Glycolipids from Sponges, I

Glycosyl Ceramide Composition of the Marine Sponge *Agelas clathrodes*

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The marine sponge *Agelas clathrodes* was analyzed for glycosphingolipids, and four cerebrosides (**1a**–**4a**) were isolated, each as a mixture of homologs. The structure of the new glycosphingolipid **1a** was elucidated by extensive NMR studies of its peracetate and by chemical analysis. The presence of unusual α-glycosphingolipids in sponges of the genus *Agelas* was confirmed.

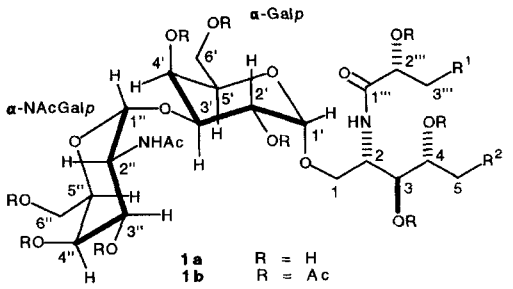
In two recent papers we reported on the isolation and structural determination of three new glycosphingolipids (GSLs), i.e. longiside, an α-digalactosyl ceramide, from the sponge *Agelas longissima*,[1] and axiceramide-A and -B isolated from the methanolic extract of the Senegalese sponge *Axinella* sp.[2]. Longiside and axiceramides belong to the quite small number of GSLs isolated from Porifera: a digalactosyl ceramide from *Haliclona japonica*[3], some β-galactosyl ceramides from *Chondropsis* sp.[4] and *Haliclona panicea*[5], a β-glucosyl ceramide from *Haliclona* sp.[6], two monoglycosyl ceramides with an α and β-galactosamine unit from *Amphimedon viridis*[6], and an α-galactosyl and a β-glucosyl ceramide from *Agelas mauritianus*[7].

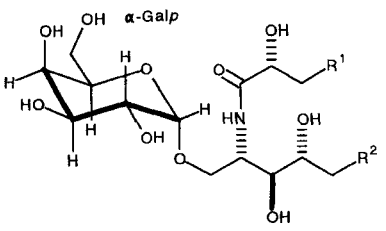
The obtained results, even though only few and concerning taxonomically quite different species were available, appear to be interesting, pointing to some peculiar structural features of the sponge GSLs. They seem to be characterized by an unusual variety of the shingoid moieties, including the normal sphingosine, the saturated phytosphingosine, and a rare C-9 branched triunsaturated sphingosine[7]. Also the sugar "head" of most sponge GSLs, which is supposed to contribute to many biological functions, is chemically uncommon being connected via an α-linkage to the ceramide part of the molecule. Some sponge GSLs were shown to be pharmacologically active compounds. In particular, agelasphins 7–13, which possess an α-galactosyl linkage, exhibit an immunostimulatory action, as well as a possibly related high in vivo antitumor activity[7].

An investigation of the glycolipid constituents of sponges has been recently initiated in our laboratory. A major purpose of such a study is to acquire a more detailed knowledge of the structure, distribution and bioactivities of spongal GSLs.

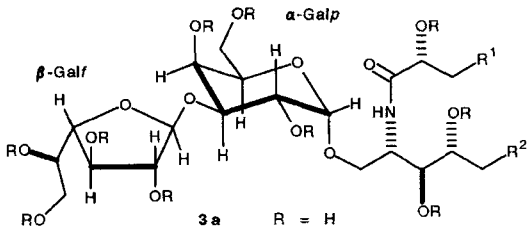
Results and Discussion

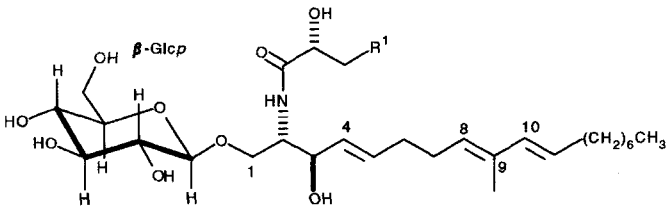
This paper reports on the results of the analysis of the glycolipidic fraction obtained from the methanolic extract of the Caribbean sponge *Agelas clathrodes* collected along





2a





R^1: see Table 2 R^2: see Table 3

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the coast of San Salvador Island. This species contains four glycosphingolipids **1a**–**4a**, three of them having an α-glycosidic linkage between the ceramide and the sugar moiety.

It should be noted that in the following discussion, even though all the GSLs from *A. clathrodes* were actually isolated as mixtures of homologs, we will refer to them as single compounds. In fact, it is now generally acknowledged that the biological activity of the GSLs mainly depends on the nature of the sugar head and the adjacent functionalized part of the ceramide[8]. In contrast, negligible importance appears to have so far been attached to the length and the branching of the alkyl chains, which are believed to serve as anchors to keep the molecule in the cellular membrane. Therefore, we think that two GSLs, differing only in the length of an alkyl chain or in the presence of an iso-branching, are not really different GSLs from a biological point of view.

Specimens of *A. clathrodes* were extracted twice with methanol and subsequently twice with chloroform, and the BuOH-soluble part of the extracts was subjected to reverse- and direct-phase column chromatography. A fraction, which was mainly composed of a mixture of glycosphingolipids, was separated by HPLC on a DIOL column, affording **1a**–**4a**. Each of these compounds gave a single spot on TLC, and appeared homogeneous by ^{1}H-NMR analysis as far as the sugar "head" is concerned. However, subsequent analysis showed that **1a**–**4a** were actually mixtures of homologs, differing in the length and in the branching of the sphingoid base and/or acyl chain. Attempts at chromatographic separation of such complex mixtures of homologs are reported in the literature[9,10], but they afforded only a small number of homogeneous fractions, while the other ones were still mixtures, which were not characterized at all, or only the major component was studied. In any case, the composition of the GSL mixture was not completely established. Therefore, we gave up any attempt to perform such a separation and carried out the structural determination of the whole mixture of homologs. Their composition in fatty acids and sphingoid bases was successively established by chemical degradation followed by GC-MS analysis.

Compound **1a**, isolated as an optically active amorphous solid, was subjected to a preliminary spectral analysis, which showed it to be a diglycosylated sphingolipid. Its ^{1}H-NMR spectrum, however, exhibits a very complex mid-field region with a number of overlapping signals. A better proton resolution was obtained in the ^{1}H-NMR spectrum of the peracetylated derivative **1b**, obtained by treatment of **1a** with Ac_2O/pyridine at room temperature. We decided therefore to continue the structure determination studies by using this derivative, taking also into account the additional advantage of the easy discrimination between ether and ester oxymethine proton resonances based on their different chemical shift ranges (δ = 3.5–4.5 and 4.7–5.7, respectively).

The negative ion FAB-MS spectrum of **1a** shows several molecular ion peaks at *m/z* 1061, 1047, 1033, and 1019, indicative of a mixture of homologs, in accordance with a molecular formula $C_{54}H_{104}N_2O_{15} + n\ CH_2$ (n = 0–3). A careful inspection of the ^{1}H- and ^{13}C-NMR spectra of **1b** and those reported for the longiside decaacetate[1] enabled the assignment of all the resonances of the ceramide portion of **1b**, which turned out to be composed of a trihydroxylated, saturated sphinganine and of a 2-hydroxy fatty acyl with the same relative configuration as the ceramide of the reference compound. Detailed analysis of the COSY-, HMQC-, and ROESY-NMR data obtained from **1b** confirmed the structure of the ceramide and allowed the identification of the sugar "head" of the molecule.

The anomeric protons (δ = 4.92, 1′-H, and 5.04, 1″-H) were readily identified from their HMQC correlation with the pertinent carbon atoms (δ = 93.9 and 96.7, respectively), resonating in a characteristic region of the ^{13}C-NMR spectrum. They were used in the analysis of the COSY-NMR spectrum as starting points for the sequential assignment of all the proton resonances within each monosaccharide unit. The high-field chemical shift of the protons 5′-H (δ = 4.04) and 5″-H (δ = 4.22) is indicative of the pyranose nature of both sugars moieties. The proton 2″-H, which is also rather shielded (δ = 4.62), shows an additional coupling with a D_2O exchangeable doublet at δ = 6.16. These features point to an acetamido group being linked to C-2″.

As a consequence of the good signal resolution of the ^{1}H-NMR spectrum of **1b**, the coupling constants of many sugar protons could be measured directly from the spectrum (see Table 1). This permitted the determination of the axial nature of 2″-H and 3″-H (both showing a large axial-axial coupling), as well as the equatorial nature of 1″-H and 4″-H. Coupling constant data did not allow the determination of the axial stereochemistry of 5″-H. This was deduced from a ROESY experiment, which displayed a distinct correlation peak between 5″-H and 3″-H attesting their 1,3-diaxial relationship. Therefore, the relevant sugar residue was identified as an α-2-amino-2-deoxygalactopyranose.

Likewise, the relative stereochemistry of 1′-H, 2′-H, 3′-H, and 4′-H, was established on the basis of the coupling constant data and that of 5′-H by means of ROESY data. In this case a quite severe signal overlapping in the region of the spectrum where the H-3′/H-5′ cross peak is located, prevented its unambiguous assignment. Anyway, further supporting data were obtained, as reported below, from a degradative analysis. Thus, the second sugar residue is an α-galactopyranose.

The ROESY correlation peaks of 1′-H with the methylene protons at C-1 demonstrate that the sugar unit directly linked to the ceramide is the galactopyranose. Similarly, in the same spectrum cross peaks of the anomeric proton 1″-H with 3′-H and 4′-H, as well as the high-field chemical shift value of 3′-H, suggest that C-3′ is glycosylated by the α-2-amino-2-deoxygalactopyranose, thus defining the gross structure of **1b**.

At this point, the determination of the complete structure of compound **1b** required the determination of the length of the alkyl chains of the sphingosine and of the α-hydroxyacyl groups, as well as the assignment of the absolute

Table 1. ^{1}H- and ^{13}C-NMR data of compound **1b** ($CDCl_3$)

Pos.	δ_H (mult., J [Hz])[a]	δ_C (mult.)[b]
1 a	3.33 (dd, 10.6, 2.1)	63.1 (CH_2)
b	3.73 (dd, 10.6, 2.7)	
2	4.21[c]	45.9 (CH)
2-N*H*	7.06 (d, 8.9)	
3	5.31 (dd, 9.6, 2.4)	68.1 (CH)
4	4.82 (m)	70.8 (CH)
5	1.58[c]	26.8 (CH_2)
1'	4.92(d, 3.7)	93.9 (CH)
2'	5.02 (dd, 10.3, 3.4)	66.8 (CH)
3'	4.16 [c]	71.3 (CH)
4'	4.46 (br. d, 3.08)	65.9 (CH)
5'	4.04[c]	64.3 (CH)
6' a	4.01[c]	59.1 (CH_2)[d]
b	4.20[c]	
1"	5.04 (d, 3.7)	96.7 (CH)
2"	4.62 (ddd, 11.6, 9.6, 3.7)	44.8 (CH)
2"N*H*	6.16 (d, 9.6)	
3"	4.96 (dd, 11.6, 3.4)	65.1 (CH)
4"	5.37 (br. d, 2.7)	64.3 (CH)
5"	4.22[c]	64.7 (CH)
6" a	4.01[c]	58.1 (CH_2)[d]
b	4.21[c]	
2'''	5.06 (dd, 7.5, 4.4)	71.6 (CH)
3'''	1.81[c]	29.3 (CH_2)
Ac's	1.99-2.22 (10 singlets)	

[a] Additional ^{1}H signals: δ = 1.50 [m, $CH(CH_3)_2$], 1.25 [broad-band, alkyl chain protons], 0.86 [t, J = 7.0, $-CH_2CH_3$], 0.84 [d, J = 6.5, $-CH_2(CH_3)_2$]. – [b] Additional ^{13}C signals: δ = 172.7–160.0 (several C), 39.4 (CH), 32.0 (CH_2), 29.7–29.4 (several CH_2), 28.0 (CH), 25.6 (CH_2), 25.1 (CH_2), 22.8 (CH_3), 22.7 (CH_2), 22.2–20.3 (several CH_3), 14.1 (CH_3). – [c] Overlapped by other signals. – [d] Resonances may be reversed.

stereochemistry of the ceramide and of the sugar units. This was accomplished by acid methanolysis of **1b** with 1 M HCl in 90% MeOH. The resulting reaction mixture was separated into fractions of different polarity. The less polar fraction A, as indicated by the ^{1}H-NMR spectrum, contained exclusively unbranched long-chain methyl esters, which could be identified and quantified by GC-MS analysis (see Table 2). The configuration at C-2 was assigned as *R* on the basis of the negative $[\alpha]_D$ of the mixture[10].

The ^{1}H-NMR spectrum indicates the fraction B to be composed of a mixture of 4-hydroxysphinganines, differing from each other in their terminal alkyl chain. This structural detail was ascertained by permanganate/periodate oxidative cleavage of C-3/C-4 bond as described in ref.[2]. The obtained carboxylic acids were methylated with diazomethane, and the resulting esters were analyzed by GC MS. The results of the analysis are summarized in Table 3. The absolute stereochemistry of the phytosphingosines was deduced from the positive $[\alpha]_D$ of the fraction[11].

The most polar fraction C was acetylated and subjected to HPLC separation, giving methyl α-D-galactopyranoside tetraacetate and methyl 2-amino-2-deoxy-α-D-galactopyranoside tetraacetate as the major products, which were identified by a comparison of their retention times, optical rotations and ^{1}H-NMR spectra with those of authentic samples.

Once the structure of compound **1b** was secured, we could completely assign the ^{1}H- and ^{13}C-NMR spectra of the parent compound **1a** (see Experimental) by means of the analysis of a COSY and a HMQC spectrum. This allowed us to demonstrate that the amino group of the 2-amino-2-deoxygalactopyranose was already acetylated in **1a**.

Compound **2a** was identified by a comparison of its NMR spectrum with that reported for agelasphin-1[7].

Table 2. Fatty acyl composition of compounds **1a**, **2a**, **3a**, and **4a**

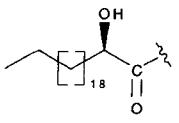
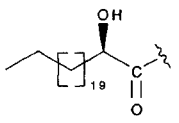
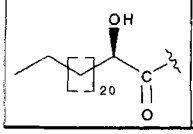
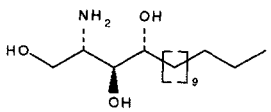
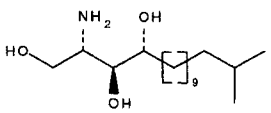
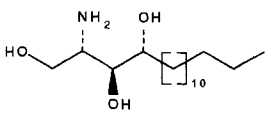
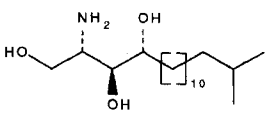
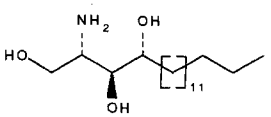
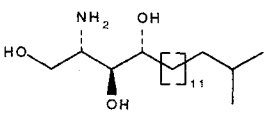
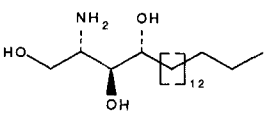
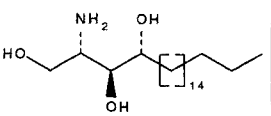
Fatty acyl	**1a**	**2a**	**3a**	**4a**
	18.6 %	12.8 %	18.6 %	19.7 %
	16.0 %	16.0 %	15.6 %	23.4 %
	65.4 %	71.2 %	65.6 %	56.9 %

Table 3. Sphinganine composition of compounds **1a**, **2a**, and **3a**

Sphinganine	**1a**	**2a**	**3a**
	27.9 %	34.0 %	28.8 %
	4.5 %	4.9 %	< 1 %
	17.0 %	21.2 %	17.1 %
	21.5 %	19.5 %	17.1 %
	8.3 %	10.8 %	7.7 %
	9.4 %	5.9 %	5.2 %
	9.0 %	3.7 %	14.4 %
	2.4 %	< 1%	9.7 %

Compound **3a** was acetylated, and the ^{1}H-NMR spectrum of its decaacetyl derivative was identical with that of longiside decaacetate[1]. In addition, the combined analysis of COSY-, HOHAHA- and ROESY-NMR spectra of the natural compound **3a** allowed complete assignment of its ^{1}H-NMR spectrum (see Experimental). Finally, compound **4a** was identified by a comparison of its ^{1}H-NMR spectrum with that reported for ophidiacerebrosides[12]. Also compounds **2a**–**4a** turned out to be mixtures of homologs as indicated by negative FAB MS. Therefore, their composition in fatty acids and sphinganines was established by degradative analysis as reported above for **1a**, and the results are compiled in Tables 2 and 3.

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Experimental

General Methods: FAB MS: VG ZAB mass spectrometer (Xe atoms of energy of 2–6 kV). – Optical rotations: Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell. – ^{1}H and ^{13}C NMR: Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26, δ_C = 77.0; [D$_6$]DMSO: δ_H = 2.50, δ_C = 39.7); methyl, methylene and methine carbon-atoms were distinguished by DEPT experiments; homonuclear ^{1}H connectivities were determined by COSY experiments; the reverse multiple-quantum heteronuclear correlation (HMQC) spectrum was recorded by using a pulse sequence developed by Bax and Subramanian[13], with a BIRD pulse 0.65 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. During the acquisition time ^{13}C broad-band decoupling was performed by using the GARP sequence[14]. – Medium-pressure liquid chromatography (MPLC): Büchi 861 apparatus with an SiO_2 column (230–400 mesh). – HPLC: Varian 2510 apparatus equipped with an RI-3 refractive index detector, and with Hibar columns. – GC MS: Hewlett-Packard 5890 gas chromatography with a mass-selective detector MSD HP 5970 MS, a split/splitness injector, and fused-silica column, 25 m × 0.20 mm HP-5 (cross-linked 25% Ph-Me silicone, 0.33 mm film thickness) was used; the temperature of the column was varied, after a delay of 5 min from the injection, from 150°C to 300°C with a slope of 5°C min^{-1}; quantitative determination was based on the area of the GLC peaks.

Extraction and Isolation of GSLs: Specimens of *Agelas clathrodes* were collected in the summer of 1992 along the coast of San Salvador Island (depth of 15 m) 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. The sponge (110 g of dry weight after extraction) was homogenized and extracted (4 × 500 ml) twice with methanol and then twice with chloroform; the combined extracts were partitioned between H_2O and *n*BuOH. The organic layer was concentrated in vacuo and afforded 3 g of a dark brown oil which was chromatographed on a column packed with RP-18 silica gel. Only three fractions (A–C) were collected [eluents: MeOH/H_2O (9:1), MeOH/EtOAc (9:1), and $CHCl_3$, respectively]. Fraction B (355 mg), containing glycolipids, was further chromatographed on a SiO_2 column, and three fractions (B1–B3) were eluted [eluents: EtOAc/hexane (9:1), EtOAc/MeOH (7:3), and MeOH, respectively]. Fraction B2 (112 mg) was mainly composed of a mixture of glycosphingolipids, which were separated by HPLC on a DIOL column [eluent: CH_2Cl_2/MeOH (95:5)], thus affording compounds **1a** (11 mg), **2a** (14 mg), **3a** (31 mg) and **4a** (33 mg), homogeneous as far as the polar head is concerned.

(2S,3S,4R)-O-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-(α-D-galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol (**1a**): White solid, $[\alpha]_D^{25}$ = +37 (*c* = 0.004 in DMSO). – Negative FAB MS: *m/z* = 1061, 1047, 1033, and 1019, [M – H]$^-$ series. – ^{1}H NMR (DMSO, 317 K): δ = 7.46 (d, *J* = 8.5 Hz, 2-NH), 7.45 (d, *J* = 8.5 Hz, 2″-NH), 5.47 (1H, d, *J* = 5.0 Hz, 2‴-OH), 4.75 (1H, d, *J* = 3.4 Hz, 1′-H), 4.74 (1H, d, *J* = 3.4 Hz, 1″-H), 4.68 (br. s, 3-OH), 4.48 (1H, t, *J* = 5.4 Hz, 6′-OH), 4.39 (1H, d, *J* = 3.7 Hz, 4″-OH), 4.33 (1H, t, *J* = 5.4 Hz, 6″-OH), 4.27 (3H, br. s, 2′-OH, 3″-OH and 4-OH), 4.14 (br. s, 4′-OH), 4.12 (overlapped, 2″-H), 4.09 (m, 2-H), 4.04 (1H, t, *J* = 6.5, 5″-H), 3.85 (1H, m, 2‴-H), 3.83 (1H, m, 4′-H), 3.79 (1H, m, 4″-H), 3.74 (overlapped, 1_a-H), 3.73 (overlapped, 2′-H), 3.69 (overlapped, 3″-H), 3.58 (overlapped, 3′-H), 3.57 (overlapped, 5′-H), 3.54 (overlapped, 6′-H_a), 3.53 (overlapped, 6″-H_a, and 6″-H_b), 3.50 (overlapped, 1-H_b), 3.46 (overlapped, 6′-H_b), 3.39 (overlapped, 3-H), 3.38 (overlapped, 4-H), 1.85 (3H, s, 2″-Ac), 1.62 (1H, m, 3‴$_a$-H), 1.58 (overlapped, 5_a-H), 1.51 (overlapped, 3‴$_b$-H), 1.42 (overlapped, 5_b-H), 1.24 (large band, alkyl chain protons), 0.86 (t, *J* = 7.0 Hz, $-CH_2CH_3$), 0.85 [d, *J* = 6.5 Hz, $-CH(CH_3)_2$]. – ^{13}C NMR (DMSO): δ = 99.4 (CH, C-1′), 95.5 (CH, C-1″), 76.3 (CH, C-5′), 74.2 (CH, C-3), 71.7, (CH, C-2‴), 71.0 (CH, C-3′), 70.7 (CH, C-5″), 70.6 (CH, C-4), 68.3 (CH, C-4″), 67.9 (CH, C-3″), 66.8 (CH_2, C-1), 66.6 (CH, C-2′), 65.6 (CH, C-4′), 60.5 (CH_2, C-6′), 60.4 (CH_2, C-6″), 49.7 (CH, C-2″), 49.3 (CH, C-2), 34.3 (CH_2, C-3‴), 31.2 (CH_2), 29.3–28.6 (several CH_2), 25.3 (CH_2), 24.5 (CH_2), 22.8 (CH_3), 22.4 (CH_3), 22.0 (CH_2), 13.8 (CH_3). – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(2S,3S,4R)-O-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-(1→3)-(α-D-galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol Peracetate (**1b**): An aliquot (5 mg) of compound **1a** was acetylated by using the standard procedure[2]. The peracetylated compound **9b** was obtained as a colorless oil, $[\alpha]_D^{25}$ = +52 (*c* = 0.004 in $CHCl_3$). – ^{1}H and ^{13}C NMR: see Table 1. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(2S,3S,4R)-O-(α-D-Galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol (**2a**) was identified by a comparison of its ^{1}H-NMR spectrum with that reported[7]. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(2S,3S,4R)-O-(β-D-Galactofuranosyl)-(1→3)-(α-D-galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol (**3a**): White solid, $[\alpha]_D^{25}$ = +28 (*c* = 0.004 in DMSO). – Negative FAB MS: *m/z* = 1020, 1006, 992, and 978, [M – H]$^-$ series. – ^{1}H NMR (DMSO, 317 K) δ = 7.50 (d, *J* = 9.5 Hz, 2-NH), 5.49 (1H, d, *J* = 4.7 Hz, 2‴-OH), 5.16 (1H, d, *J* = 5.8 Hz, 2″-OH), 5.02 (1H, br. s, 1″-H), 4.86 (1H, d, *J* = 6.8 Hz, 3″-OH), 4.72 (1H, d, *J* = 3.7 Hz, 1-H), 4.62 (br. s, 3-OH), 4.58 (1H, d, *J* =

5.4 Hz, 5″-OH), 4.53 (1 H, d, J = 6.4 Hz, 2′-OH), 4.56 (overlapped, 6′-OH), 4.55 (overlapped, 6″-OH), 4.28 (1 H, br. s, 4-OH), 4.10 (overlapped, 2-H), 4.07 (d, J = 3.4 Hz, 4′-OH), 3.90 (overlapped 2″-H), 3.90 (overlapped, 4″-H), 3.88 (overlapped, 4′-H), 3.87 (overlapped, 2‴-H), 3.80 (1 H, m, 3″-H), 3.73 (overlapped, 1-H_a), 3.69 (overlapped, 2′-H), 3.64 (1 H, t, J = 6.0 Hz, 5′-H), 3.58 (1 H, dd, J = 10.5 and 2.7 Hz, 3′-H), 3.54 (overlapped, 5″-H), 3.53 (overlapped, 1-H_b), 3.53 (overlapped, 6′-H_a), 3.46 (overlapped, 6′-H_b), 3.42 (overlapped 3-H), 3.41 (6″-H_a, and 6″-H_b), 3.40 (overlapped, 4-H), 1.85 (3 H, s, 2″-Ac), 1.61 (1 H, m, 3‴-H_a), 1.52 (overlapped, 5-H_a), 1.48 (overlapped, 3‴-H_b), 1.43 (overlapped, 5-H_b), 1.24 (large band, alkyl-chain protons), 0.86 (t, J = 7.0 Hz, $-CH_2CH_3$), 0.85 [d, J = 6.5 Hz, $-CH(CH_3)_2$]. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(2S,3S)-O-(β-D-Galactofuranosyl)-(1→3)-(α-D-galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol Peracetate (**3b**): An aliquot (5 mg) of compound **1a** was acetylated by using the standard procedure[2]. The peracetylated compound **9b** shows spectroscopic properties identical with those of an authentic sample of longiside decaactate[1]. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(4E,8E,10E,2S,3S)-O-(β-D-Glucopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-9-methyl-4,8,10-octadecatriene-1,3,4-triol (**4a**) was identified by a comparison of its ^{1}H-NMR spectrum with that reported[12]. – Composition in fatty acids: Table 2.

Methanolysis of GSLs: A small amount (2–5 mg) of the GSL was dissolved in 1 ml of 1 N HCl in 91% MeOH and the obtained solution was kept for about 12 h at 80 °C in a sealed tube. The reaction mixture was dried under nitrogen, dissolved in a small quantity of $CHCl_3$ and the solution was passed through a SiO_2 column (70–230 mesh). Elution with 15 ml of 0.1% pyridine in $CHCl_3$ gave a mixture of α-hydroxy acid methyl esters (fraction A), and subsequent elution with 0.1% pyridine in MeOH afforded sphinganines and methyl glycosides. The mixture was partitioned between $CHCl_3$ and H_2O/MeOH (8:2), the organic and aqueous layers were separated and concentrated to give a mixture of sphinganines (fraction B) and a mixture of methyl glycosides (fraction C), respectively.

Analysis of Fatty Acid Methyl Esters: Fractions A from compounds **1b** ($[\alpha]_D^{25}$ = −3, c = 0.001 in $CHCl_3$), **2a** ($[\alpha]_D^{25}$ = −3, c = 0.002 in $CHCl_3$), **3b** ($[\alpha]_D^{25}$ = −2, c = 0.002 in $CHCl_3$), and **4a** ($[\alpha]_D^{25}$ = −3, c = 0.002 in $CHCl_3$) were analyzed by GC MS and their components identified by a comparison of their retention times and mass spectra with those of authentic samples.

Analysis of Sphinganines: Fractions B from compounds **1b** ($[\alpha]_D^{25}$ = +7, c = 0.001 in $CHCl_3$), **2a** ($[\alpha]_D^{25}$ = +10, c = 0.001 in $CHCl_3$), and **3b** ($[\alpha]_D^{25}$ = +8, c = 0.001 in $CHCl_3$) were subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described in ref.[2], and the resulting carboxylic acids were methylated with diazomethane and the obtained esters analyzed by GC MS. The results are compiled in Table 3, expressed in terms of original sphinganines. The sphinganine from **4a**, isolated as a pure compound, was acetylated and identified by a comparison of its ^{1}H NMR and mass spectral data with those reported for (4*E*,8*E*,10*E*,2*S*,3*S*)-2-acetamido-1,3-diacetoxy-9-methyl-4,8,10-octadecatriene[7].

Analysis of Methyl Glycosides from Compound **1b**: Fraction C from compound **1b** was acetylated and subjected to HPLC separation (column: RP-18, 250 × 4 mm; eluent: H_2O/MeOH 1:1), affording methyl α-D-galactopyranoside tetraacetate and methyl 2-amino-2-deoxy-α-D-galactopyranoside tetraacetate. All the methyl glycosides were identified by a comparison of their retention times, ^{1}H-NMR spectra and optical rotations with those of authentic samples prepared from D-galactose and 2-amino-2-deoxy-D-galactose under the same conditions employed for the methanolysis of GSLs.

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