New Glycosphingolipids from the Marine Sponge *Aplysinella rhax* and Their Potential as Nitric Oxide Release Inhibitors

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Several new glycosphingolipids were isolated by means of reversed-phase HPLC from the less-polar fraction of the methanol extract of the sponge *Aplysinella rhax*. Their structures, showing very close similarity, were elucidated by extensive NMR experiments including $^1H,^1H$ (COSY, TOCSY and ROESY) and $^1H,^{13}C$ (HMQC and HMBC) spectroscopy and chemical evidence. The molecular masses of the glycosphingolipids were determined by negative-ion fast-atom bombardment mass spectrometry, while HREIMS and HRFABMS provided important information for the identification of the fatty acids and sphingoid bases. The mixture of glycosphingolipids exhibited a good inhibitory activity on LPS-induced NO_2^- release by J774 A.1 macrophages.

Introduction

Aplysinella rhax was collected in the framework of a collaborative program on the search for new biologically active compounds from marine natural products. Our investigation of the MeOH extract of this sponge led to the isolation of three new glycosphingolipids (**4**, **8**, **12**) and the related nearly homogeneous glycosphingolipids **1**, **2**, **6**, **7** and **10**. The structure elucidation of the homogeneous glycosphingolipids was conducted due to the interest and importance attached to the determination of the composition of mixtures of glycolipids.[1] They possess the same branched trisaccharide moiety — 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside — bound to the primary alcoholic function of a ceramide composed of homogeneous phytosphingosines and 2-hydroxy fatty acids.

Results and Discussion

The chloroform-soluble part of the methanol extract of the freeze-dried specimens of *Aplysinella rhax*, collected in the shallow waters of New Caledonia, was subjected to normal-phase column chromatography to furnish a glycolipid mixture. Before isolation by reversed-phase HPLC and structure elucidation of the components, the basic structures of their sugar and ceramide moieties were determined. A negative-ion fast-atom bombardment spectrum showed a series of molecular ion peaks as anionized cluster ions $[M - H]^-$ at m/z = 1178, 1192, 1206, 1220, 1234, 1248, 1262, together with fragment ion peaks due to the cleavage of the saccharide moiety, indicative of a mixture of homologous glycolipids. The ^1H and ^{13}C NMR spectra measured in [D_5]pyridine showed the presence of two acetyls [δ_H = 2.04 (s, 3 H) and 2.06 (s, 3 H), δ_C = 23.6], three amides [δ_H = 8.35 (d, J = 8.4 Hz, 1 H), δ_C = 175.5; δ_H = 9.08 (d, J = 8.9 Hz, 2 H), δ_C = 171.2 and 171.6], several oxygen-bearing methine/methylene protons, including three anomeric protons [δ_H = 4.74 and 5.15 (d, J = 7.0 Hz), δ_C = 101.6 and 101.7; δ_H = 5.66 (d, J = 2.0 Hz), δ_C = 100.1] and a large methylene signal (δ_H = 1.12−1.32, δ_C = 30.1). Furthermore, in the ^{13}C NMR spectrum, the characteristic signals[2,3] due to the phytosphingosine-type ceramide possessing unbranched 2-hydroxy fatty acids and a trisaccharide moiety at C-1 were observed at δ_C = 14.3 ($-CH_2\mathit{CH_3}$), 22.9 [$-CH(\mathit{CH_3})_2$], 35.5 (C-3′), 51.2 (C-2), 69.4 (C-1), 72.5 (C-4), 72.5 (C-2′), 75.9 (C-3), 175.5 (C-1′). When the glycosphingolipids mixture was methanolyzed with 2.5 N HCl/MeOH a mixture of fatty acid methyl esters together with a mixture of long-chain bases was obtained. After acid hydrolysis with 1 N HCl, trimethylsilyl ethers of *N*-acetylglucosamine, *N*-acetylgalactosamine and fucose were detected in a 1:1:1 ratio in the GLC analysis after comparing the GC retention times of each sugar with those of authentic samples prepared by the same manner.[4]

The mixture of fatty acid methyl esters was characterized by HREIMS and ^1H NMR spectroscopy as methyl 2-hydroxyeicosanoate, methyl 2-hydroxyheneicosanoate; methyl

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2-hydroxydocosanoate; methyl 2-hydroxytricosanoate; methyl 2-hydroxytetracosanoate, methyl 2-hydroxypentacosanoate and methyl 2-hydroxyesacosanoate. The configuration at C-2′ was assigned as *R* on the basis of the negative $[\alpha]_D$ value of the mixture.[5] On the basis of their HRFAB spectra and by comparison of their ^{1}H NMR spectra and optical rotations of tetracetylate derivatives[6,7] the mixture of long-chain bases was identified as (2*S*,3*S*,4*R*)-2-amino-15-methyl-1,3,4-hexadecanetriol, (2*S*,3*S*,4*R*)-2-amino-16-methyl-1,3,4-heptadecanetriol and (2*S*,3*S*,4*R*)-2-amino-17-methyl-1,3,4-octadecanetriol.

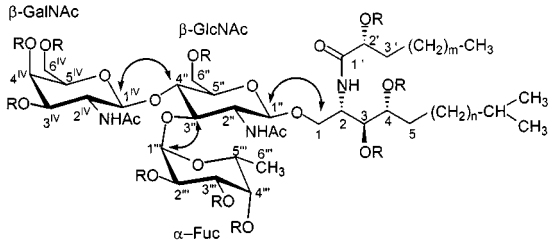
Therefore the glycosphingolipid mixture was suggested to be a molecular species composed of a phytosphingosine-type ceramide[8] possessing three monosaccharides (fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine) and an unbranched 2-hydroxy fatty acid. Because the presence of overlapping signals in the ^{1}H NMR spectrum, the glycosphingolipid mixture was subjected to reversed-phase HPLC chromatography, and further spectroscopic investigations on the sugar moiety were then performed on the peracetylated derivatives.[9−11]

By reversed-phase HPLC the glycosphingolipid mixture could be separated into twelve peaks.[6,12] Of the twelve components recovered, eight of them (**1, 2, 4, 6−8, 10** and **12**), each displaying a quasi molecular ion peak $[M - H]^-$ in the negative-ion FABMS spectrum (see Exp. Sect.), were each hydrolyzed with methanolic hydrochloric acid and the fatty acid methyl ester and long-chain base moieties were characterized by EI and FAB mass spectrometry, respectively. Unfortunately, a single fatty acid methyl ester and a single long chain base were detected only from the compounds **4**, **8** and **12**, therefore the glycosphingolipids **1**, **2**, **6**, **7** and **10** that were thought to be pure compounds were still mixtures of isomers possessing the same molecular mass but different fatty acid/long-chain base combinations.

The composition in fatty acids and phytosphingosines of each compound is described in the Exp. Sect. and shown in Figure 1.

Compounds **4**, **8** and **12** were acetylated under the usual conditions (Ac_2O/pyridine, room temperature, overnight). The ^{1}H NMR spectrum (Table 1) of the sugar portion of the peracetylated derivative **8a** measured in [D]chloroform exhibited signals for three anomeric protons at δ_H = 4.29 (d, J = 8.5 Hz), 4.77 (d, J = 7.7 Hz) and 5.33 (d, J = 4.0 Hz); one methyl doublet at δ_H = 1.27 (J = 6.4 Hz) and two exchangeable doublets at δ_H = 5.83 (J = 9.6 Hz) and 6.17 (J = 8.1 Hz). Three anomeric carbons signals (δ_C = 95.3, 98.7 and 101.5) were also observed in the ^{13}C NMR spectrum.

The ^{1}H and ^{13}C NMR chemical shift assignments of the sugars (Table 1) were made by COSY,[13] TOCSY,[14] ROESY,[15] HMQC[16] and HMBC[17] experiments. The COSY experiment allowed the sequential assignment of most of the resonances for each pyranosyl sugar ring, starting from the anomeric signals. Complete assignments were achieved by a combination of COSY and TOCSY results. Indeed, the TOCSY experiment clearly showed correlations for the H-1′′ to H_2-6′′ spin system of *N*-acetylglucosamine.



Compound			
1	R = H	m = 16, n = 10	(70%)
		m = 17, n = 9	(30%)
2	R = H	m = 16, n = 11	(10%)
		m = 17, n = 10	(60%)
		m = 18, n = 9	(30%)
4	R = H	m = 18, n = 10	(100%)
4a	R = Ac		
6	R = H	m = 18, n = 11	(19%)
		m = 19, n = 10	(56%)
		m = 20, n = 19	(25%)
7	R = H	m = 19, n = 11	(20%)
		m = 20, n = 10	(70%)
		m = 21, n = 9	(10%)
8	R = H	m = 19, n = 11	(100%)
8a	R = Ac		
10	R = H	m = 20, n = 11	(30%)
		m = 21, n = 10	(60%)
		m = 22, n = 9	(10%)
12	R = H	m = 22, n = 10	(100%)
12a	R = Ac		

Figure 1. New glycosphingolipids from the marine sponge *Aplysinella rhax*; bent double-headed arrows indicate significant HMBC correlations

The coherence transfer to H-5′′′ of fucose and to H-5^{IV} of *N*-acetylgalactosamine was not observed because of the small couplings H-4′′′/H-5′′′ and H-4^{IV}/H-5^{IV}. A proton−carbon one bond chemical shift correlation experiment by heteronuclear multiple quantum coherence (HMQC) correlated all the proton resonances with those of their corresponding carbon atoms (Table 1). The positions of the glycosidic linkages were established by a combination of HMBC and ROESY experiments. A long-range correlation between C-1 (δ_C = 66.2) of the ceramide and H-1′′ of *N*-acetylglucosamine (δ_H = 4.77) indicated that *N*-acetylglucosamine (GlcNAc) was connected to C-1 of the ceramide. This interpretation was confirmed by a strong ROESY cross peak between H-1 and H-1′′. The linkage of the *N*-acetylgalactosamine (GalNAc) at C-4′′ of the *N*-acetylglucosamine was indicated by the cross peaks C-4′′ GlcNAc (δ_C = 74.5)/H-1^{IV} GalNAc (δ_H = 4.29) in the HMBC spectrum. Similarly, the linkage of the fucose (Fuc) at the C-3′′ of the *N*-acetylglucosamine was indicated by the cross peaks H-1′′′ Fuc (δ_H = 5.33)/C-3′′ GlcNAc (δ_C = 72.6) and C-1′′′ Fuc (δ_C = 95.3)/H-3′′ GlcNAc (δ_H = 4.30). The β-configurations for the GlcNAc, GalNAc were deduced from their $^3J_{H1-H2}$ coupling constants (7−8 Hz) and correlations between H-1′′−H-5′′ and H-1^{IV}−H-5^{IV} in the

Table 1. NMR spectroscopic data for compounds **4a**, **8a** and **12a** ($CDCl_3$, 500 MHz)

		8a		**4a**[a]	**12a**[a]
	Position	δ_H [b]	δ_C	δ_C	δ_C
Ceramide					
	1	3.63 (dd, J = 11.0, 3.7 Hz), 3.73[c]	66.2	66.2	66.4
	2	4.25[c]	47.9	47.8	48.0
	2-NH	7.01 (d, J = 8.8 Hz)	–	–	–
	3	5.04[c]	68.0	68.0	68.2
	4	4.91[c]	72.6	72.5	72.7
	5	1.57[c]	28.0	27.9	28.2
	2′	5.05[c]	74.2	74.2	74.4
	3′	1.83[c]	31.4	31.5	31.6
	$-CH_2CH_3$	0.88 (t, J = 6.6 Hz)	14.0	14.0	14.1
	$-CH(CH_3)_2$	0.86 (d, J = 6.3 Hz)	22.6	22.7	22.8
	$-COCH_3$	1.97–2.22 (12 singlets)			
β-GlcNAc					
	1′′	4.77 d (J = 7.7 Hz)	98.7	98.7	99.0
	2′′	3.22 m	57.5	57.4	57.6
	2′′-NH	6.17 d (J = 8.1 Hz)	–	–	–
	3′′	4.30[c]	72.6	72.6	72.7
	4′′	3.70[c]	74.5	74.4	74.6
	5′′	3.55[c]	73.1	73.1	73.1
	6′′	4.22 (dd, J = 12.1, 4.4 Hz), 4.40 (br. d, J = 12.1 Hz)	61.8	61.7	61.9
α-Fuc					
	1′′′	5.33 (d, J = 4.0 Hz)	95.3	95.3	95.2
	2′′′	5.03[c]	72.5	72.5	72.6
	3′′′	5.20 (dd, J = 11.0, 3.3 Hz)	67.9	67.9	68.0
	4′′′	5.38 (br. d, J = 3.3 Hz)	71.3	71.3	71.4
	5′′′	4.95[c]	64.0	64.1	64.2
	CH_3	1.27 (d, J = 6.4 Hz)	15.3	15.2	15.2
β-GalNAc					
	1^{IV}	4.29 (d, J = 8.5 Hz)	101.5	101.4	101.6
	2^{IV}	4.15[c]	50.2	50.2	50.3
	2^{IV}-NH	5.83 (d, J = 9.6 Hz)	–	–	–
	3^{IV}	4.95[c]	70.1	70.0	70.1
	4^{IV}	5.36 (br. d, J = 3.3 Hz)	65.8	65.8	65.9
	5^{IV}	3.77 (t, J = 6.2 Hz)	70.6	70.6	70.7
	6^{IV}	4.28[c], 4.51 (dd, J = 11.4, 6.2 Hz)	60.5	60.4	60.6

[a] ^1H NMR ($CDCl_3$, 500 MHz): Almost the same spectrum as that of **8a**. [b] Assignments confirmed by two-dimensional experiments (COSY, TOCSY, ROESY, HMQC and HMBC). Additional ^1H NMR signals: δ_H = 1.50 [m, $-CH(CH_3)_2$], and 1.25 [broad band, alkyl chain protons]. [c] Submerged by other signals.

ROESY spectrum. The α-configuration for the Fuc was derived from its $^3J_{H1-H2}$ coupling constant (4.0 Hz).

On the basis of this, and assuming that GlcNAc, GalNAc and Fuc belong to the commonly found D series, the structures of the homogeneous glycosphingolipids were determined to be: **4**: 1-*O*-{2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxydocosanoylamino]-16-methyl-1,3,4-heptadecanetriol; **8**: 1-*O*-{2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxy-tricosanoylamino]-17-methyl-1,3,4-octadecanetriol; and **12**: 1-*O*-{2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-[α-D-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxy-esacosanoylamino]-16-methyl-1,3,4-heptadecanetriol.

Nitric oxide (NO), a short lived mediator, is synthesized by a family of enzymes termed NO-synthase (NOS).[18] Two types of NOS are recognized: constitutive isoforms (endothelial NOS and neuronal NOS) and an inducible isoform for which mRNA translation and protein synthesis are required.[18,19] Inducible NOS (iNOS) is regulated by inflammatory mediators (LPS, cytokines),[20] and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response.[21–23]

We measured the production of NO_2^- (a stable metabolite of NO) as a parameter of macrophage activation and iNOS induction. Unstimulated J774 A.1 cells generated undetectable (< 5 nmol/mL) amounts of NO_2^-. Stimulation of the cells with LPS (1 μg/mL) produced a significant release of NO_2^- (33.5 ± 1 nmol/mL). When the cells were incubated in the presence of the glycosphingolipid mixture a significant inhibition of LPS-induced NO_2^- release was observed.

In fact, the test compound given at 10 and 30 μg/mL significantly ($P < 0.01$) inhibited LPS-induced NO_2^- release by 15% and by 39%, respectively, while at 3 μg/mL it was inactive.

Experimental Section

General: All NMR measurements were performed on a Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer, using the UXNMR software package. The ^{1}H-detected one-bond and multiple-bond ^{13}C multiple-quantum coherence experiments (HMQC and HMBC, respectively) utilized a 5-mm probe with reverse geometry. The magnitude of the delay for optimizing one bond correlations in the HMQC spectrum and suppressing them in the HMBC spectrum was 3.5 ms, and the evolution delay for long-range couplings in the latter was set to 60 ms. Optical rotations were measured on a Perkin−Elmer 141 polarimeter. Fast ion bombardment mass spectra (FABMS) were recorded in a triethanolamine matrix on a VG PROSPEC instrument (Cs^+ ions with an energy of 4 kV). EIMS was performed using a VG PROPEC instrument (70 eV).

GLC analyses were performed on a Carlo Erba Fractovap 4160 for capillary column (SPB−1, 25 m; helium carrier flow 5 mL min^{-1}). Conditions of HPLC: column Phenomenex Luna C-18 (3 μ, 150 × 4.60 mm; flow rate 1 mL min^{-1}) Waters Model 510 pump equipped with U6 K injector and a differential refractometer, model 401.

Animal Material: The sponge was collected by divers at a depth of between 8 and 15 m at Luganville, New Caledonia in 1996. It was identified as *Aplysinella rhax*, (class Demospongiae, order Verongida, family Aplysinidae). Voucher specimen R1657 has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia. The sponge was frozen immediately after collection and freeze-dried to yield 536 g of dry mass.

Extraction and Isolation: The freeze-dried sponge (536 g) was extracted with MeOH (2 × 3 L) at room temperature. The combined extracts were taken to dryness and the glassy material (60 g) was subjected to Kupchan's partitioning scheme[24] to give four extracts: *n*-hexane (4.3 g), CCl_4 (1.3 g), $CHCl_3$ (8.0 g) and *n*BuOH (3.2 g). The CCl_4 extract was chromatographed on a silica gel column with a MeOH/CH_2Cl_2 solvent system. The 30% MeOH/CH_2Cl_2 fraction (150 mg) was separated by reversed-phase HPLC, using MeOH as solvent, to yield the twelve components: **1** (t_R = 16.8 min; 3.7 mg), **2** (t_R = 19.2 min; 3.0 mg), **3** (t_R = 20.8 min; 0.6 mg), **4** (t_R = 22.4 min; 4.0 mg), **5** (t_R = 24.0 min; 0.5 mg), **6** (t_R = 25.6 min; 1.4 mg), **7** (t_R = 27.6 min; 1.3 mg), **8** (t_R = 30.0 min; 5.4 mg), **9** (t_R = 32.4 min; 1.2 mg), **10** (t_R = 34.4 min; 2.6 mg), **11** (t_R = 37.2 min; 0.9 mg), **12** (t_R = 39.2 min; 3.5 mg).

Compounds 1, 2, 4, 6−8, 10 and 12: FABMS (−ve ion): (**1**): *m*/*z* = 1178 [M − H]$^-$; (**2**): *m*/*z* = 1192 [M − H]$^-$; (**4**): *m*/*z* = 1206 [M − H]$^-$; (**6**): *m*/*z* = 1220 [M − H]$^-$; (**7**): *m*/*z* = 1234 [M − H]$^-$; (**8**): *m*/*z* = 1234 [M − H]$^-$; (**10**): *m*/*z* = 1248 [M − H]$^-$; (**12**): *m*/*z* = 1262 [M − H]$^-$.

Compound 4: $[\alpha]_D^{25}$ = +2.6 (*c* = 0.07, MeOH). FABMS (−ve ion): *m*/*z* = 1206 [M − H]$^-$, 1060 [M − Fuc − H]$^-$, 1003 [M − GlcNAc − H]$^-$, 857 [M − GlcNAc − Fuc − H]$^-$, 654 [M − GlcNAc − Fuc − GalNAc − H]$^-$. $C_{62}H_{116}N_3O_{19}$ HRFABMS: *m*/*z* = 1206.8194 [M − H]$^-$ (Δ = 0.9 mDa).

Compound 8: $[\alpha]_D^{25}$ = +2.8 (*c* = 0.09, MeOH). FABMS (−ve ion): *m*/*z* = 1234 [M − H]$^-$, 1088 [M − Fuc − H]$^-$, 1031 [M − GlcNAc − H]$^-$, 885 [M − GlcNAc − Fuc − H]$^-$, 682 [M − GlcNAc − Fuc − GalNAc − H]$^-$. $C_{64}H_{120}N_3O_{19}$ HRFABMS: *m*/*z* = 1234.8510 [M − H]$^-$ (Δ = 0.6 mDa).

Compound 12: $[\alpha]_D^{25}$ = +1.8 (*c* = 0.05, MeOH). FABMS (−ve ion): *m*/*z* = 1262 [M − H]$^-$, 1116 [M − Fuc − H]$^-$, 1059 [M − GlcNAc − H]$^-$, 913 [M − GlcNAc − Fuc − H]$^-$, 710 [M − GlcNAc − Fuc − GalNAc − H]$^-$. $C_{66}H_{124}N_3O_{19}$ HRFABMS: *m*/*z* = 1262.8821 [M − H]$^-$ (Δ = 0.8 mDa).

Acetylation of Compounds 4, 8 and 12 to Yield the Peracetylated Derivatives 4a, 8a and 12a: Compounds **4**, **8** and **12** (each 3.0 mg) were acetylated with Ac_2O/C_5H_5N (1:1, 1 mL) at room temp. for 24 h to yield compounds **4a**, **8a** and **12a**. The reaction mixtures were concentrated in vacuo and the residues were chromatographed on a DIOL column, eluting with hexane/EtOAc (6:4), to afford **4a**, **8a** and **12a**.

Compounds 4a, 8a and 12a: For ^{1}H and ^{13}C NMR data in $CDCl_3$, see Table 1.

Acid Methanolysis of the Glycosphingolipid Mixture: A 6.2 mg portion of the glycosphingolipid mixture in anhydrous 2 N HCl/MeOH (1 mL) was heated at 80 °C in a stoppered reaction vial for 15 h. After cooling, the reaction mixture was extracted with *n*-hexane, and the hexane layer was concentrated to give a mixture of the methyl esters of α-hydroxy $C_{20}-C_{26}$ fatty acids. $[\alpha]_D^{25}$ = −30 (*c* = 0.20, $CHCl_3$). ^{1}H NMR (CD_3OD): δ = 0.91 (t, *J* = 7.1 Hz, 3 H, terminal CH_3), 1.3−1.4 (m), 1.58 (m, 2 H), 1.75 (m, 2 H), 3.75 (s, 3 H, $COOCH_3$), 4.16 (dd, *J* = 7.7, 4.4 Hz, 1 H); methyl *n*-2-hydroxyeicosanoate {EIMS: *m*/*z* = 342 [M$^+$]. HREIMS ($C_{21}H_{42}O_3$): 342.3127 (Δ = 0.7 mDa), 283 [M$^+$ − 59]}; methyl *n*-2-hydroxyheneicosanoate {EIMS: *m*/*z* = 356 [M$^+$]. HREIMS ($C_{22}H_{44}O_3$): 356.3284 (Δ = 0.6 mDa), 297 [M$^+$ − 59]}; methyl *n*-2-hydroxydocosanoate {EIMS: *m*/*z* = 370 [M$^+$]. HREIMS ($C_{23}H_{46}O_3$): 370.3439 (Δ = 0.7 mDa), 311 [M$^+$ − 59]}; methyl *n*-2-hydroxytricosanoate {EIMS: *m*/*z* = 384 [M$^+$]. HREIMS ($C_{24}H_{48}O_3$): 384.3609 (Δ = −0.6 mDa), 325 [M$^+$ − 59]}; methyl *n*-2-hydroxytetracosanoate {EIMS: *m*/*z* = 398 [M$^+$]. HREIMS ($C_{25}H_{50}O_3$): 398.3767 (Δ = −0.7 mDa), 339 [M$^+$ − 59]}; methyl *n*-2-hydroxypentacosanoate {EIMS: *m*/*z* = 412 [M$^+$]. HREIMS ($C_{26}H_{52}O_3$): 412.3910 (Δ = 0.6 mDa), 353 [M$^+$ − 59]}; methyl *n*-2-hydroxyesacosanoate {EIMS: *m*/*z* = 426 [M$^+$]. HREIMS ($C_{27}H_{54}O_3$): 426.4069 (Δ = 0.4 mDa), 367 [M$^+$ − 59]}.

The MeOH layer was evaporated to dryness in vacuo and the residue was subjected to silica gel column chromatography with a MeOH/$CHCl_3$ solvent system. The MeOH/$CHCl_3$ (1:4) fraction gave a mixture of sphingosine bases; 2-amino-15-methyl-1,3,4-hexadecanetriol {FABMS: *m*/*z* = 304 [M + H]$^+$. HRFABMS ($C_{17}H_{38}NO_3$): 304.2846 (Δ = 0.5 mDa)}; 2-amino-16-methyl-1,3,4-heptadecanetriol {FABMS: *m*/*z* = 318 [M + H]$^+$. HRFABMS ($C_{18}H_{40}NO_3$): 318.3015 (Δ = −0.7 mDa)}; 2-amino-17-methyl-1,3,4-octadecanetriol {FABMS: *m*/*z* = 332 [M + H]$^+$. HRFABMS ($C_{19}H_{42}NO_3$): 332.3158 (Δ = 0.6 mDa)}.

Acid Hydrolysis of the Glycosphingolipid Mixture and GLC Analysis of Sugars: A solution of the glycosphingolipid mixture (1.2 mg) in 1 N HCl (0.5 mL) was stirred at 60 °C for 4 h. After cooling, the solution was extracted with EtOAc, neutralized with Ag_2CO_3, filtered and the filtrate was freeze-dried. The residue was heated with 1-(trimethylsilyl)imidazole and pyridine and examined by GLC using an SPB-1 (Supelco) column (0.32 mm × 25 m). Temperatures of injector and detector were 200 °C and 230 °C, respectively. A temperature gradient system was used for the oven; the initial temperature was maintained at 100 °C for 2 min and then raised to

230 °C at the rate of 5 °C min^{-1} with the following results: t_R [min] = 15.6; 16.4 (1 mol) fucose; 24.4 (0.87 mol) *N*-acetylgalactosamine; 24.8 (0.78 mol) *N*-acetylglucosamine.

Acetylation of a Mixture of Sphingosine Bases: A mixture of the sphingosine bases was heated with Ac_2O/C_5H_5N (1:1) at 70 °C for 2 h. The reaction mixture was diluted with water and extracted with chloroform. The $CHCl_3$ extract was subjected to silica gel column chromatography to furnish a mixture of tetracetylated sphingosine bases. $[\alpha]_D^{25}$ = + 28.2 (*c* = 0.22, $CHCl_3$). FABMS *m*/*z* = 472, 486, 550 $[M + H]^+$. ^1H NMR ($CDCl_3$): δ_H = 0.86 (d, *J* = 6.7 Hz, 6 H), 1.24 (alkyl-chain protons, br), 1.69 (m, 1 H), 2.03 (s, 3 H), 2.06 (s, 6 H), 2.08 (s, 3 H), 3.99 (dd, *J* = 11.5, 4.5 Hz, 1 H), 4.28 (dd, *J* = 11.5, 5.0 Hz, 1 H), 4.49 (m, 1 H), 4.91 (dt, *J* = 9.0, 3.1 Hz, 1 H), 5.09 (dd, *J* = 8.2, 3.1 Hz, 1 H), 5.93 (d, *J* = 8.5 Hz, 1 H).

Acid Methanolysis of Each Glycosphingolipid: A 0.9 mg portion of **8** in anhydrous 2 N HCl/MeOH (1 mL) was heated at 80 °C in a stoppered reaction vial overnight. The reaction mixture was extracted with *n*-hexane, and the extract was then evaporated to dryness under N_2 to give methyl *n*-2-hydroxytricosanoate: EIMS: *m*/*z* = 384 $[M^+]$.

The compounds **1, 2, 4, 6, 7, 10** and **12** were treated similarly and the results were as follows:

The fatty acid methyl esters of **1** are methyl *n*-2-hydroxyeicosanoate (70%) and methyl *n*-2-hydroxyheneicosanoate (30%).

The fatty acid methyl esters of **2** are methyl *n*-2-hydroxyeicosanoate (10%) and methyl *n*-2-hydroxyheneicosanoate (60%), methyl *n*-2-hydroxydocosanoate (30%).

The fatty acid methyl ester of **4** is methyl *n*-2-hydroxydocosanoate (100%).

The fatty acid methyl esters of **6** are methyl *n*-2-hydroxydocosanoate (19%), methyl *n*-2-hydroxytricosanoate (56%) and methyl *n*-2-hydroxytetracosanoate (25%).

The fatty acid methyl esters of **7** are methyl *n*-2-hydroxytricosanoate (20%), methyl *n*-2-hydroxytetracosanoate (70%) and methyl *n*-2-hydroxypentacosanoate (10%).

The fatty acid methyl esters of **10** are methyl *n*-2-hydroxytetracosanoate (30%), methyl *n*-2-hydroxypentacosanoate (60%) and methyl *n*-2-hydroxycosanoate (10%).

The fatty acid methyl ester of **12** is methyl *n*-2-hydroxyhexacosanoate (100%).

The MeOH layer of compound **8** was neutralized with Ag_2CO_3, filtered and the filtrate concentrated in vacuo to give 2-amino-17-methyl-1,3,4-octadecanetriol. FABMS: *m*/*z* = 332 $[M + H]^+$.

Compounds **1, 2, 4, 6, 7, 10** and **12** were treated similarly and the results were as follows:

The long chain bases of **1** are 2-amino-16-methyl-1,3,4-heptadecanetriol (70%) and 2-amino-15-methyl-1,3,4-hexadecanetriol (30%).

The long chain bases of **2** are 2-amino-17-methyl-1,3,4-octadecanetriol (10%), 2-amino-16-methyl-1,3,4-heptadecanetriol (60%) and 2-amino-15-methyl-1,3,4-hexadecanetriol (30%).

The long chain base of **4** is 2-amino-16-methyl-1,3,4-heptadecanetriol (100%).

The long chain bases of **6** are 2-amino-17-methyl-1,3,4-octadecanetriol (19%), 2-amino-16-methyl-1,3,4-heptadecanetriol (56%) and 2-amino-15-methyl-1,3,4-hexadecanetriol (25%).

The long chain bases of **7** are 2-amino-17-methyl-1,3,4-octadecanetriol (20%), 2-amino-16-methyl-1,3,4-heptadecanetriol (70%) and 2-amino-15-methyl-1,3,4-hexadecanetriol (10%).

The long chain bases of **10** are 2-amino-17-methyl-1,3,4-octadecanetriol (30%), 2-amino-16-methyl-1,3,4-heptadecanetriol (60%) and 2-amino-15-methyl-1,3,4-hexadecanetriol (10%).

The long chain base of **12** is 2-amino-16-methyl-1,3,4-heptadecanetriol (100%).

Cell Culture: The murine monocyte/macrophages cell line J774 A.1 was from ECACC. J774 A.1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Biowhittaker) and cultured at 37 °C in humified 5% CO_2/95% air. The cells were plated in 24-well culture plates (Falcon) at a density of 2.5×10^6 cells/mL/well and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and the cells were activated by lipopolysaccharide (LPS 1 μg/mL) from *E. coli* (Fluka). The glycosphingolipid mixture was added to the cells 2 h before LPS challenge. After 24 h of incubation the culture medium was removed, centrifuged and the supernatant was used for the determination of nitrite (NO_2^-) production. Cell viability (> 95%) was determined with the MTT assay.[25]

NO_2^- assay: NO_2^- levels in culture media from J774 A.1 macrophages were measured 24 h after LPS challenge with the Griess reaction as previously described.[26] Results are expressed as nmol/mL of NO_2^- and represent the mean ± S.E.M. of *n* experiments run in triplicate.

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