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Glycolipids from Sponges. Part 9:[1] Plakoside C and D, Two Further Prenylated Glycosphingolipids from the Marine Sponge *Ectyoplasia ferox*

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Abstract—Plakoside C (**3a**) and D (**4a**), two further glycosphingolipids with a prenylated galactose and cyclopropane-containing alkyl chains, have been isolated from the Caribbean sponge *Ectyoplasia ferox*. Structures **3a** and **4a** have been determined on the basis of spectral data and micro-scale chemical degradation.

Introduction

Marine sponges are being shown to be a rich source of unusual glycolipids,[2] many of them possessing interesting bioactivities.[3] Our research group, which is actively involved in this field, has recently reported the presence in the Caribbean sponge *Plakortis simplex* of plakoside A (**1a**) and B (**2a**), two unique glycosphingolipids with a prenylated sugar hydroxyl group and cyclopropane-containing alkyl chains, which are immunosuppressors with a non-cytotoxic mechanism of action.[4] Analysis of the extract of another species from the Caribbean sea, *Ectyoplasia ferox*, led to the isolation of two more complex glycosphingolipids, structurally related to plakosides. This paper describes isolation and structure determination of these two new glycolipids, which have been named plakoside C (**3a**) and D (**4a**) (Fig. 1).

Results

Specimens of *E. ferox* were extracted, in sequence, with methanol and chloroform, and the extract partitioned between water and *n*-BuOH. Glycolipids were separated from other metabolites by subjecting the organic extract to subsequent RP-18 and SiO_2 column chromatography. The glycolipid fraction was acetylated with Ac_2O/Py and subjected to direct-phase HPLC purification, yielding a

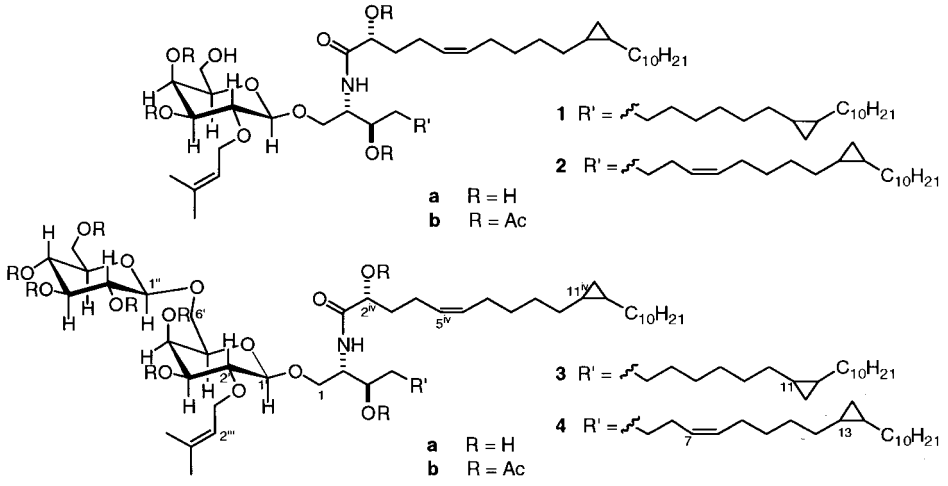

Figure 1. Structures **1**–**4**.

Keywords: marine metabolites; glycolipids; natural products; sponges.

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fraction composed of a mixture of peracetylated plakoside C and D. This mixture was deacetylated with Et_3N/MeOH, and pure compounds **3a** (2.0 mg) and **4a** (1.2 mg) were obtained by reversed-phase HPLC.

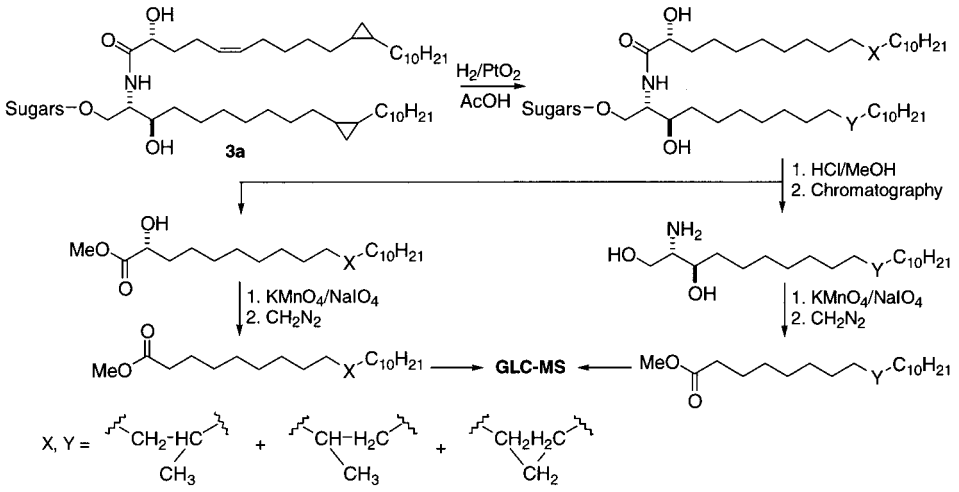
High-resolution negative-ion FAB mass spectrum of plakoside C (**3a**) showed a pseudomolecular $[M-H]^-$ ion peak at *m/z* 1108.8285, in accordance with the molecular formula $C_{63}H_{115}NO_{14}$ (calcd 1108.8239). Inspection of its ^1H NMR spectrum (pyridine-d_5) showed compound **3a** to be closely related to plakoside A. The characteristic signals of the ceramide of plakoside A (eight cyclopropane protons in the high field region of the spectrum at δ −0.29, 2H; δ 0.65, 2H; and δ 0.71, 4H) as well as those of the 3,3-dimethylallyl group [a 6H singlet at δ 1.56 (methyl groups) and a 1H triplet at δ 5.66 (olefinic protons)] were also present in the proton spectrum of compound **3a**. However, plakoside C showed additional signals in the middle-field region of the spectrum, and two anomeric carbon atoms (δ 105.3 and 105.1) rather than one in the ^{13}C NMR spectrum. These data suggested that plakoside C could be a glycosylated analogue of plakoside A.

Determination of the nature of the additional sugar and elucidation of its linkage to the rest of the molecule was achieved by analysis of 2D NMR spectral data. The proton spectrum of compound **3a** in pyridine-d_5 was characterized by broad signals and extensive signal overlapping. Therefore, all the 2D NMR experiments were performed on the peracetyl derivative (**3b**) of plakoside C, so as to take advantage of the better signal dispersion in the ^1H spectrum of this derivative and of the possibility to distinguish between alkoxymethine and acetoxymethine protons on the basis of the chemical shift.[2]

Table 1. NMR data of plakoside C pentaacetate (**3b**) ($CDCl_3$)

Pos.		δ_H (mult, *J* (Hz))	δ_C (mult)	
1	a	4.10 (dd, 11.4, 1.5)	69.0	(CH_2)
	b	3.54 (dd, 11.4, 3.6)		
2		4.33 (m)	50.9	(CH)
2-N*H*		6.69 (d, 9.3)		
3		5.00 (m)	72.6	(CH)
4	a	1.65 (m)	31.2	(CH_2)
	b	1.55 (m)		
5		1.32[a]	25.0	(CH_2)
6-8, 15-19, 15^{IV}-19^{IV}		1.26[a]	29.9-29.3	(CH_2)
9, 14, 9^{IV}, 14^{IV}		1.37[a]	29.9	(CH_2)
10, 13, 10^{IV}, 13^{IV}	a	1.37[a]	28.6	(CH_2)
	b	1.13 (m)		
11, 12, 11^{IV}, 12^{IV}		0.63 (m)	15.8	(CH)
20, 20^{IV}		1.26[a]	31.8	(CH_2)
21, 21^{IV}		1.28[a]	22.7	(CH_2)
22, 22^{IV}		0.88 (t, 7.1)	14.1	(CH_3)
23, 23^{IV}	a	0.56 (ddd, 8.3, 8.3, 4.3)	11.0	(CH_2)
	b	−0.34 (ddd, 5.1, 5.1, 4.3)		
1′		4.29 (d, 7.9)	104.1	(CH)
2′		3.48 (dd, 10.1, 8.0)	76.2	(CH)
3′		4.89 (dd, 10.1, 3.3)	72.8	(CH)
4′		5.30 (br. d, 3.3)	67.8	(CH)
5′		3.78 (br. t, 6.6)	71.8	(CH)
6′	a	3.73[a]	66.2	(CH_2)
	b	3.73[a]		
1″		4.58 (d, 8.0)	100.1	(CH)
2″		4.93 (dd, 9.5, 8.0)	71.0	(CH)
3″		5.16 (t, 9.5)	72.6	(CH)
4″		5.07 (t, 9.5)	68.2	(CH)
5″		3.69 (m)	72.0	(CH)
6″	a	4.23 (m)	62.0	(CH_2)
	b	4.13 (dd, 13.5. 1.5)		
1‴	a	4.23 (m)	69.9	(CH_2)
	b	4.23 (m)		
2‴		5.34[a]	120.7	(CH)
3‴			137.6	(C)
4‴		1.75 (br. s)	25.7	(CH_3)
5‴		1.68 (br. s)	18.0	(CH_3)
1^{IV}			169.7	(C)
2^{IV}		5.02 (m)	73.9	(CH)
3^{IV}		1.89 (m)	31.9	(CH_2)
4^{IV}		2.12 (m)	22.6	(CH_2)
5^{IV}		5.31[a]	127.4	(CH)
6^{IV}		5.42 (m)	131.5	(CH)
7^{IV}		2.00 (m)	27.3	(CH_2)
			170.4-169.8(C)	
Ac′s		2.13, 2.12, 2.09, 2.07, 2.04, 2.02, 2.01, 1.99 (s)	21.0-20.6	(CH_3)

[a] Submerged by other signals.



Scheme 1. Micro-scale procedure for degradation of plakoside C.

The analysis of the COSY and HMQC spectra of compound **3b** allowed us to identify the resonances of all the proton and carbon atoms of the sphinganine and the fatty acid residue. All of them were nearly coincident with the corresponding signals of plakoside A pentaacetate **1a** (see Table 1), showing that the same functional groups, with the same relative stereochemistry, were present on the ceramide part of the molecule. The chemical shift and multiplicities of the signals of the prenylated galactose were also very similar, except for the oxymethylene group at position 6′. Protons H_2-6′ (both resonating at δ 3.73) were significantly shielded compared to the corresponding protons of plakoside A (δ 4.09 and 4.11), suggesting that the oxymethylene is no longer acetylated, while the carbon atom C-6′ (δ 66.2) was shifted downfield by 4.8 ppm. Both these data were clearly indicative of the second sugar residue being linked at position 6 of the first galactose.

The anomeric proton of the additional sugar was identified by its correlation peak in the HMQC spectrum with the anomeric carbon at δ 100.1 (C-1″), and resonated as a doublet at δ 4.58 (J=8.0 Hz). Using this proton as a starting point, examination of the COSY spectrum allowed us to assign H-2″ (δ 4.93, dd, J=9.5 and 8.0 Hz), H-3″ (δ 5.16, t, J=9.5 Hz), H-4″ (δ 5.07, t, J=9.5 Hz), H-5″ (δ 3.69, m) and, finally, the methylene protons at C-6″ (H-6″a, δ 4.23, m; H-6″b, δ 4.13, dd, J=13.5 and 1.5 Hz). As shown by the large coupling constants between all the oxymethine protons, the sugar is in the pyranose form with all the ring protons in the axial orientation: that is, the sugar residue is a β-glucopyranoside. Further evidence for the (1→6) linkage between the two sugars came from the ROESY spectrum, which evidenced prominent correlation peaks of H-1″ with H-5′ and H_2-6′.

All the remaining NMR data were in accordance with plakoside C being a 6′-*O*-glucopiranoside of plakoside A. However, although the mass spectrum showed that the ceramide part of plakoside C has the same mass as that of plakoside A, spectral data alone could not provide definitive evidence that the length of each of the two alkyl chains of the ceramide, and the position of the cyclopropane rings in the chains were the same in the two compounds. Therefore, we used for compound **3a** the degradation procedure that we developed for plakoside A (reductive cleavage of cyclopropane rings, acidic methanolysis, oxidative cleavage to carboxylic acid methyl esters, and GC–MS analysis), as reported in Scheme 1. Not surprisingly, the results were identical, and methyl 9-methyl- and 10-methyleicosanoate were obtained from the sphinganine, while the 2-hydroxy fatty acid gave methyl 10-methyl- and 11-methylheneicosanoate.

Once the structure of the peracetylated plakoside C (**3b**) was secured, a retrospective analysis of the NMR data of the parent compound **3a** allowed the assignment of all the signals in its ^{1}H and ^{13}C NMR spectra (see Experimental), thus providing further support for the proposed structure.

Plakoside D (**4a**) showed a pseudomolecular ion peak in the negative-ion FAB mass spectrum at m/z 1134.8431, accounting for the molecular formula $C_{65}H_{117}NO_{14}$. Therefore, plakoside D has two more carbon atoms and one more unsaturation than plakoside C. The ^{1}H and ^{13}C NMR spectra of compound **4a** are very similar to those of plakoside C (**3a**), except for an additional 2H multiplet at δ 5.51 in the proton spectrum and two additional sp^2 methine carbon resonances in the carbon spectrum (δ 130.3 and 130.2). These signals were at the same chemical shift as the signals of the double bond at position 7 of plakoside B (**2a**). These data strongly suggested that plakoside D (**4a**) has the same sugar chain as plakoside C (**3a**), while its ceramide is like that of plakoside B (**2a**). When the degradation procedure described in Scheme 1 was performed on plakoside C and D it gave methyl 11-methyl- and 12-methyldocosanoate from the sphinganine and methyl 10-methyl- and 11-methylheneicosanoate from the fatty acid, thus showing that the position of the cyclopropane rings was actually the same in **2a** and **4a**.

Discussion

Plakoside C (**3a**) and D (**4a**) from *E. ferox* were shown to be the 6′-*O*-β-glucopyranosides of, respectively, plakoside A (**1a**) and B (**2a**), that have been isolated from *P. simplex* and

are the first and until now the only example of glycosphingolipids with a prenylated sugar. The presence of small amounts of similar compounds in taxonomically distant sponges may raise doubts on the actual organism responsible for their biosynthesis, and in these cases the bacterial (or more generally dietary) origin of the metabolites is often hypothesized. We cannot provide any evidence either against or in favor of this possibility at the moment, aside from the observation that plakosides A and B are consistently present in all of several samples of *P. simplex* we have analyzed (all from Bahamas, but collected in different places and years), while plakosides C and D were also present in another sample of *E. ferox* collected near the coast of Eleutheria Island.

As for the possible immunosuppressive activity of the new plakosides, this will be examined in the frame of a more general project, which is in progress in our laboratory, for the study of immunomodulating activity of several natural and synthetic glycolipids.

Experimental

General methods

FABMS spectra were performed in a triethanolamine matrix on a VG Prospec-Autospec (Fisons) mass spectrometer. Optical rotations were measured at 589 nm on a Perkin–Elmer 192 polarimeter using a 10 cm microcell. ^{1}H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H=7.26, δ_C=77.0; C_5D_5N: δ_H=8.71, 7.56, and 7.19, δ_C=149.8, 135.3, and 123.4). Homonuclear ^{1}H connectivities were determined by the COSY experiment. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the interpulse delays were adjusted for an average $^1J_{CH}$ of 142 Hz. GC–MS spectra were performed on a Hewlett–Packard 5890 gas chromatograph with a mass selective detector MSD HP 5970 MS, a split/splitness injector, and a fused-silica column, 25 m×0.20 mm HP-5 (cross-linked 25% Ph Me silicone, 0.33 mm film thickness). The temperature of the column was varied, after a delay of 5 min from the injection, from 220 to 300°C with a slope of 3°C min^{-1}. High performance liquid chromatographies (HPLC) were achieved on a Varian 2510 apparatus equipped with an Varian Star 9040 refractive index detector.

Collection, extraction and isolation

Specimens of *Ectyoplasia ferox* were collected in the Summer of 1998 along the coast of Grand Bahama Island and identified by Prof M. Pansini (University of Genoa). They were frozen immediately after collection and kept frozen until extraction. Reference specimens were deposited at the Istituto di Zoologia, University of Genoa, Italy (Ref. No. 98-01). The sponge (61 g of dry weight after extraction) was homogenized and extracted with methanol (3×2 L) and then with chloroform (3×2 L); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated in vacuo and afforded 9.0 g of a dark brown oil, which was chromatographed on a column packed with RP-18 silica gel. A fraction (1.92 g) eluted with $CHCl_3$ (9:1), containing glycolipids, was further chromatographed on a SiO_2 column. The glycolipid fraction (75 mg), eluted with EtOAc/MeOH (7:3) was acetylated with Ac_2O in pyridine for 12 h, and separated by HPLC on a SiO_2 column [eluent: *n*-hexane/EtOAc (6:4)], thus affording a mixture (9.5 mg) containing peracetylated plakoside C and D together with other glycolipids. Further direct-phase HPLC purification [eluent: *n*-hexane/*i*-PrOH (9:1)] gave a fraction (5.8 mg) entirely composed of peracetylated plakoside C and D, plus other minor homologues. Plakosides were deacetylated by keeping them in a mixture of 1 mL of MeOH and 200 μL of Et_3N for 72 h at 65°C, and subjected to reversed-phase HPLC using MeOH as eluent, giving pure **3a** (2.0 mg) and **4a** (1.1 mg).

Plakoside C (3a). Amorphous solid; $[\alpha]_D^{25}$=+4 (*c* 0.2, MeOH); HRFABMS (negative ion mode, triethanolamine matrix) *m/z* 1108.8285 ($[M-H]^-$, $C_{63}H_{114}NO_{14}$ gives 1108.8239); ^{1}H NMR (pyridine-d_5) δ 8.29 (1H, d, *J*=9.5 Hz, NH-2), 5.66 (1H, br. t, *J*=6.5 Hz, H-2‴), 5.60 (1H, m, H-5^{IV}), 5.52 (1H, m, H-6^{IV}), 4.97 (1H, d, *J*=7.8 Hz, H-1″), 4.82 (1H, m, H-1a), 4.70 (1H, m, H-2), 4.69 (overlapped, H-1‴a), 4.68 (1H, d, *J*=7.6 Hz, H-1′), 4.63 (overlapped, H-2^{IV}), 4.62 (1H, dd, *J*=10.1 and 6.1 Hz, H-6′a), 4.55 (1H, dd, *J*=11.7 and 7.3 Hz, H-1‴b), 4.50 (1H, br. d, *J*=11.1 Hz, H-6″a), 4.36 (1H, br. s, H-4′), 4.35 (overlapped, H-6″ b), 4.31 (overlapped, H-6′b), 4.24 (overlapped, H-3″ and H-4″), 4.17 (overlapped, H-3), 4.05 (overlapped, H-1b, H-2′, and H-2″), 3.99 (overlapped, H-5′), 3.94 (1H, m, H-3′), 3.90 (1H, m, H-5″), 2.60 (2H, m, H_2-4^{IV}), 2.33 (1H, m, H-3^{IV}a), 2.18 (overlapped, H-3^{IV}b and H-7^{IV}), 1.93 (overlapped, H-4a and H-5a), 1.89 (overlapped, H-4b), 1.60 (overlapped, H-5b), 1.56 (6H, br. s, H_3-4‴ and H_3-5‴), 1.41 (overlapped, H-10a, H-13a, H-10^{IV}a, H-13^{IV}a), 1.34 (overlapped, H_2-9, H_2-14, H_2-9^{IV}, H_2-14^{IV}, and H-8^{IV}), 1.25 (large band, long-chain methylene protons), 1.20 (overlapped, H-10b, H-13b, H-10^{IV}b, and H-13^{IV}b), 0.85 (6H, t, *J*=7.5 Hz, H_3-22 and H_3-22^{IV}), 0.71 (4H, m, H-11, H-12, H-11^{IV}, and H-12^{IV}), 0.65 (2H, m, H-23a and H-23^{IV}a), −0.23 (2H, m, H-23b and H-23^{IV}b); ^{13}C NMR (pyridine-d_5) δ 174.9 (C, C-1^{IV}), 134.9 (C, C-3‴), 130.8 (CH, C-6^{IV}), 129.6 (CH, C-5^{IV}), 123.1 (CH, C-2‴), 105.3 (CH, C-1′), 105.1 (CH, C-1″), 79.7 (CH, C-2′), 78.5 (CH, C-3″), 75.2 (CH, C-2″), 75.0 (CH, C-5′), 74.4 (CH, C-3′), 72.0 (CH, C-5″), 71.9 (CH, C-2^{IV}), 71.6 (CH, C-4″), 71.6 (CH, C-3), 69.9 (CH_2, C-1), 69.8 (CH, C-4′), 69.5 (CH_2, C-6′), 69.5 (CH_2, C-1‴), 62.6 (CH_2, C-6″), 54.3 (CH, C-2), 35.7 (CH_2, C-3^{IV}), 34.8 (CH_2, C-4), 32.8 (CH_2, C-20 and C-20^{IV}), 30.1–29.5 (several CH_2), 28.9 (CH_2, C-10, 13, 10^{IV}, 13^{IV}), 27.6 (CH_2, C-8^{IV}), 27.6 (CH_2, C-7^{IV}), 26.5 (CH_2, C-5), 25.6 (CH_3, C-4‴), 23.7 (CH_2, C-4^{IV}), 22.8 (CH_2, C-21 and C-21^{IV}), 18.1 (CH_3, C-5‴), 16.1 (CH, C-11, C-12, C-11^{IV} and C-12^{IV}), 14.2 (CH_3, C-22, C-22^{IV}), 11.3 (CH_2, C-23, C-23^{IV}).

Plakoside C pentaacetate (3b). Colorless oil; $[\alpha]_D^{25}$=0±1 (*c* 0.25, $CHCl_3$); ^{1}H and ^{13}C NMR: Table 1.

Plakoside D (4a). Amorphous solid, $[\alpha]_D^{25}=+4$ (*c* 0.1, MeOH); HRFABMS (negative ion mode, triethanolamine matrix) *m/z* 1134.8431 ($[M-H]^-$, $C_{65}H_{116}NO_{14}$ gives 1134.8396); ^1H NMR (pyridine-d_5): same resonances as those reported for **3a**, except for δ 5.52 (overlapped, H-7), 5.50 (overlapped, H-8) δ 2.18 (overlapped, H_2-6 and H_2-9), δ 1.99 (overlapped, H-4a and H-5a), 1.90 (1H, m, H-4b), 1.69 (1H, m, H-5b); ^{13}C NMR (pyridine-d_5): same resonances as those reported for **3a**, except for δ 130.2 and 130.3 (CH, C-7 and C-8), 34.4 (CH_2, C-4), 26.7 (CH_2, C-5).

Degradation analysis of plakoside C and D

Plakoside C and D (200 μg of each compound) were subjected to the degradation procedure previously described, and reported in Scheme 1. All the fatty acid methyl esters produced were identified by comparison of their relative retention times and mass spectra with those reported.[4,5] The α-hydroxy fatty acid of plakoside C (**3a**) gave methyl docosanoate (21%) and a mixture of methyl 10-methylheneicosanoate and methyl 11-methylheneicosanoate (79%); the α-hydroxy fatty acid of plakoside D (**4a**) gave methyl docosanoate (18%) and a mixture of methyl 10-methylheneicosanoate and methyl 11-methylheneicosanoate (82%); the sphinganine of plakoside C (**3a**) gave methyl heneicosanoate (17%) and a mixture of methyl 9-methyleicosanoate and methyl 10-methylheicosanoate (83%); the sphinganine of plakoside D (**4a**) gave methyl tricosanoate (20%) and a mixture of methyl 11-methyldocosanoate and methyl 12-methyldocosanoate (80%).

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References

1. Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A. *Tetrahedron* **2000**, *56*, 1393–1395.
2. Fattorusso, E.; Mangoni, A. Marine Glycolipids; In *Progress in the Chemistry of Organic Natural Products*, Hertz, W., Kirby, G. W., Moore, R. E., Steiglich, W., Tamm, Ch., Eds.; Springer: Wien, 1997; pp 215–301.
3. Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 271–276.
4. Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A. *J. Am. Chem. Soc.* **1997**, *119*, 12465–12470.
5. McCloskey, J. A.; Law, H. L. *Lipids* **1967**, *2*, 225–228.