Luteophanol A, a New Polyhydroxyl Compound from Symbiotic Marine Dinoflagellate *Amphidinium* sp.

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Luteophanol A (**1**), a new polyhydroxy linear carbon-chain compound, has been isolated from the cultured marine dinoflagellate *Amphidinium* sp. which was isolated from the Okinawan marine acoel flatworm *Pseudaphanostoma luteocoloris*. The structure was elucidated by extensive analyses of 2D NMR spectra including PFG-HMBC, HSQC-HOHAHA, and INADEQUATE as well as FABMS/MS data. Luteophanol A (**1**) possesses a sulfate ester, two tetrahydropyrans, and nineteen hydroxyl groups on C_{57}-linear aliphatic chain with one exo-methylene and two methyl branches.

Marine dinoflagellates of the genus *Amphidinium* have been recognized as a source of novel bioactive secondary metabolites with unique chemical structures.[2−5] We previously isolated a series of cytotoxic macrolides, amphidinolides,[6] from dinoflagellates *Amphidinium* sp., which were isolated from Okinawan marine acoel flatworms of the genus *Amphiscolops*. In further search for unique natural products from dinoflagellates *Amphidinium* sp., we investigated another strain of *Amphidinium* sp. (strain number, Y-52), which was isolated from the inside of the cells of the Okinawan marine acoel flatworm *Pseudaphanostoma luteocoloris*. Here we describe the isolation and structure elucidation of a new polyhydroxyl compound, luteophanol A (**1**), consisting of C_{57} linear aliphatic chain possessing one sulfate ester and two tetrahydropyran rings.

The host invertebrate *P. luteocoloris* was collected at Zanpa, Okinawa, and the isolated dinoflagellate *Amphidinium* sp. (Y-52) was mass cultured unialgally at 25 °C for 2 weeks in seawater medium enriched with 1% ES supplement.[7] The cultured algal cells were harvested by centrifugation and extracted with MeOH−toluene (3:1). The extract was partitioned between toluene and water, and the aqueous phase was successively extracted with chloroform, ethyl acetate, and 1-butanol. The 1-butanol-soluble fraction was subjected to separation by two gel filtration columns on HP-Cellulofine (MeOH/H_2O, 1:1) and Sephadex LH-20 (MeOH), followed by purification with reversed-phase MPLC (ODS, 45% MeOH) to afford luteophanol A (**1**, 0.004%, wet weight), as a colorless amorphous solid, $[\alpha]^{29}_D$ −7.6° (*c* 1, MeOH). Electrospray ionization (ESI) MS of **1** prominently showed a quasi-molecular ion peak at *m/z* 1253 $(M - H)^-$, and its molecular formula was inferred as $C_{60}H_{102}O_{25}S$ from the HRFABMS data [*m/z* 1253.6352, $(M - H)^-$, Δ +4.0 mmu]. The UV and IR absorption spectra were indicative of the presence of conjugated diene chromophore (λ_{max} 232 nm), hydroxyl groups (ν_{max} 3400 cm^{-1}), and sulfate ester (ν_{max} 1230 cm^{-1}). The presence of sulfate ester in **1** was also suggested by intense fragment ions observed in the negative ion FABMS [*m/z* 80 (SO_3^-) and 97 (HSO_4^-)] and was further confirmed by ion chromatography of sulfate ions liberated by solvolysis.[8] The ^1H and ^{13}C NMR data (Table 1) suggested that **1** contains two sp^2 quaternary carbons, two sp^2 methylenes, twenty-three oxymethines, one oxymethylene, one sp^3 methine, and two methyl groups. The number of hydroxyl groups was suggested from the ^{13}C NMR deuterium-induced shift experiment using CD_3OH/C_5D_5N (2:1) and CD_3OD/C_5D_5N (2:1) as solvents. Of 24 signals observed for oxygenated carbons (δ_C 68−82), four oxymethines and one oxymethylene did not show the deuterium-induced upfield shifts. These four unchanged oxymethines (C-26, C-30, C-37, and C-41) were suggested to constitute two ether rings from the 2D NMR spectral data described below, and the remaining oxymethylene (C-1) was deduced to bear the sulfate ester.

The extensive 2D NMR experiments of **1** including DQF-COSY, HOHAHA, E-COSY,[9] ROESY, HSQC, pulse field gradient[10]-HMBC (PFG HMBC), and HSQC-HOHAHA[11] spectra were carried out in CD_3OD/C_5D_5N (2:1) solution for structure elucidation of **1**. In addition, INADEQUATE[12] spectrum was recorded using the ^{13}C-enriched sample of **1**, which was obtained by culturing this microalga with addition of $NaH^{13}CO_3$, to provide supporting data for assigning direct carbon−carbon bonds. Interpretation of these 2D NMR spectral data of

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Chart 1

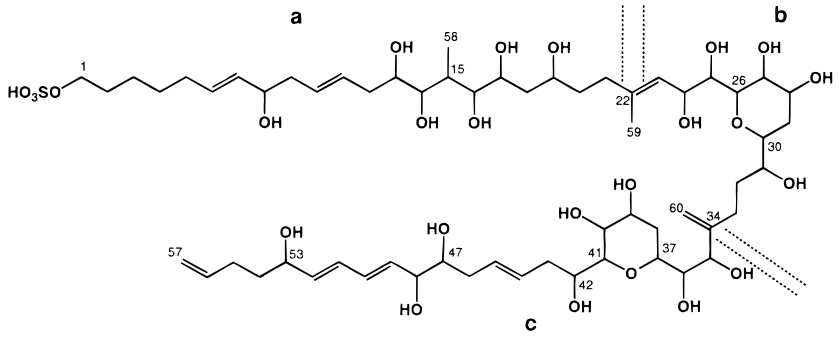

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Table 1. ^{1}H and ^{13}C NMR Data of Luteophanol A (1) in CD_3OD/C_5D_5N (2:1)

position	^{13}C	^{1}H		position	^{13}C	^{1}H	
1	69.5	4.10[a]	t	31	75.3	3.74	m
2	31.1	1.64[a]	m	32	33.4	1.72	m
3	27.1	1.35[a]	m			2.11	m
4	30.7	1.34[a]	m	33	28.9	2.25	m
5	33.9	1.94[a]	m			2.65	m
6	132.7	5.60	m	34	152.6		
7	135.1	5.51	dd	35	77.4	4.40	brd
8	74.1	4.08	m	36	76.0	3.54	brd
9	43.0	2.27[a]	m	37	71.4	4.24	dd
10	130.8	5.63	m	38 eq	32.6	1.67	m
11	131.6	5.74	m	ax		2.35	m
12	39.0	2.25[a]	m	39	68.1	4.22	m
		2.66	m	40	69.5	4.33	brs
13	73.3	3.71	m	41	81.5	4.02	brd
14	80.3	3.75	m	42	72.8	4.23	m
15	35.9	2.60	m	43	43.0	2.33[a]	m
16	81.0	3.74	m	44	130.8	5.63	m
17	73.1	3.91	m	45	130.8	5.63	m
18	42.0	1.67	m	46	43.0	2.33[a]	m
		2.10	m	47	68.3	4.23	dd
19	72.4	3.99	m	48	75.0	4.63	m
20	37.6	1.66	m	49	130.3	5.82	m
		1.70		50	135.5	5.90	m
21	37.5	2.13	m	51	130.3	5.84	m
		2.26	m	52	131.5	5.75	m
22	139.5			53	73.0	4.18	m
23	127.5	5.67	d	54	38.5	1.60	m
24	68.6	4.76	m			1.65	m
25	73.3	3.88	dd	55	31.6	2.15[a]	m
26	80.0	4.26	brd	56	140.4	5.82	m
27	69.7	4.33	brs	57	116.0	4.94	d
28	68.2	4.17	dd			5.00	
29 eq	31.5	1.96	dt	58	7.8	1.14[b]	d
ax		2.04	m	59	18.2	1.75[b]	s
30	76.6	3.64	m	60	113.6	5.03	s
						5.16	s

[a] 2H. [b] 3H.

1 led to the following three partial structures: (**a**) from C-1 to C-21, (**b**) from C-23 to C-33, and (**c**) from C-35 to C-57.

Partial Structure a. The proton-connectivities for the five subunits ($\mathbf{a_1}$−$\mathbf{a_5}$) were evident from the DQF-COSY spectrum (Figure 1): from H_2-1 to H_2-3 ($\mathbf{a_1}$); from H_2-4 to H-8 ($\mathbf{a_2}$); from H-11 to H-13 ($\mathbf{a_3}$); from H_3-58 to H-15 ($\mathbf{a_4}$); and from H-17 to H_2-21 ($\mathbf{a_5}$). The connectivities from C-3 ($\mathbf{a_1}$) to C-4 ($\mathbf{a_2}$) was assigned by PFG HMBC spectrum of **1**, which showed cross-peaks due to H_2-3/C-4, H_2-4/C-3, and H_2-5/C-3. The connectivities from C-8 ($\mathbf{a_2}$) to C-11 ($\mathbf{a_3}$) were revealed by PFG HMBC correlations for H-8/C-9, H-8/C-10, H_2-9/C-10, H-10/C-11, and H-10/C-12. A secondary methyl group was located on C-15 ($\mathbf{a_4}$), which was shown by the DQF-COSY cross-peak for H_3-58/H-15. The connectivities from C-13 ($\mathbf{a_3}$) to C-16 through subunit $\mathbf{a_4}$ were revealed by PFG HMBC correlations (H-13/C-14, H_3-58/C-14, and H_3-58/C-16). The C-16 was connected to the C-17−C-21 moiety ($\mathbf{a_5}$) by HSQC-HOHAHA correlations (H-16/C-17, H-16/C-18, and H-16/C-19). The C-18 methylene was assigned to a methylene located between two hydroxy-bearing carbons (i.e., 2-position of 1,3-diol) by characteristic NMR chemical shifts (δ_C 42.0 and δ_H 1.67, 2.10).[13] Thus, the partial structure **a** was elucidated.

Partial Structure b. For partial structure **b**, connectivities from H-23 to H-26 (subunit $\mathbf{b_1}$) and from H-27 to H-33 (subunit $\mathbf{b_2}$) were evident from DQF-COSY cross-peaks (Figure 2). These subunits $\mathbf{b_1}$ and $\mathbf{b_2}$ were connected by the PFG HMBC spectrum, which showed cross-peaks due to H-26/C-24, H-26/C-25, H-26/C-27, H-26/C-28, and H-26/C-30, also suggesting the presence of a tetrahydropyran ring. The coupling constants ($J_{26,27}$ = *ca.* 0 Hz, $J_{27,28}$ = *ca.* 0 Hz, $J_{28,29eq}$ = 3.3 Hz, $J_{28,29ax}$ = 13.3 Hz, and $J_{29ax,30}$ = 13.3 Hz) and a ROESY cross-peak (H-28/H-30) suggested that H-28 and H-30 were axially oriented on the tetrahydropyran ring and H-27 was equatorial.

Partial Structure c. The DQF-COSY spectrum suggested the proton connectivities from H-35 to H-42 (subunit $\mathbf{c_1}$), which was further supported by cross-peaks observed in the HSQC-HOHAHA (H-36/C-35, H-36/C-37, and H-37/C-39) and PFG HMBC (H-37/C-41, $H\text{-}38_{ax}$/C-37, $H\text{-}38_{ax}$/C-40, H-41/C-39, H-41/C-40, and H-41/C-42) spectra to suggest that this subunit ($\mathbf{c_1}$) contains another tetrahydropyran ring. The ROESY correlations (H-37/H-39) and the ^{1}H NMR coupling constants ($J_{37,38eq}$ = 3.8 Hz, $J_{37,38ax}$ = 10.8 Hz, $J_{38ax,39}$ = 13.3 Hz, $J_{39,40}$ = *ca.* 0 Hz, and $J_{40,41}$ = *ca.* 0 Hz) suggested that H-37 and H-39 protons on the tetrahydroxypyran ring were axially oriented and H-40 was equatorial. The ^{1}H NMR signals for the C-42−C-47 part (subunit $\mathbf{c_2}$), viz., H-42/H-47, H_2-43/H_2-46, and H-44/H-45, were observed in duplicate, respectively, due to symmetrical structure of this subunit ($\mathbf{c_2}$), and the DQF-COSY spectrum showed correlations from H-42 to H-44 and from H-47 to H-45, simultaneously. Thus, the symmetrical bis-homoallyl alcohol structure was assigned for this subunit ($\mathbf{c_2}$). The DQF-COSY spectrum also revealed correlations from H-47 to H_2-55 (subunit $\mathbf{c_3}$) as well as the cross-peak due to H-56/H_2-57 (subunit $\mathbf{c_4}$). Although the DQF-COSY correlation was obscure between H_2-55 and H-56, the connection between C-55 ($\mathbf{c_3}$) and C-56 ($\mathbf{c_4}$) was suggested by the HMBC correlation of H_2-57/C-55, thus accounting for the partial structure **c**, viz., from C-35 to one end of the molecule (C-57).

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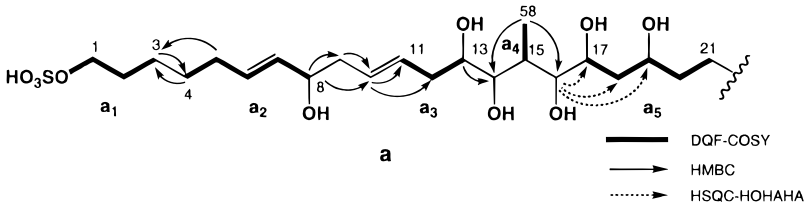
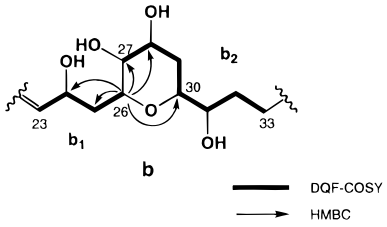


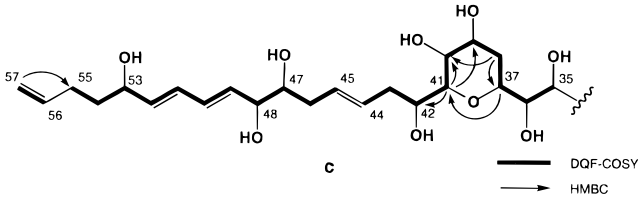
a

Figure 1. Partial structure **a**.



b

Figure 2. Partial structure **b**.



c

Figure 3. Partial structure **c**.

Assembly of Partial Structures. Three partial structures **a**−**c** had to be connected through two sp^2 quaternary carbons, C-22 and C-34, bearing a methyl and an exo-methylene group, respectively. The PFG HMBC correlations of **1** for H_3-59/C-21, H_3-59/C-22, H_3-59/C-23, and H_2-21/C-23 suggested that units **a** and **b** were connected through the quaternary carbon C-22. On the other hand, the PFG HMBC spectrum showed cross-peaks due to H_2-60/C-33, H_2-60/C-34, H_2-60/C-35, and H-35/C-33, indicating that **b** and **c** units were connected through the quaternary carbon C-34. The whole structure of luteophanol A was thus constructed as **1**.[14] The geometries of disubstituted olefins were all assigned as *E*, from the the coupling constants, the ROESY spectrum, and the ^{13}C NMR chemical shifts of the allylic carbons.[15] The Δ^{22}-trisubstituted olefin had also *E*-configuration as revealed by the ^{13}C NMR chemical shift of C-59 (δ_C 18.2).

FABMS/MS Study. Tandem mass spectrometry experiments were carried out to provide further proof for the total structural elucidation. Negative ion FABMS/MS spectra of **1** [precursor ion, *m*/*z* 1253 $(M - H)^-$] showed characteristic patterns for charge-remote fragmentations,[16] probably attributable to the presence of the C-1 sulfate ester group. Particularly, product ion peaks generated by fissions at α and β positions to hydroxyl groups or those due to cleavages at allylic or homoallylic positions were prominently observed (Figure 4). In regard to the substructure C-42−C-57 (**c**$_2$, **c**$_3$, and **c**$_4$), the substantial product ion peaks (*e.g.*, *m*/*z* 1211, 1197, 1167, 1115, 1085, 1055, 1015, 1001, and 971) corroborated the C-42−C-47 symmetrical structure (**c**$_2$) as well as the presence of a conjugated diene (C-49−C-52) and the terminal butenyl group (C-54−C-57) as shown in Figure 4. In the negative ion FABMS of **1**, an intense ion at *m*/*z* 565 was characteristically observed. The high resolution FABMS analysis of this ion revealed its composition as $C_{26}H_{45}O_{11}S$, thus assigning it to an fragment ion generated by allylic and 1,2-diol fission at the C-24/C-25 position. The FABMS/MS spectrum selecting the *m*/*z* 565 ion as a precursor ion was also recorded to give a series of intense product ion peaks (*e.g.*, *m*/*z* 481, 423, 393, 363, 335, 305, 275, 221, and 151)[17] consistently supporting the structure of the C-1−C-24 part of **1**.

Discussion. From all of these observations, we concluded the planar structure of luteophanol A as **1**.[18] The central portion (the C-15−C-42 moiety) of luteophanol A (**1**) is structurally common to that of amphidinols,[4a,5] potent antifungal metabolites previously isolated from dinoflagellates *Amphidinium* sp. Luteophanol A (**1**), however, possesses different structural features from those of amphidinols in both ends of the molecule. Particularly, amphidinols comprise a hydrophobic polyene portion in one end of the molecule, whereas the corresponding portion of luteophanol A (**1**) contains three hydroxyl groups (C-47, C-48, and C-53) with no conjugated triene, which may make this side of molecule less hydrophobic. Luteophanol A (**1**) showed no antifungal activity, but exhibited weak antimicrobial activity against Gram-positive bacteria (MIC values: *Staphylococcus aureus*, 33 μg/mL; *Sarcina lutea*, 33 μg/mL; *Bacillus subtilis*, 66 μg/mL).

Experimental Section

General Methods. For each 2D NMR experiment except INADEQUATE a total of 512 increments of 2k data points were collected. The DQF-COSY, HOHAHA, E-COSY, ROESY, HSQC, and HSQC-HOHAHA spectra were in the phase-sensitive mode. The PFG-HMBC were recorded using standard pulse sequence with *z*-axis PFG. Sine bell shaped gradient pulses were used with a 5:3:4 ratio and 1 ms duration, and maximum strength was 25.0 G/cm. For long-range C−H coupling, a 50 ms delay time was used. For INADEQUATE experiment, a total of 128 increments of 2k data points were collected. ESIMS spectrum was recorded using sample dis-

(14) Interpretation of the correlations observed in the INADEQUATE spectrum was also consistent with the gross structure of **1**. Particularly, it clearly revealed connectivities between the quaternary carbons (C-22 and C-34) and their neighboring carbons, viz., cross-peaks for C-22/C-21, C-22/C-23, and C-22/C-59; C-34/C-33, C-34/C-35, and C-34/C-60.

(15) The 6*E*, 10*E*, and 49*E*-configurations were assigned from the proton coupling constants detected in the ^1H NMR and E-COSY spectrum ($J_{6,7}$ = 15.5, $J_{10,11}$ = 15.6, and $J_{49,50}$ = 15.3), and the 51*E*-configuration was inferred from the ROESY cross-peak observed for H-50/H-52. The ^{13}C NMR chemical shift (δ_C 43.0) of allylic carbons (C-43 and C-46) implied 44*E* since the ^{13}C chemical shifts of allylic carbons located between a secondary homoallylic alcohol and a disubstituted-olefin were observed at δ_C 41−43 for *E*-olefins and at δ_C 35−39 for *Z*-olefins: Gustafson, K.; Roman, M.; Fenical, W. *J. Am. Chem. Soc.* **1989**, *111*, 7519−7524.

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(17) See, Supporting Information.

(18) A preliminary account of this work was presented at the 38th Symposium on the Chemistry of Natural Products, Sendai, Japan, 14−16 October, 1996; Abstracts, pp 445−450. The proposed structure of luteophanol A in the symposium abstract was corrected as **1** in this paper.

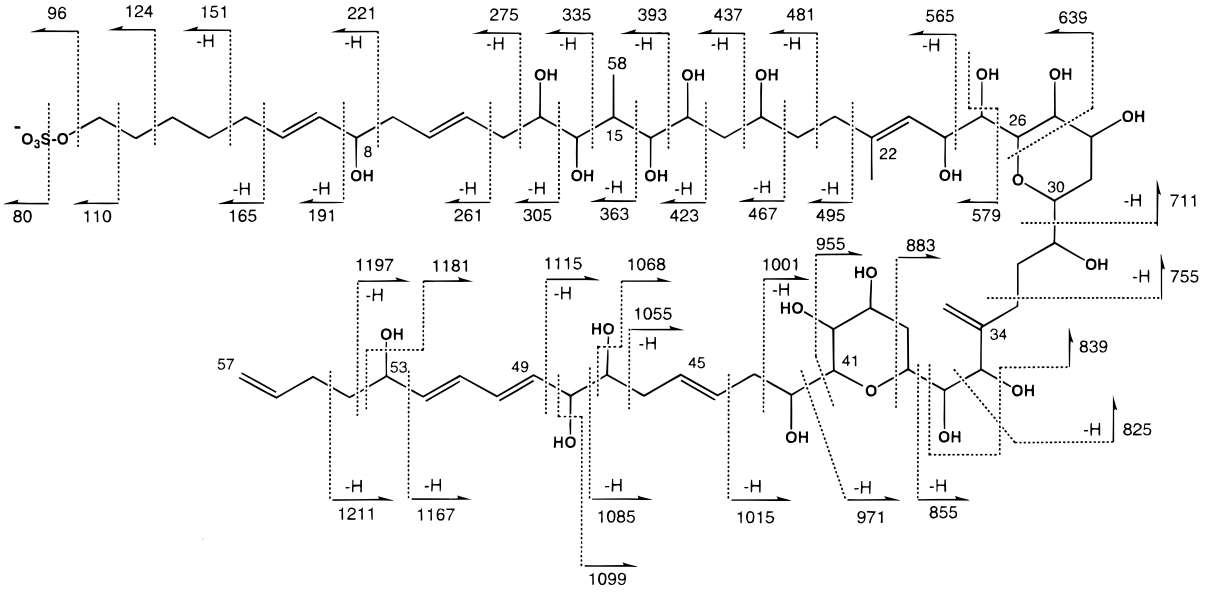

Figure 4. Fragmentation patterns observed in negative ion FAB-MS/MS spectra of luteophanol A (**1**) (precursor ion *m*/*z* 1253).

solved in MeOH with a flow rate of 2 μL/min. The MS/MS spectra were obtained on a tandem mass spectrometer (BEBE geometry) equipped with Xenon FAB gun (6 kV). The mass spectrometer was operated at an accelerating voltage of 10 kV and in the negative ion mode. MS/MS experiments were performed on B/E-linked scan mode of MS_2 via the collision cell in the third field-free region, floated at 5 kV or not floated. Ar collision gas was used with the pressure to reduce the selected precursor ion intensity by 80%. The sample (10 μg) in MeOH solution was mixed with the glycerol matrix, and the mixture was applied to a FAB target and used for analysis.

Isolation. The harvest cells of the cultured dinoflagellate *Amphidinium* sp. (strain number Y-52, 10-μm wide and 10-μm long; 450 g, wet weight, from 1700 L of culture) were extracted with toluene/MeOH (3:1, 500 mL × 3). After addition of 1 M NaCl (750 mL), the mixture was extracted with toluene (250 mL × 4). The aqueous layer was partitioned successively with $CHCl_3$ (250 mL × 4), EtOAc (250 mL × 4), and *n*-BuOH (250 mL × 4). The *n*-BuOH-soluble fraction was evaporated under reduced pressure to give a residue (3.05 g), which was partially (1.30 g) subjected to a gel filtration on HP Cellulofine (Seikagaku Kogyo, 2.0 × 100 cm; MeOH/H_2O, 1:1). The fraction eluting from 160 to 240 mL (328 mg) was separated by gel filtration on Sephadex LH-20 (Pharmacia, 2.0 × 100 cm; MeOH), and the fraction (223 mg) eluted from 80 to 200 mL was purified by reversed-phase MPLC (CPO-HS-221-20, Kusano Kagakukikai, 22 × 100 mm, 20 μm; flow rate: 2.5 mL/min; detection: UV at 230 nm and refractive index; eluent: 45% MeOH) to afford luteophanol A (**1**, 8.1 mg, t_R 10.4 min).

Luteophanol A (1). Colorless amorphous solid; $[\alpha]^{29}_D$ −7.6° (*c* 1, MeOH); UV (MeOH) λ_{max} 232nm (ϵ 2700); IR (film) ν_{max} 3370, 2900, 1640, 1400, and 1230 cm^{-1}; ^1H and ^{13}C NMR (Table 1); ESI-MS *m*/*z* 1253 $(M - H)^{-1}$; FABMS *m*/*z* 1253 $(M - H)^-$ and 565; HRFABMS *m*/*z* 1253.6352 (calcd for $C_{60}H_{101}O_{25}S$ (M − H): 1253.6312) and *m*/*z* 565.2683 (calcd for $C_{26}H_{45}O_{11}S$: 565.2656).

Cultivation with $NaH^{13}CO_3$. Prior to inoculation of the seed alga, $NaH^{13}CO_3$ (50 mg/L) was added to the seawater medium. A total volume of 200 L of cultures (2 L × 100 bottles) was incubated for 2 weeks under the conditions previously described.[6a] The harvested algal cells (61.9 g) were extracted with MeOH (150 mL × 3) and MeOH/H_2O (2:1, 150 mL). After evaporation under reduced pressure, the residue (3.4 g) was partitioned between hexane (200 mL) and 1 M NaCl aqueous solution (200 mL), and the aqueous portion was subsequently extracted with $CHCl_3$ (200 mL) and *n*-BuOH (200 mL × 3). The *n*-BuOH soluble material (1.3 g) was subjected to a gel filtration on HP Cellulofine (2.0 × 100 cm; MeOH/H_2O, 1:1), and the fraction eluting from 160 to 240 mL (1.1 g) was separated by gel filtration on Sephadex LH-20 (2.0 × 100 cm; MeOH) to give a fraction containing **1** (17.3 mg), which was used for measurment of the INADEQUATE spectrum.

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Supporting Information Available: NMR and MS spectral data of **1** (16 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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