoflagellates, unicellular phytoplanktons, produce various metabolites notable from the viewpoint of chemical structure and bioactivity; e.g. ciguatoxin, maitotoxin, and palytoxin.[1–3] Since dinoflagellate toxins are accumulated in higher organisms through the food chain or through symbiotic relationships, the identification of the true origin of marine toxins is difficult.[1,4]

Zooxanthellatoxins (ZTs) are compounds from a symbiotic marine dinoflagellate, *Symbiodinium* sp. (strain Y-6), belonging to the zooxanthellae, well-known symbionts distributed in a wide range of marine invertebrates. The ZTs contain a 62-membered lactone structure and exhibit potent vasoconstrictive activity.[5–8] The relationship between their bioactivities and unique structures is a challenging subject.

In the course of our studies on the function and distribution of ZTs, we found a structurally related compound to ZTs, named zooxanthellamide A (**1**), from one species of the genus *Symbiodinium* (strain HA3-5) isolated in the free-living state from an Hawaiian tide pool.[9,10]

2. Results and discussion

A 70% ethanolic extract of *Symbiodinium* sp. (strain HA3-5) was partitioned between EtOAc and water, and the water layer was extracted with *n*-BuOH. The *n*-BuOH extract was subjected first to polystyrene then to DEAE column chromatography and finally HPLC, to obtain zooxanthellamide A (**1**, 0.0035% from wet cells).

Zooxanthellamide A (**1**), isolated as a colorless amorphous solid, showed a bivalent pseudo-molecular ion at *m*/*z* 1356.72 and a tervalent pseudo-molecular ion at *m*/*z* 904.14 in the negative ESI-TOF-MS spectrum, suggesting a molecular weight of 2715.4. The IR spectrum indicated the presence of an hydroxy group (3385 cm^{-1}), a secondary amide group (1640 and 1560 cm^{-1}) and sulfate groups (1250 cm^{-1}). Structural elucidation was performed mainly by NMR experiments. The ^{13}C NMR and DEPT spectra in CD_3OD revealed 128 signals consisting of three carbonyl carbons, two sp^2 quaternary carbons, 22 sp^2 methines, two acetal carbons, one sp^3 quaternary carbon, 43 oxymethines, 6 sp^3 methines, 39 sp^3 methylenes and 10 methyl groups. It was deduced that 38 carbons (C3, C5, C7, C11, C12, C15, C17, C21, C4′, C5′, C8′, C10′, C14′, C20′, C24′, C27′, C29′, C34′, C35′, C36′, C37′, C39′, C43′, C44′, C47′, C50′, C51′, C53′, C67′, C68′, C71′, C72′, C75′, C76′, C77′, C3″, C4″ and C5″) were hydroxy-bearing carbons based on the deuterium shifts observed between the ^{13}C chemical shifts in CD_3OD and CD_3OH. Detailed analysis with 2D NMR experiments including COSY-90, DQF-COSY, HOHAHA, NOESY, FG-HMQC, and FG-HMBC led to elucidation of the structure of **1**. The ^1H and ^{13}C NMR data are summarized in Table 1. As this compound consists of three segments based on a continuous carbon framework, we detail the structural analysis in terms of these three segments.

2.1. Segment A

From the DQF-COSY and HOHAHA spectra, proton

Keywords: zooxanthellamide A; dinoflagellate; *symbiodinium*; polyhydroxy compound.

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Table 1. ^{1}H and ^{13}C NMR data for Zooxanthellamide A (**1**) in CD_3OD

Position	δ_C	δ_H (multiplicity,[a] J in Hz)	Position	δ_C	δ_H (multiplicity,[a] J in Hz)
1	180.42	–	38′	41.68	1.76, 1.73
2	45.56	2.36, 2.28	39′	71.40	3.83
3	69.01	4.08	40′	34.90	1.85, 1.58
4	45.06	1.62	41′	25.75	1.84, 1.74
5	70.08	3.97	42′	82.73	4.64
6	44.39	1.65, 1.55	43′	73.59	4.07
7	70.91	3.83	44′	76.97	3.36 (dd, 9.3, 3.3)
8	41.55	2.25, 2.20	45′	32.79	1.97
9	130.66	5.75 (dd, 15.2, 7.7)	46′	38.55	1.73, 1.48
10	133.18	5.56 (dd, 15.2, 6.6)	47′	71.89	4.15
11	76.83	3.92	48′	136.63	5.70
12	76.26	3.94	49′	131.91	5.71
13	131.42	5.68	50′	76.86	3.92
14	136.00	5.68	51′	72.47	3.73
15	71.95	4.26	52′	41.22	1.54
16	41.90	1.64, 1.54	53′	69.29	3.82
17	74.00	3.59	54′	39.38	1.51, 1.48
18	43.83	2.22	55′	23.21	1.60, 1.50
19	133.95	5.59	56′	37.61	1.49, 1.37
20	134.56	5.47 (dd, 15.5, 6.6)	57′	65.46	3.91 (ddd, 13.9, 10.6, 4.2)
21	73.35	4.05	58′	38.21	a 1.44, b 1.36 (dt, 10.6, 4.8)
22	41.87	2.29	59′	26.68	1.96
23	131.40	5.68	60′	41.57	a 1.56, b 1.46
24	133.21	6.08 (dd, 15.0, 10.5)	61′	98.51	–
25	133.30	6.20 (dd, 15.0, 10.5)	62′	37.12	a 1.38, b 1.54
26	128.20	5.57	63′	20.13	a 1.94, b 1.53
27	41.69	3.86	64′	32.72	1.55, 1.20
1′	174.22	–	65′	67.54	3.98 (ddd, 14.0, 10.7, 2.9)
2′	71.80	4.17 (dd, 6.7, 4.8)	66′	42.33	1.70, 1.43 (ddd, 14.0, 12.0, 4.8)
3′	32.55	2.12, 2.08	67′	68.89	4.03
4′	71.40	3.67 (dd, 6.4, 4.8)	68′	81.06	3.05 (dd, 7.6, 1.9)
5′	72.16	–	69′	33.81	2.03
6′	77.80	3.96 (dd, 9.6, 3.6)	70′	38.47	2.02, 1.46
7′	36.02	1.76, 1.68	71′	70.86	3.82
8′	70.42	3.82	72′	77.40	3.20 (dd, 8.0, 1.9)
9′	44.88	1.67	73′	69.01	4.19
10′	72.29	4.22	74′	31.10	1.96, 1.48
11′	136.22	5.49 (dd, 15.6, 8.9)	75′	67.17	4.11
12′	129.02	5.62	76′	70.42	3.58 (d, 2.9)
13′	39.31	2.26, 2.14	77′	99.58	–
14′	77.98	4.00	78′	46.81	3.64, 3.31 (d, 13.7)
15′	138.56	–	1″	175.60	–
16′	126.90	5.99 (d, 10.6)	2″	41.34	2.52 (d, 6.6)
17′	129.59	6.33 (dd, 15.0, 10.6)	3″	71.07	4.15
18′	131.26	5.66	4″	77.23	3.31
19′	42.22	2.36, 2.27 (dd, 15.0, 5.0)	5″	69.48	4.06
20′	69.16	4.37 (dt, 8.4, 6.4)	6″	40.29	1.89, 1.58
21′	131.65	5.23 (d, 8.4)	7″	76.61	4.64
22′	135.64	–	8″	44.44	1.71
23′	48.97	2.19	9″	30.55	1.57
24′	71.07	4.23	10″	38.80	1.35, 1.16
25′	134.37	5.66	11″	27.68	1.35, 1.29
26′	134.21	5.67	12″	33.36	1.26
27′	71.54	4.25	13″	23.75	1.31
28′	46.02	1.66	14″	14.47	0.89 (t, 7.1)
29′	68.59	3.77	18 Me	16.73	1.02
30′	46.09	1.43, 1.39	5′ Me	20.34	1.15 (s)
31′	34.39	2.44 (m)	15′ Me	12.40	1.72 (s)
32′	138.16	5.57 (dd, 15.2, 7.0)	22′ Me	17.17	1.71 (s)
33′	131.03	5.68	31′ Me	22.03	1.01
34′	72.80	4.34 (d, 6.4)	45′ Me	18.16	1.04 (d, 7.0)
35′	74.89	3.54	59′ Me	21.83	1.22 (d, 7.3)
36′	73.19	3.54	69′ Me	18.67	1.00
37′	70.16	4.10	9″ Me	19.97	0.92 (d, 6.4)

The ^{13}C and ^{1}H NMR spectra were recorded at 150 MHz and 600 MHz, respectively.
[a] Multiplicity; s: singlet; d: doublet; t: triplet; m: multiplet, unspecified: overlapping signals.

connectivities from H2 to H11, H12 to H13, and H14 to H27 were established. The connectivity from C1 to C2 was revealed by the cross peaks of H2/C1 and H3/C1 in the FG-HMBC spectrum. The connectivities from C11 to C12 and C13 to C14 were assigned by the FG-HMBC spectrum, which showed cross peaks due to H10/C12 and H15/C13, respectively. The methyl group on C18 was confirmed by the DQF-COSY cross peak of 18-Me/H18.

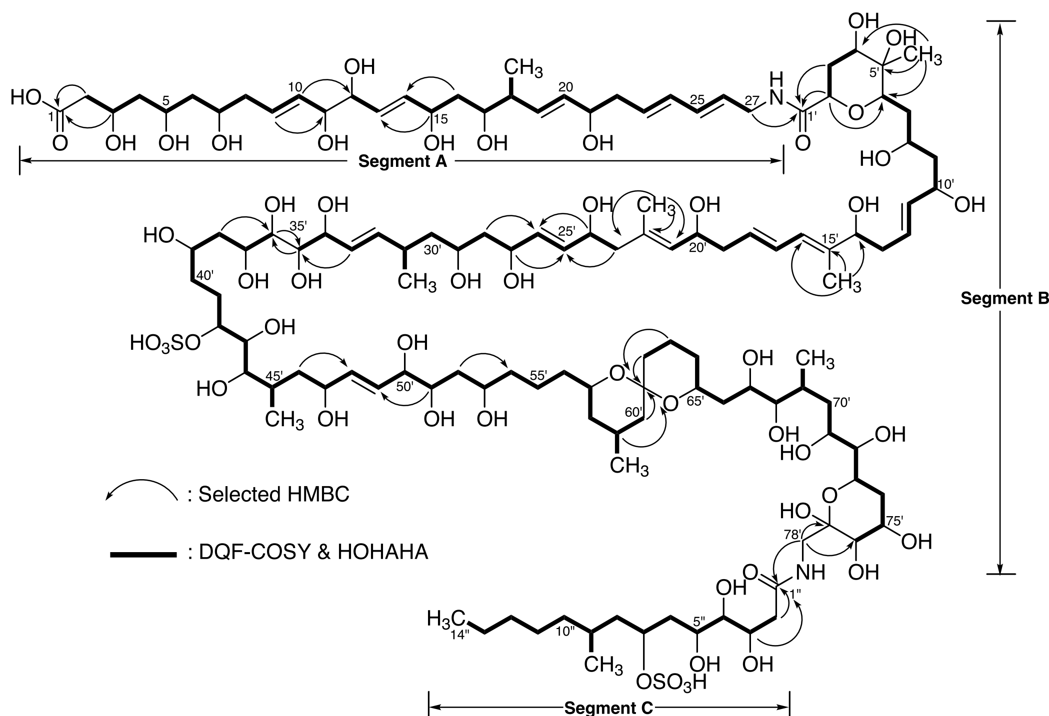

Figure 1. Structure and 2D NMR correlations of zooxanthellamide A (**1**).

2.2. Segment B

This segment is the longest of the three. Using DQF-COSY and HOHAHA spectra it was possible to trace the proton sequences from H2′ to H4′, H6′ to H14′, H16′ to H21′, H23′ to H35′, H36′ to H60′, and H62′ to H76′. C1′ was connected to C2′ by the FG-HMBC cross peaks of H2′/C1′ and H3′/C1′. The connectivities from C4′ to C6′, C14′ to C16′, and C21′ to C23′ were revealed by the FG-HMBC correlations from the tertiary methyl protons of 5′-Me, 15′-Me, and 22′-Me, respectively, to the neighboring carbons. The connectivity from C35′ to C36′ was also determined by the FG-HMBC cross peaks of H35′/C36′ and H36′/C35′. The connectivities relating to the two acetal carbons C61′ (δ_C 98.51) and C77′ (δ_C 99.58) were evident from the FG-HMBC cross peaks of H59′/C61′, H60′/C61′, H62′/C61′, H63′/C61′, and H78′/C77′. The connectivity from C76′ to C77′ was determined by the FG-HMBC correlation from H78′ to C76′. The location of the secondary methyl groups were confirmed at positions, C31′, C45′, C59′, and C69′, by the DQF-COSY and FG-HMBC correlations relating to these methyl protons. From these findings, the presence of two tetrahydropyran rings and one spiro ring was suggested in segment B. One tetrahydropyran ring (C2′ to C6′) was confirmed by the FG-HMBC cross peak of H2′/C6′. The relative configuration of this tetrahydropyran ring was estimated by conformational analysis based on NOE correlations and coupling constants. Although the information from the NOE also indicated the possibility of a chair conformation, the middle values of coupling constants (4.8–6.7 Hz) suggested a twist-boat conformation as shown in Figure 2. The other tetrahydropyran ring (C73′ to C77′) was not revealed by FG-HMBC correlations but was deduced from proton coupling constants and NOE correlations. Thus, the large and small coupling constants (*J*H73′/H74′a=12.2 Hz, *J*H74′a/H75′=12.5 Hz, and *J*H73′/H74′b=*J*H75′/H76′=2.9 Hz) and the NOE correlation of H73′/H75′ indicated the existence of this tetrahydropyran ring, which should adopt a chair conformation as shown in Figure 2. The presence of a dioxaspiro[5,5]undecane system (C57′ to C65′) was confirmed, as shown in Figure 2, by the following: the NOE correlations of H57′/59′–Me, H57′/H62′b, and H63′a/H65′; the proton coupling constants of *J*H57′/H58′a=10.6 Hz and *J*H64′a/H65′=10.7 Hz; and the *W* couplings observed for 59′-Me/H58′a and 59′-Me/H60′a in the COSY-90 spectrum.

2.3. Segment C

The proton connectivities from H2″ to H14″ were revealed by the DQF-COSY and HOHAHA spectra. The connectivities from C1″ to C2″ were determined by the FG-HMBC correlations of H2″/C1″ and H3″/C1″. The position of the C9″ methyl group was determined by the DQF-COSY cross peak for 9″-Me/H9″.

2.4. Whole structure of zooxanthellamide A

The FG-HMBC correlations of H27/C1′ and H78′/C1″ suggested that segments A and B, and segments B and C were connected. The geometries of the double bonds of C9=C10, C19=C20, C23=C24, C25=C26, C11′=C12′, C17′=C18′ and C32′=C33′ were determined to be all *E* by the proton coupling constants of 15.0–15.6 Hz. The geometries of two trisubstituted double bonds (C15′=C16′ and C21′=C22′) were assigned as all *E* by NOE correlations of H14′/H16′, 15′-Me/H17′, H20′/22′-Me, and H21′/H23′. The double bond C48′=C49′ was shown to have *E* geometry by the NOE correlations of H47′/H49′ and H48′/H50′. The geometries of C13=C14 and C25′=C26′ could not be determined by proton coupling constants or NOE experiments, as the chemical shifts of these olefinic

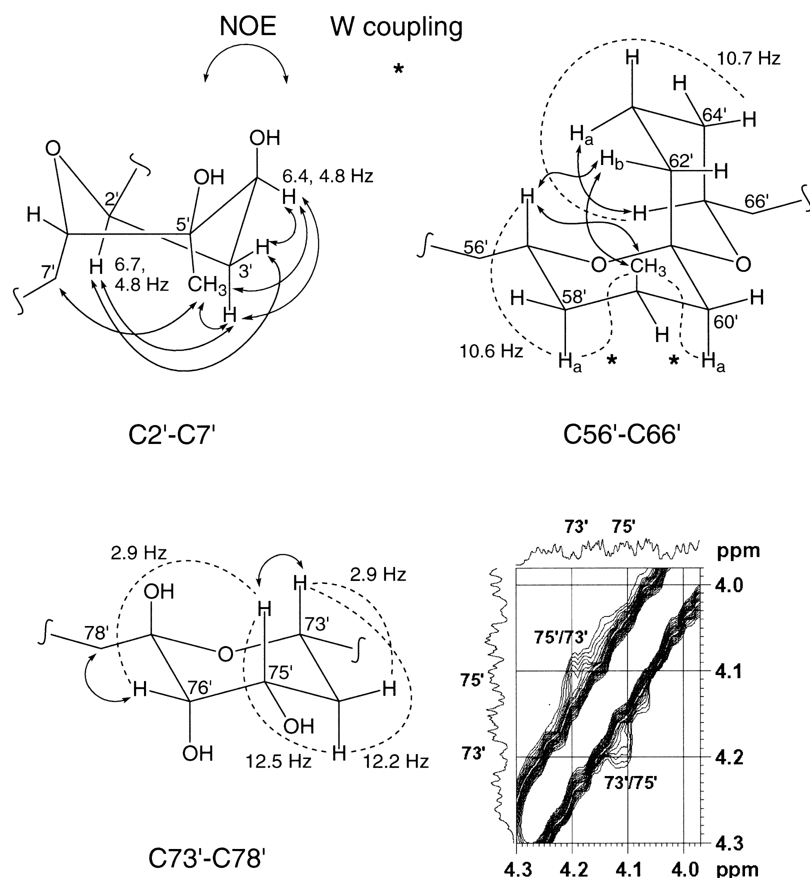

Figure 2. Relative stereochemistry of the spiro and two tetrahydropyran rings with a partial NOESY spectrum of zooxanthellamide A (**1**). The *J* values were obtained from a COSY-90 spectrum.

protons were indistinguishable from each other. The chemical shifts of ^{1}H and ^{13}C of C13=C14 were comparable to those of the double bond C48′=C49′ in the same structural environment. The high similarity of both values showed that C13=C14 has *E* geometry. The olefin C25′=C26′ was expected to have *E* geometry on the basis of the ^{13}C chemical shifts of allylic position C27′ (δ_C 71.54) that was quite similar to those of others C15 (δ_C 71.95) and C10′(72.29) in the same circumstances. The ^{13}C chemical shifts of allylic positions of *Z* olefins are generally observed at higher fields by 4–6 ppm than those of *E* olefins.[11] For example, the hydroxylated allylic positions of a *Z* olefin resonant at 68.2–68.4 ppm[12] and those of an *E* olefin at 72.3–72.4 ppm.[13] The difference between the molecular weight of 2715 from the MS and that of 2555 from the NMR analysis was explained by the presence of two sulfate groups. The sulfate groups were determined to be at C42′ and C7″ by the relatively low-field chemical shift of δ_H 4.64 as well as by insufficiency of deuterium shifts between the ^{13}C data in CD_3OD and CD_3OH. The possibility of phosphates rather than the sulfates being present could be excluded by the lack of spin coupling $^{2}J_{C-P}$ at C42′ and C7″. Thus, the structure of zooxanthellamide A (**1**) with partial stereochemistry of the ring systems is shown in Figures 1 and 2.

In summary, during the search for ZTs-related compounds, we isolated zooxanthellamide A (**1**) from a cultured marine dinoflagellate, *Symbiodinium* sp. (strain HA3-5). This compound possesses a smaller molecular weight than that of ZTs. The structure of **1** differs considerably from that of ZTs in that, unlike ZTs, **1** does not possess bisepoxide and exomethylene. In ZTs, the amide and sulfate groups exist singly, whereas there is a pair of both of these groups in **1**. In contrast, their partial resemblance might suggest that these metabolites arise from a similar biosynthetic pathway. In a test for vasoconstrictive activity, a positive control, ZT-A, was active as reported,[5] whereas **1** showed no activity (data not shown). The existence of a lactone structure like ZTs might be important for vasoconstrictive activity. Further studies including structural analysis of **1** and its congeners as well as the structure–activity relationship are in progress.

3. Experimental

3.1. General

UV spectrum was recorded on a JASCO V-530 spectrometer and was reported as wavelength (nm). The IR spectrum was determined with a JASCO FT/IR-8300 spectrophotometer and was reported in wave number (cm^{-1}). Optical rotation was measured on a JASCO DIP-370 polarimeter. The ESI-TOF-MS spectrum was obtained on an Applied Biosystems Mariner Biospectrometry Workstation in the negative mode. High-resolution ESI-TOF-MS was performed using polypropylene glycol as an internal standard. NMR spectra were obtained by a JEOL Alpha-600 (600 MHz for ^{1}H) or a Bruker AMX2-600 (600 MHz for ^{1}H) spectrometer. Chemical shifts (δ) of ^{1}H NMR were

given in parts per million (ppm) relative to the solvent peak of δ 3.30 (residual CD_2HOD) as an internal standard, and coupling constants (J) were in Hz. Chemical shifts (δ) of ^{13}C NMR were given in parts per million (ppm) relative to the solvent peak of δ 49.0 (CD_3OD) as an internal standard. HPLC was carried out using a JASCO PU-880 pump system equipped with a JASCO UV-875 UV/VIS detector.

3.2. Culture

The dinoflagellate *Symbiodinium* sp. (strain HA3-5) was cultured in a 3 L glass bottles containing 2 L of seawater with 20 mL ES supplement under 12 h light and 12 h dark conditions at 25°C. The supplement consists of the following in 20 mL of distilled water: $NaNO_3$ (70 mg), Na_2glycerophosphate·$5.5H_2O$ (15 mg), Fe–EDTA·$3H_2O$ (1 mg), $Na_2EDTA\cdot 2H_2O$ (5 mg), Tris (100 mg), H_3BO_3 (1 mg), thiamine·HCl (100 mg), biotin (1 mg), vitamin B_{12} (0.2 mg), $MnCl_2$ (200 mg), $ZnCl_2$ (25 mg), and $CoCl_2$ (5 mg). After 6 weeks, the culture media were removed by decantation and the cells were torn off with a brush and collected by filtration. The cells were kept at −80°C until use.

3.3. Extraction and isolation

The frozen cells (130.3 g from 198 L of culture) were immersed in 70% EtOH (200 mL) and then homogenized by an ULTRA-TURRAX T25 (Janke & Kunkel GmbH & Co. KG IKA-Labortechnik, Germany), soaked for 3 days, and centrifuged. The supernatant was collected and the extraction process was applied to the precipitates twice more with 70% EtOH (200 mL each). The combined extracts were evaporated in vacuo, the residue then suspended in water (110 mL) and extracted with EtOAc (200 mL×3) and then *n*-BuOH (200 mL×3).

The *n*-BuOH soluble fraction (907 mg) was applied to a polystyrene column (56 cm^3 of MCI CHP-20P 75–150 μm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan) and eluted with water (230 mL), 20% EtOH (230 mL), 40% EtOH (280 mL), 60% EtOH (170 mL), 80% EtOH (230 mL) and EtOH (230 mL), sequentially. The 40% EtOH eluate (172.8 mg) was applied to a DEAE Sephadex A-25 column (16 cm^3, Pharmacia Biotech Wikstroms, Sweden) and eluted with 33 mM phosphate buffer (pH 6.9, 130 mL) and then the same buffer with 0.2 M NaCl (80 mL). Each fraction was passed through a polystyrene column to remove inorganic salts. The 0.2 M NaCl eluate (40.1 mg) was purified by HPLC on a YMC-Pack D-ODS-5 (20 mm ϕ×250 mm, YMC Ltd, Kyoto, Japan) with 70% MeOH containing 20 mM NH_4OAc at a flow rate of 8.0 mL min^{-1} to give zooxanthellamide A (**1**) (t_R= 11.4 min, 4.6 mg, 0.0035%): a colorless amorphous solid; $[\alpha]_D^{29}=+1.9$ (c 0.36, MeOH); UV (MeOH) λ_{max} 232 (ε 49000) nm; IR (KBr) 3385, 1640, 1560, 1250, 1215, 1060, 970 cm^{-1}; ESIMS m/z 1356.72 $[M-2H]^{2-}$, 904.14 $[M-3H]^{3-}$; HRESIMS found m/z 904.1318 $[M-3H]^{3-}$, deconvoluted 2712.3954, calcd for $C_{128}H_{219}N_2O_{54}S_2$ [M−3H] 2712.3877. For ^1H and ^{13}C NMR data see Table 1.

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References

1. Shimizu, Y. *Chem. Rev.* **1993**, *93*, 1685–1698.
2. Yasumoto, T.; Murata, M. *Chem. Rev.* **1993**, *93*, 1897–1909.
3. Murata, M.; Yasumoto, T. *Nat. Prod. Rep.* **2000**, *17*, 293–314.
4. Mebs, D. *Toxicon* **1998**, *36*, 1519–1522.
5. Nakamura, H.; Asari, T.; Ohizumi, Y.; Kobayashi, J.; Yamasu, T.; Murai, A. *Toxicon* **1993**, *31*, 371–376.
6. Nakamura, H.; Asari, T.; Murai, A.; Kan, Y.; Kondo, T.; Yoshida, K.; Ohizumi, Y. *J. Am. Chem. Soc.* **1995**, *117*, 550–551.
7. Nakamura, H.; Asari, T.; Fujimaki, K.; Maruyama, K.; Murai, A.; Ohizumi, Y.; Kan, Y. *Tetrahedron Lett.* **1995**, *36*, 7255–7258.
8. Moriya, T.; Ishida, Y.; Nakamura, H.; Asari, T.; Murai, A.; Ohizumi, Y. *Eur. J. Pharmacol.* **1998**, *350*, 59–65.
9. Carlos, A. A.; Baillie, B. K.; Kawachi, M.; Maruyama, T. *J. Phycol.* **1999**, *35*, 1054–1062.
10. Baillie, B. K.; Belda-Baillie, C. A.; Maruyama, T. *J. Phycol.* **2000**, *36*, 1153–1161.
11. Pretsch, E.; Clerc, T.; Seibl, J.; Simon, W. *Table of Special Data for Structure Determination of Organic Compounds*, 2nd ed; Springer: Berlin, 1989; pp C10–C15.
12. Bloch, R.; Brillet, C.; Kuhn, P.; Mandville, G. *Heterocycles* **1994**, *38*, 1589–1594.
13. Guerrero, A.; Casals, I.; Busquets, M.; Leon, Y.; Manresa, A. *Biochim. Biophys. Acta* **1997**, *1347*, 75–81.