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Oreacerebrosides: Bioactive Cerebrosides with a Triunsaturated Sphingoid Base from the Sea Star *Oreaster reticulatus*

Valeria Costantino,[a] Caterina de Rosa,[a] Ernesto Fattorusso,[a] Concetta Imperatore,[a] Alfonso Mangoni,*[a] Carlo Irace,[b] Carmen Maffettone,[b] Domenica Capasso,[c] Livia Malorni,[d] Rosanna Palumbo,[d] and Carlo Pedone[d]

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The sea star *Oreaster reticulatus* contains, along with the known ophidiacerebrosides C–E, nine new glycosphingolipids named oreacerebrosides A–I. Their structures were elucidated by a combination of spectroscopic methods and chemical degradation. All compounds contain a 4,8,10-triunsaturated sphingoid base. Oreacerebrosides A–C have a β-glucopyranoside as the sugar residue, as found in ophidiacerebrosides C–E and in all known compounds of this type; in contrast, oreacerebrosides D–I are the first examples of β-galactosylceramides containing this unusual sphingoid base. Four representative glycosphingolipids were tested for cytotoxic activity on rat glioma C6 cells and were shown to be mildly cytotoxic; the glucosylceramides were more active than the galactosylceramides. In addition, oreacerebroside I, but not ophidiacerebroside E, was shown to exert proangiogenic activity and was able to increase VEGF-induced human endothelial cell proliferation.

Introduction

Glycosphingolipids are a large group of biomolecules containing two basic structural units: a sugar and a ceramide. The hydrophobic ceramide portion involves a sphingoid base and an amide-linked fatty acyl chain.[1] In general, glycosphingolipids exhibit a wide range of biological functions that might be related to the amphipathic nature of the molecule.[2]

Glycosphingolipids and other sphingosine derivatives have been isolated from a number of marine sources, including sea stars,[3–7] sea anemones,[8] sponges,[9,10] corals,[9] tunicates,[11] and plants.[12] Studies on the isolation and structure elucidation of glycosphingolipids from sponges have been in progress in our laboratory for many years. We have now analyzed the glycolipid fraction from the starfish *Oreaster reticulatus* (Linnaeus, 1758), and we have isolated several glycosphingolipids, including ophidiacerebrosides C–E and nine new compounds named oreacerebrosides A–I. Ophidiacerebrosides C–E are known compounds containing a sphingosine unit with a methyl branch and a conjugated diene and a β-glucose as the sugar unit.[13] In this paper, we wish to report the analysis of the glycolipidic fraction from the starfish *Oreaster reticulatus* and the structure elucidation of oreacerebrosides A–I, along with an evaluation of the cytotoxic and proangiogenic activity of the isolated compounds.

[a] Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II",
Via D. Montesano 49, 80131 Napoli, Italy
Fax: +39-081-678-552
E-mail: alfonso.mangoni@unina.it
[b] Dipartimento di Farmacologia Sperimentale, Università di Napoli "Federico II",
Via D. Montesano 49, 80131 Napoli, Italy
[c] Dipartimento delle Scienze Biologiche, Università di Napoli "Federico II",
Via Mezzocannone 8, 80134 Napoli, Italy
[d] CNR – Institute of Biostructure and Bioimaging (IBB),
Via Mezzocannone 16, 80134 Napoli, Italy

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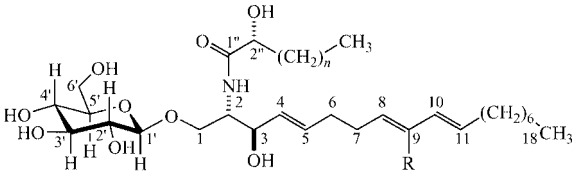
Results and Discussion

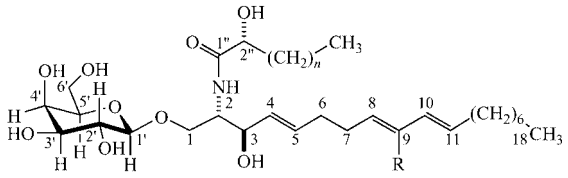
Specimens of *Oreaster reticulatus* were collected in Sweeting Cay along the coasts of Grand Bahama Island (Bahamas) and extracted with MeOH and $CHCl_3$. By following our usual procedure,[14] the extract was partitioned between water and *n*BuOH, and the organic phase was subjected to sequential column chromatography on RP18 and normal silica gel, which afforded a crude glycolipid mixture that was acetylated with Ac_2O in pyridine.

This mixture was purified by repeated HPLC on silica columns with the use of *n*-hexane/EtOAc and *n*-hexane/*i*PrOH as the eluents to afford a mixture of peracetylated glycosphingolipids. These compounds were then deacetylated with MeONa/MeOH to give the natural products. Separation by HPLC on a silica column gave two fractions each of which consisted of a mixture of homologous glycolipids; the first fraction consisted of six glycosphingolipids, **1a**–**f**, each containing a glucopyranose residue, and the sec-

ond fraction was composed of glycosphingolipids **2a**–**f**, each containing a galactopyranose residue. The two fractions were subjected to purification by HPLC on RP18 columns to afford pure **1a**, **1f**, **2a**, and **2f** and inseparable mixtures of **1b/1d**, **1c/1e**, **2b/2d**, and **2c/2e** (Figure 1).



Oreacerebroside A (**1a**)	R = H	*n* = 19
Oreacerebroside B (**1b**)[a]	R = H	*n* = 20
Oreacerebroside C (**1c**)[b]	R = H	*n* = 21
Ophidiacerebroside C (**1d**)[a]	R = CH_3	*n* = 19
Ophidiacerebroside D (**1e**)[b]	R = CH_3	*n* = 20
Ophidiacerebroside E (**1f**)	R = CH_3	*n* = 21



Oreacerebroside D (**2a**)	R = H	*n* = 19
Oreacerebroside E (**2b**)[c]	R = H	*n* = 20
Oreacerebroside F (**2c**)[d]	R = H	*n* = 21
Oreacerebroside G (**2d**)[c]	R = CH_3	*n* = 19
Oreacerebroside H (**2e**)[d]	R = CH_3	*n* = 20
Oreacerebroside I (**2f**)	R = CH_3	*n* = 21

Figure 1. Cerebrosides for *O. reticulatus*. Compounds marked with the same superscript were isolated together as an inseparable mixture.

Finally, the possibility that the acetylation/deacetylation sequence in some way modified the structure of compounds **1a**–**f** and **2a**–**f**, for example, by removing an acetyl group already present in the molecule, was ruled out by examining retrospectively the ^{1}H NMR spectrum of the crude glycolipid fraction taken prior to the acetylation reaction. All the resonances of compounds **1a**–**f** and **2a**–**f** were clearly visible in the spectrum.

Structure Elucidation of Compound 1f

The high-resolution ESI mass spectrum of compound **1f**, recorded after dissolving the sample in MeOH with 1 mM LiCl, showed a prominent peak from the lithiated [M + Li]$^+$ pseudomolecular ion at *m*/*z* = 844.6844, in accordance with the molecular formula $C_{49}H_{91}NO_9$. Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **1f** with those described for ophidiacerebroside E (isolated from the sea star *Ophidiaster ophidiamus*[13]) showed that the sugar residue was the same, and the ceramide was composed of the same 4,8,10-triunsaturated, 9-methyl branched sphingoid base and 2-hydroxy fatty acid, with the same relative stereochemistry. What remained to be established for the complete elucidation of the structure of **1f** was the length of the alkyl chains of the ceramide. In fact, even though the mass spectra indicated that the ceramide contained 43 carbon atoms, NMR spectroscopic data did not provide any information on how they were distributed between the sphingosine and the fatty acid. This was achieved by using tandem mass spectrometry. The MS/MS spectrum of **1f** was recorded by using the [M + Li]$^+$ pseudomolecular ion at *m*/*z* = 844 as the parent ion, because it has been reported[15] that the lithiated adduct produced under these conditions gives rise to very informative fragments. The spectrum contained three intense peaks at *m*/*z* = 682 [M + Li – 162]$^+$, 478 [M + Li – 366]$^+$, and 416 [M + Li – 428]$^+$. The former peak was due to loss of the dehydrated sugar molecule; more significantly, the second peak is accounted for by the loss of a C_{24} 2-hydroxy fatty acyl group (with transfer of one H atom to the sphingosine), which indicates that the long chain base was a C_{19} sphingosine, as in ophidiacerebroside E. This was confirmed by the peak at *m*/*z* = 416, which originated from the precursor ion by loss of the sugar unit and 17 of the 19 carbon atoms of the sphingosine (Figure 2). All the above data showed that compound **1f** has the same planar structure and relative configuration as ophidiacerebroside E, but not that they have the same absolute configuration.

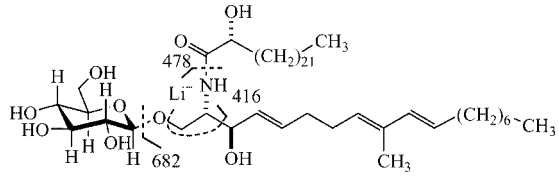


Figure 2. The most characteristic fragment ions in the ESI MS/MS spectrum of compound **1f**.

The optical rotation of ophidiacerebroside E is not reported in the original paper, so we compared the optical rotation of **1f**, $[\alpha]_D^{25}$ = +8.6, with that of phalluside 3, $[\alpha]_D^{25}$ = +9.4, a cerebroside from the ascidian *Phallusia fumigata* that differs from ophidiacerebroside E only in the length of the 2-hydroxy fatty acid residue.[16] This confirmed that compound **1f** is ophidiacerebroside E.

Structure Elucidation of Oreacerebroside A (1a)

The molecular formula of compound **1a**, $C_{46}H_{85}NO_9$, was obtained from its high-resolution ESI mass spectrum. It showed a strong [M + Li]$^+$ pseudomolecular ion peak at *m*/*z* = 802.6396, which indicated that **1a**, lacking a C_3H_6 unit, was a lower homolog of **1f**. The NMR spectroscopic data of **1a** clearly indicated that its structure (including relative stereochemistry) was very similar to that of **1f**, but it lacked the methyl group at C-9, as ascertained by the absence in the ^{1}H NMR spectrum of the singlet at δ = 1.76 ppm and the presence of signals for the six olefinic protons of the three disubstituted double bonds. These data, together with a careful analysis of the COSY and the HMBC spectra, enabled us to locate the double bonds at the 4-, 8-, and 10-positions as in compound **1f**. The length of the ceramide alkyl chains was determined by using a tan-

dem MS experiment. The fragmentation pattern observed in the MS/MS spectrum of **1a**, recorded with the use of the $[M + Li]^+$ pseudomolecular ion at m/z = 802 as the precursor ion, was similar to that of **1f**. The two peaks at m/z = 464 $[M + Li - 338]^+$ and 388 $[M + Li - 414]^+$ showed that in **1a** the ceramide portion was composed of an unbranched C_{18} sphingosine and a C_{22} 2-hydroxy fatty acid. The structure of oreacerebroside A is very similar to that of phalluside 4 isolated from the ascidian *Phallusia fumigata*:[16] the two cerebrosides have the same structure except for the C_{22} 2-hydroxy fatty acid in **1a**, which is a C_{18} 2-hydroxy fatty acid in phalluside 4.

Structure Elucidation of Compounds 1b–e

The high-resolution ESI mass spectrum of the mixture of compounds **1b** and **1d** showed a single $[M + Li]^+$ ion peak at m/z = 816.6533, in accordance with the molecular formula $C_{47}H_{87}NO_9$, showing that the two compounds were isomers and contained an additional CH_2 moiety relative to **1a**. The ^1H NMR spectrum of this mixture looked like the superimposition of the ^1H NMR spectra of **1a** and **1f**, which indicates that one of the two compounds contained in this mixture had a C-9 methyl branched sphingosine and the other one had an unbranched sphingosine. This was confirmed by the MS/MS spectrum of the mixture that was recorded by using the $[M + Li]^+$ pseudomolecular ion peak at m/z = 816 as the precursor ion. The spectrum contained two intense fragment peaks at m/z = 478 and 464 originating from the loss of the acyl group from the respective parent ions, and two fragment peaks at m/z = 402 and 388 originating from the loss of the sugar unit and part of the sphingoid base. These data suggested that one isomer (**1d**) contained a ceramide composed of the same C_{19} methyl branched sphingosine as in **1f** and a C_{22} 2-hydroxy fatty acid, and the other isomer (**1b**) contained a ceramide composed of the same C_{18} unbranched sphingosine as in **1a** and a C_{23} 2-hydroxy fatty acid. Compounds **1d** and **1b** were present in the mixture in a 2:1 ratio, as estimated from the relative integration of the signal for 10-H for **1d** and that of 9-H/10-H for **1b**.

Similarly, the mixture of compounds **1c** and **1e** proved to be composed of isomers containing an additional CH_2 unit in the fatty acid residue compared to **1b** and **1d**, respectively, on the basis of the following evidence. The high resolution ESI mass spectrum showed a single $[M + Li]^+$ ion peak at m/z = 830.6685, which corresponds to the molecular formula $C_{48}H_{89}NO_9$. In the ^1H NMR spectrum of the mixture, all the chemical shifts and multiplicities were identical to those in the spectrum of **1b** and **1d**. Finally, in the MS/MS spectrum of the mixture, recorded by using the lithiated pseudomolecular ion at m/z = 830 as the precursor ion, the fragment peaks at m/z = 478 $[M + Li - 352]^+$ and 464 $[M + Li - 366]^+$ (loss of fatty acyl chain) were the same as those in the spectrum of **1b** and **1d**, whereas those at m/z = 416 $[M + Li - 414]^+$ and 402 $[M + Li - 428]^+$ (loss of the sugar and part of the sphingoid base) were 14 amu above the corresponding peaks in the spectrum of **1b** and **1d**. Compounds **1e** and **1c** were estimated to be present in the mixture in a 5:1 ratio.

Structure Elucidation of Oreacerebrosides D–I (2a–f)

The high-resolution ESI mass spectrum of compound **2f** showed a prominent $[M + Li]^+$ pseudomolecular ion peak at m/z = 844.6868 corresponding to the molecular formula $C_{49}H_{91}NO_9$, which indicated that it was isomeric with **1f**. In the ^1H NMR spectrum of **2f**, resonances for the ceramide were almost superimposable to those present in the spectrum of **1f**, but the signals of the sugar unit were remarkably different. Moreover, the MS/MS spectrum of **2f** showed the same fragmentation pattern as that of compound **1f**, which confirmed that compounds **1f** and **2f** possess the same ceramide. Therefore, the difference between the two compounds could be located in the monosaccharide unit.

The sugar linked to the ceramide was identified as a β-galactopyranoside by NMR spectroscopy. First of all, the anomeric proton (δ = 4.84 ppm, d, J = 7.7 Hz) was identified from its correlation peak with the anomeric carbon at δ = 106.0 ppm in the HSQC spectrum. Starting from this proton, all the ^1H and ^{13}C spectroscopic signals of the sugar were assigned by using the COSY and HSQC spectra, and the vicinal proton–proton coupling constants were determined (Table 1). The *galacto* configuration of the sugar, as well as its β anomeric configuration, was established on the basis of the ring proton coupling constants ($J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 9.4 Hz, $J_{3,4}$ = 2.8 Hz), which showed that 1′-H, 2′-H, and 3′-H were axial and that 4′-H was equatorial. A ROESY correlation peak between 1′-H and 5′-H also indicated their 1,3-diaxial relationship. Finally, the linkage of this sugar to the ceramide was demonstrated by the three-bond ^{13}C–^1H couplings of C-1′ with 1a-H and 1b-H observed in an HMBC spectrum.

Compounds **2a**–**e** were shown to be the respective analogues of compounds **1a**–**e**, which contain a β-galactopyranoside instead of a β-glucopyranoside as the sugar residue, by comparing the ^1H and ^{13}C NMR spectra of compounds **2a**–**e** with those of **2f**, and their ESI MS and ESI MS/MS spectra with those of **1a**–**e**.

Finally, the absolute configuration of the sugar in compounds **2a**–**f** was determined by microscale chemical degradation of a small amount of their mixture, which had not been subjected to reversed-phase HPLC separation. The mixture (100 μg) was subjected to acidic methanolysis in HCl/MeOH, and the resulting reaction mixture was separated by partitioning between $CHCl_3$ and water/MeOH into an aqueous phase containing methyl glycosides and an organic phase containing 2-hydroxy fatty acid methyl esters and sphinganines. The methyl glycosides were subjected to perbenzoylation and subsequent HPLC separation. The HPLC chromatogram of the reaction product contained one prominent peak, which was collected and identified on the basis of its retention time and ^1H NMR spectrum rela-

Table 1. ^{1}H (700 MHz) and ^{13}C NMR (175 MHz) spectroscopic data of **1f** and **2f** (in [D_5]pyridine).

Position	**1f** δ_H (mult., *J* [Hz])	δ_C (mult.)	**2f** δ_H (mult., *J* [Hz])	δ_C (mult.)
1a	4.71 (dd, 10.3, 3.5)	69.9 (CH_2)	4.79[a]	69.9 (CH_2)
1b	4.23[a]		4.20 (dd, 9.9, 3.2)	
2	4.80 (m)	54.4 (CH)	4.81[a]	54.2 (CH)
2-N*H*	8.36 (d, 8.7)	–	8.33 (d, 8.7)	–
3	4.76 (m)	72.0 (CH)	4.71 (m)	72.1 (CH)
3-O*H*	6.89 (d, 4.9)	–	6.82 (br.s)	–
4	6.00 (dd, 15.4, 6.7)	132.0 (CH)	5.98 (dd, 15.5, 6.6)	132.0 (CH)
5	5.91 (dt, 15.4, 6.7)	131.8 (CH)	5.86 (dt, 15.5, 6.3)	131.7 (CH)
6	2.16 (m)	32.5 (CH_2)	2.14 (m)	32.9 (CH_2)
7	2.23 (m)	28.1 (CH_2)	2.23 (m)	28.2 (CH_2)
8	5.50 (t, 6.9)	129.7 (CH)	5.50 (t, 7.0)	129.8 (CH)
9	–	133.9 (C)	–	135.5 (C)
10	6.20 (d, 15.5)	135.1 (CH)	6.21 (d, 15.6)	135.0 (CH)
11	5.64 (dt, 15.5, 6.9)	127.7 (CH)	5.65 (dt, 15.6, 6.9)	127.7 (CH)
12	2.11 (m)	33.0 (CH_2)	2.12 (m)	33.1 (CH_2)
13	1.36[a]	29.8 (CH_2)	1.37[a]	29.9 (CH_2)
14–15	1.26 (m)	29.8 (CH_2)	1.26[a]	29.8 (CH_2)
16	1.21[a]	31.9 (CH_2)	1.21[a]	31.8 (CH_2)
17	1.24[a]	22.7 (CH_2)	1.24[a]	22.8 (CH_2)
18	0.85 (t, 7.0)	14.1 (CH_3)	0.85 (t, 6.8)	14.1 (CH_3)
19	1.76 (s)	12.5 (CH_3)	1.77 (br.s)	12.5 (CH_3)
1′	4.92 (d, 7.9)	105.4 (CH)	4.84 (d, 7.7)	106.0 (CH)
2′	4.04 (ddd, 7.9, 7.9, 3.3)	74.9 (CH)	4.49 (br.t, 8.6)	72.4 (CH)
2′-O*H*	7.21[a]	–	7.16 (br.s)	–
3′	4.21[a]	78.2 (CH)	4.14 (dd, 9.4, 2.8)	75.2 (CH)
3′-O*H*	7.18[a]	–	6.90 (br.s)	–
4′	4.22[a]	71.3 (CH)	4.55 (br.d, 2.8)	70.0 (CH)
4′-O*H*	7.18[a]	–	6.43 (br.s)	–
5′	4.21[a]	78.3 (CH)	4.04 (t, 6.0)	77.0 (CH)
6′a	4.51 (m)	62.4 (CH_2)	4.43[a]	62.3 (CH_2)
6′b	4.36 (ddd, 11.6, 5.8, 5.8)		4.43[a]	
6′-O*H*	6.38 (br.s)	–	6.57 (br.s)	–
1′′	–	175.3 (C=O)	–	175.7 (C=O)
2′′	4.58 (m)	72.2 (CH)	4.57 (dt, 8.1, 3.4)	72.3 (CH)
2′′-O*H*	7.65 (d, 5.3)	–	7.66 (br.s)	–
3′′a	2.21 (m)	35.5 (CH_2)	2.20 (m)	35.4 (CH_2)
3′′b	2.01 (m)		2.00 (m)	
4′′a	1.80 (m)	25.6 (CH_2)	1.80 (m)	25.8 (CH_2)
4′′b	1.71 (m)		1.71 (m)	
5′′	1.37 (m)	29.8 (CH_2)	1.37 (m)	29.9 (CH_2)
6′′-21′′	1.26 (m)	29.8 (CH_2)	1.26 (m)	29.8 (CH_2)
22′′	1.21[a]	31.9 (CH_2)	1.21[a]	31.9 (CH_2)
23′′	1.24[a]	22.7 (CH_2)	1.24[a]	22.7 (CH_2)
24′′	0.84 (t, 7.0)	14.1 (CH_3)	0.84 (t, 7.0)	14.1 (CH_3)

[a] Overlapped with other signals.

tive to those of an authentic sample of methyl tetra-*O*-benzoyl-α-D-galactopyranoside prepared with the same procedure from D-galactose. Finally, the CD spectra of the glycosides derived from compounds **2a**–**f** matched those of the synthetic D-galactosides, which demonstrates that the absolute stereochemistry of the two glycosides is the same.

Cytotoxic Activity

Ophidiacerebrosides C–E (**1d**–**f**) have been reported to be cytotoxic against L1210 murine leukemia cells.[13] The cytotoxic activities of compounds **1a**, **1f**, **2a**, and **2f** (the four glycosphingolipids isolated in their pure forms) were investigated on rat glioma C6 cells by evaluation of cell growth. To this aim, the cells were continuously treated for 48 h with 250 μM of each compound, and the MTT assay procedure was then performed. The results are shown in Table 2. All compounds exhibited significant cytotoxic activity against C6 cells, but compounds **1a** and **1f** displayed

Table 2. Effect of compounds **1a**, **1f**, **2a**, and **2f** (250 μM) on cell growth and viability.

Compound	% Inhibition[a]
1a	41.3 ± 4.5[c]
1f	37.3 ± 2.7[c]
2a	25.3 ± 4.7[b]
2f	24.0 ± 4.5[b]

[a] Results are expressed as mean inhibition vs. control ± SEM (n = 8) of two independent experiments. [b] $p \leq 0.01$ vs. control cells. [c] $p \leq 0.001$ vs. control cells.

a remarkably higher cytotoxic effect, which led to a decrease in mitochondrial activity by about 40% versus control cells (compared to about 25% for **2a** and **2f**). These results suggest that the nature of the sugar residue (β-glucopyranoside vs. β-galactopyranoside) is important for the cytotoxic activities of these types of compounds, and the glucosylceramides are more active. In contrast, the presence of a methyl branch at C-9 does not affect the activity significantly.

Enhancement of VEGF-Induced HUVEC Proliferation

Glycosphingolipids have been reported as modulators of signaling of angiogenic growth factors, among which vascular endothelial growth factor (VEGF) is the most important.[17] Identifying new molecules able to interfere with the formation of new blood vessels is an attractive goal in pharmaceutical studies, both in chronic ischemia and heart failure and in cancer therapy.

For this purpose, the proangiogenic activity of orecerebroside I (**2f**) and ophidiacerebroside E (**1f**) was assessed on human endothelial cells. Human umbilical vein endothelial cells (HUVEC) were preincubated for 1 h in endothelial basal medium (EBM) containing low serum (1% FCS) with **2f** or **1f** (10 μM) and then cultured in EBM with or without 20 ng/mL VEGF for 72 h. As shown in Figure 3, VEGF showed a significant effect on cell number (37%) compared to the control, whereas **2f** alone caused a small (16%), but not statistically significant, increase. The combination of preincubation with **2f** and exposure to VEGF increased cell number significantly both over the control (56%) and over VEGF alone (14%). On the contrary, no effect was observed on VEGF-induced proliferation of HUVEC preincubated with **1f** (data not shown). These results confirm the importance of the sugar residue in the modulation of the biological activity. Cell viability was unaffected by the presence of **1f** and **2f** both in proliferating and quiescent conditions, which suggests that these compounds are not cytotoxic at 10 μM (data not shown). Possible mechanisms for explaining the observed activity may involve the stabilization of VEGF/VEGFR complex or the VEGF bound to its receptors.

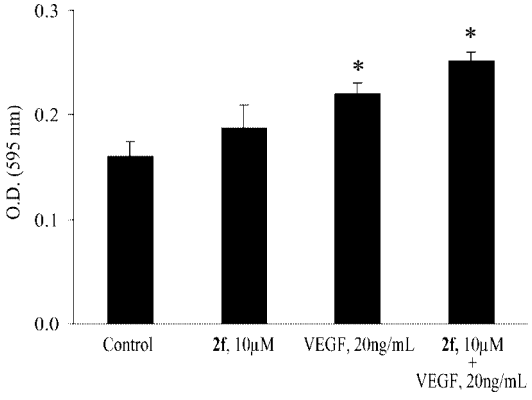


Figure 3. Compound **2f** increases the mitogenic activity induced by VEGF on HUVEC. Data are reported as mean ± SEM of three independent experiments carried out in triplicate. The asterisks indicate $p < 0.05$ (two-tailed paired Student's t-test).

Conclusions

Marine organisms often contain cerebrosides with a typical 4,8,10-triunsaturated sphingoid base (either 9-methyl branched or unbranched). All previously reported compounds of this type have a β-glucopyranoside as the sugar residue, as found in oreacerebrosides A–C. In contrast, oreacerebrosides D–I are the first examples of glycosphingolipids containing this triunsaturated sphingoid base associated with a β-galactopyranoside instead of a β-glucopyranoside. The nature of the sugar residue appears to be relevant to the biological activity of these glycosphingolipids. Thus, β-glucopyranosides showed stronger cytotoxicity, although this activity was weak in an absolute sense for all compounds. β-Galactopyranoside **2f** was able to improve the biological response to the angiogenic factor VEGF, whereas corresponding β-glucopyranoside **1f** was not active.

Experimental Section

General Experimental Procedure: ESI-MS was performed with an Applied Biosystems API 2000 triple quadrupole mass spectrometer, whereas high-resolution ESI-MS was performed with a Micromass QTOF spectrometer. The spectra were recorded by infusing samples into the ESI source as methanolic solutions containing 1 mM LiCl. Optical rotations were measured at 589 nm with a Perkin–Elmer 192 polarimeter and the use of a 10-cm microcell. CD spectra were recorded with a Jasco J-710 spectrophotometer and the use of a 1-cm cell. NMR spectra were recorded with Varian Unity Inova 700 and 500 NMR spectrometers; chemical shifts were referenced to residual solvent signals (CD_3OD: δ_H = 3.31 ppm, δ_C = 49.0 ppm; [D_5]pyridine: δ_H = 8.71, 7.56, and 7.19 ppm, δ_C = 149.9, 135.6, and 123.5 ppm). Homonuclear ^1H connectivities were determined by COSY experiments. Through-space ^1H–^1H interactions were studied by a ROESY experiment with a mixing time of 500 ms. The reverse-detected, gradient-enhanced single-quantum heteronuclear correlation (HSQC) spectra were optimized for an average $^1J_{CH}$ of 140 Hz. The gradient-enhanced multiple-bond heteronuclear correlation (HMBC) experiments were optimized for a $^3J_{CH}$ of 8 Hz. High-performance liquid chromatography (HPLC) was carried out with a Varian Prostar 210 apparatus equipped with a Varian 350 refractive index detector or a Varian 325 UV detector.

Collection, Extraction and Isolation: Specimens of *O. reticulatus* (phylum Echinodermata, class Asteroidea, order Valvatida, family Oreasteridae) were collected by scuba (depth 23 m) during the third "Pawlik expedition" (June 2003) along the coast of Grand Bahama Island (Bahamas), and stored at –20 °C until extraction. One frozen sample of *O. reticulatus* (volume of fresh material 2.4 L, dry weight after extraction 431 g), was blended in MeOH, then extracted in sequence with MeOH (4 × 2.5 L), MeOH/$CHCl_3$, 2:1 (2.5 L), MeOH/$CHCl_3$, 1:2 (2.5 L), and $CHCl_3$ (2 × 3 L). The MeOH and MeOH/$CHCl_3$ extracts were partitioned between BuOH and water. The BuOH phase was concentrated in vacuo and combined with the $CHCl_3$ extract. The total lipophilic extract (18.82 g) was subjected to reverse-phase chromatography on a column packed with RP-18 silica gel by elution with a gradient of

H_2O/MeOH and then $CHCl_3$. The fraction eluted with $CHCl_3$ (5.05 g) was further partitioned between $CHCl_3$/H_2O/MeOH (8:3:4). The organic layer (4.94 g) was dried, and subsequently chromatographed on a column packed with silica (*n*-hexane/EtOAc, 9:1 to MeOH). The fraction eluted with EtOAc/MeOH (7:3) (615 mg) was only composed of glycolipids. This fraction was acetylated with Ac_2O in pyridine at 25 °C for 18 h. The acetylated glycolipids were subjected to HPLC separation on a silica column (*n*-hexane/EtOAc, 7:3) to afford a mixture (45.9 mg) containing compounds **1a**–**f**, **2a**–**f**, and other glycolipids. Further normal-phase HPLC purification of this fraction (*n*-hexane/*i*PrOH, 95:5) gave 21.2 mg of a mixture composed only of compounds **1a**–**f** and **2a**–**f**. This mixture was dissolved in MeOH (2.5 mL), and a solution of MeONa in MeOH (0.4 M, 100 μL) was added. The reaction was allowed to proceed for 18 h at 25 °C, and the reaction mixture was dried under an atmosphere of nitrogen. The residue was then partitioned between water and chloroform. After removal of the solvent, the organic layer (16.9 mg) was subjected to HPLC separation on a silica column (*n*-hexane/*i*PrOH, 7:3) to give 6.1 mg of **1a**–**f** and 7.4 mg of **2a**–**f**. Reverse-phase HPLC separation of the former fraction (**1a**–**f**) on an RP-18 column (MeOH) gave 0.8 mg of pure oreacerebroside A (**1a**), 1.4 mg of an inseparable mixture of oreacerebroside B and ophidiacerebroside C (**1b/1d**), 2.2 mg of an inseparable mixture of oreacerebroside C and ophidiacerebroside D (**1c/1e**), and 1.6 mg of pure ophidiacerebroside E (**1f**). Likewise, reverse-phase HPLC separation of the latter fraction under the same conditions gave 1.1 mg of pure oreacerebroside D (**2a**), 2.1 mg of an inseparable mixture of oreacerebroside E and oreacerebroside G (**2b/2d**), 1.9 mg of an inseparable mixture of oreacerebroside F and oreacerebroside H (**2c/2e**), and 1.3 mg of pure oreacerebroside I (**2f**).

Oreacerebroside A (1a): Colorless oil. $[\alpha]_D^{25}$ = +5.6 (*c* = 0.1, PrOH). ^{1}H NMR (700 MHz, [D$_5$]pyridine): δ = 2.06, (q, *J* = 14.5, 7.2 Hz, 12-H), 2.16 (m, 7-H), 5.65 (m, 8-H, 11-H), 6.13 (m, 9-H, 10-H) ppm, all other signals were identical to those in the spectrum of **1f**. ^{13}C NMR (175 MHz, [D$_5$]pyridine): δ = 32.5 (C-7), 32.6 (C-12), 131.0 (C-9, C-10), 131.5 (C-8), 132.6 (C-11) ppm, all other signals were identical to those in the spectrum of **1f**. HRMS (ESI): calcd. for $C_{46}H_{85}LiNO_9$ [M + Li]$^+$ 802.6384; found 802.6396.

Oreacerebroside B (1b) and Ophidiacerebroside C (1d) (1b/1d, 1:2): Colorless oil. ^{1}H NMR (500 MHz, [D$_5$]pyridine) and ^{13}C NMR (125 MHz, [D$_5$]pyridine): superimposition of the spectra of **1a** and **1f**. HRMS (ESI): calcd. for $C_{47}H_{87}LiNO_9$ [M + Li]$^+$ 816.6541; found 816.6533.

Oreacerebroside C (1c) and Ophidiacerebroside D (1e) (1c/1e, 1:5): Colorless oil. ^{1}H NMR (500 MHz, [D$_5$]pyridine) and ^{13}C NMR (125 MHz, [D$_5$]pyridine): superimposition of the spectra of **1a** and **1f**. HRMS (ESI): calcd. for $C_{48}H_{89}LiNO_9$ [M + Li]$^+$ 830.6697; found 830.6685.

Ophidiacerebroside E (1f): Colorless oil. $[\alpha]_D^{25}$ = +5.8 (*c* = 0.1, PrOH). ^{1}H and ^{13}C NMR spectroscopic data in CD_3OD match those in ref.[13] ^{1}H NMR (700 MHz, [D$_5$]pyridine) and ^{13}C NMR (175 MHz, [D$_5$]pyridine): see Table 1. HRMS (ESI): calcd. for $C_{49}H_{91}LiNO_9$ [M + Li]$^+$ 844.6854; found 844.6844.

Oreacerebroside D (2a): Colorless oil. $[\alpha]_D^{25}$ = +8.6 (*c* = 0.2, PrOH). ^{1}H NMR (700 MHz, [D$_5$]pyridine): δ = 2.06 (q, *J* = 14.3, 7.1 Hz, 12-H), 2.15 (m, 7-H), 5.65 (m, 8-H, 11-H), 6.13 (m, 9-H, 10-H) ppm, all other signals were identical to those in the spectrum of **2f**. ^{13}C NMR (175 MHz, [D$_5$]pyridine): δ = 32.4 (C-7), 32.6 (C-12), 130.9 (C-9), 130.9 (C-10), 131.4 (C-8), 132.6 (C-11) ppm, all other signals were identical to those in the spectrum of **2f**. HRMS (ESI): calcd. for $C_{46}H_{85}LiNO_9$ [M + Li]$^+$ 802.6384; found 802.6371.

Oreacerebroside E (2b) and Oreacerebroside F (2d) (2b/2d, 1:2): Colorless oil. ^{1}H NMR (500 MHz, [D$_5$]pyridine) and ^{13}C NMR (125 MHz, [D$_5$]pyridine): superimposition of the spectra of **2a** and **2f**. HRMS (ESI): calcd. for $C_{47}H_{87}LiNO_9$ [M + Li]$^+$ 816.6541; found 816.6550.

Mixture of Oreacerebroside G (2c) and Oreacerebroside H (2e) (2c/2e, 1:5): Colorless oil. ^{1}H NMR (500 MHz, [D$_5$]pyridine) and ^{13}C NMR (125 MHz, [D$_5$]pyridine): superimposition of the spectra of **2a** and **2f**. HRMS (ESI): calcd. for $C_{48}H_{89}LiNO_9$ [M + Li]$^+$ 830.6697; found 830.6687.

Oreacerebroside I (2f): Colorless oil. $[\alpha]_D^{25}$ = +8.9 (*c* = 0.2, PrOH). ^{1}H NMR (700 MHz, [D$_5$]pyridine) and ^{13}C NMR (175 MHz, [D$_5$]-pyridine): see Table 1. HRMS (ESI): calcd. for $C_{49}H_{91}LiNO_9$ [M + Li]$^+$ 844.6854; found 844.6868.

Methanolysis of 2a–f: A mixture of oreacerebrosides D–I (100 μg) was dissolved in of HCl (1 N in 91% MeOH, 500 μL), and the resulting solution was kept for about 12 h at 80 °C in a sealed tube. The reaction mixture was dried under an atmosphere of nitrogen and partitioned between $CHCl_3$ and H_2O/MeOH (8:2). The aqueous layer was concentrated to give a mixture of methyl glycosides (fraction A), whereas the organic layer contained a mixture of α-hydroxy acid methyl esters and sphinganines.

Absolute Stereochemistry of Methyl Glycosides from Compounds 2a–f: Fraction A from methanolysis of mixture of **2a**–**f** was benzoylated with benzoyl chloride (20 μL) in pyridine (500 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH and after 30 min was dried under an atmosphere of nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column: Luna silica, 5 μ; eluent: *n*-hexane/*i*PrOH, 99:1, flow 1 mL/min). The chromatogram contained one prominent peak, which was identified as methyl 2,3,4,6-tetra-*O*-benzoyl-α-D-galactopyranoside by comparison of its retention time, ^{1}H NMR spectrum, and CD spectrum with those of an authentic sample prepared from D-galactose.

Cell Culture and Assay for Cytotoxic Activity: Rat glioma cells (C6 line) were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L) and supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. For biological assays, glioma cells were washed, collected by trypsine and then inoculated in 96-microwell culture plates at density of 10^4 cells/well. Cells were allowed to grow for 24 h, then the medium was replaced with fresh medium and cells were treated for a further 48 h with test compounds. In detail, a DMSO solution (2 μL) containing a test compound was added to cell culture medium to give a final concentration of 250 μM of the compound of interest; DMSO alone (vehicle, 2 μL) was added into control cells (1% *v*/*v* final concentration). After incubation, cell growth was evaluated with an MTT assay procedure, which measures the level of mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) as substrate.[18] The assay is based on the redox ability of living mitochondria to convert dissolved MTT into insoluble formazan. Briefly, the medium was removed and the cells were incubated with an MTT solution (5 mg/mL, 20 μL/well) for 1 h in a humidified 5% CO_2 incubator at 37 °C. The incubation was stopped by removing the MTT solution and adding DMSO (100 μL/well) to solubilize the formazan.[19] The absorbance was monitored at 550 nm by using a Perkin–Elmer LS 55 Luminescence Spectrometer (Perkin–Elmer Ltd, Beaconsfield, UK). Statistical significance among the means was determined by the ANOVA fol-

lowed by the Newman–Keuls test; p values ≤ 0.01 (**) and ≤ 0.001 (***) were considered statistically significant.

Cell Culture and Viability Assay on HUVEC: Human umbilical vein endothelial cells (HUVEC) were grown in EBM supplemented with 2% FCS, VEGF 0.5 ng/mL, heparin 22.5 μg/mL, EGF 0.1 ng/mL, bFGF 1 ng/mL, hydrocortisone 1 μg/mL, amphotericin B 50 ng/mL, gentamycin 50 μg/mL (EGM). Cells were maintained at 37 °C in humidified air containing 5% CO_2. The culture medium was changed every 2 d and cell cultures were split weekly. All experiments were performed using subconfluent cultures of passages 3–8. For viability assays, HUVEC were seeded at 2×10^3 cells/well in 96 well plates in EGM and incubated for 24 h. Then, cells were treated with compounds 10 μM or vehicle in EBM 1% FCS for 1 h. The medium was replaced with EBM 1% FCS with or without $VEGF_{165}$ (20 ng/mL) and the cells were incubated for a further 72 h.

For evaluation of the cytotoxicities of the compounds, the cells were incubated in EGM and EBM 1% FCS for 72 h in the presence of **1f** or **2f** (10 μM). Cell viability was evaluated with crystal violet, which correlates optical density with cell number, according to the procedure described by Gilles et al.[20] In detail, cells were washed with PBS and fixed by adding 10% formalin solution (100 μL). After 15 min, cells were washed with deionized water and stained with 100 μL of 0.1% crystal violet solution in water for 30 min. Excess dye was removed by washing with deionized water and plates were air-dried prior to bound dye solubilization in 200 μL of 10% acetic acid. The optical density of dye extracts was measured at 595 nm using a BioRad microplate Reader Model 680.

Statistical significance was carried out by means of the two-tailed paired Student's t-test. A p value of 0.05 was used as the limit for statistical significance. All experiments were performed in triplicate and repeated at least 3 times.

Supporting Information (see footnote on the first page of this article): ^{1}H NMR, COSY, HSQC, and HMBC spectra of compounds **1a**, **1f**, **2a**, and **2f**. ESI MS/MS spectra of compounds **2a**, **2f** and **2b/2d**.

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