Glycolipids from Sponges, II[◇]

Glycosyl Ceramide Composition of the Marine Sponge *Agelas longissima*

Francesco Cafieri, Ernesto Fattorusso*, Alfonso Mangoni, and Orazio Taglialatela-Scafati

Dipartimento di Chimica delle Sostanze Naturali,
Via D. Montesano 49, 80131 Napoli, Italy

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A specimen of the marine sponge *Agelas longissima* was analyzed for glycosphingolipids. Three cerebrosides **1a–3a** were isolated, each as a mixture of homologs. The structure of the new glycosphingolipid **1a** was elucidated by extensive NMR studies of its peracetate derivative and chemical analysis. Compound **2a**, previously isolated from another specimen of *A. longissima*, exhibits in the new specimen a remarkably different alkyl chain composition.

Glycosphingolipids (GSLs) are ubiquitous membrane constituents in animals, plants and also lower forms of life, and they are believed to play fundamental roles in membrane phenomena such as cell-cell adhesion, cell-cell recognition, antigenic specificity, and other kinds of transmembrane signalling. Changes in their composition that accompany development and differentiation of organisms suggest that GSLs may be involved here as well[1]. By a chemical point of view, spongal GSLs appear to be a quite peculiar class of molecules in terms of the structure of their carbohydrate moieties and ceramide portions. According to the relatively little knowledge available so far, they seem to differ significantly from the deeply studied vertebrate GSLs, and the presence, particularly in sponges of the genus *Agelas*, of some GSLs with the unusual α-glycosidic linkage between the aglycone and the sugar moiety is worthy of note.

On account of the increasing interest in spongal GSLs and considering also the small number of species investigated so far, we started a systematic study of glycolipids from porifera[2,3]. Concerning the first results of such a work we recently reported[2] on the chemical investigation of a specimen of the marine sponge *Agelas longissima* (*long. 1*), a Demospongia of the order Poecilosclerida collected in the Caribbean Sea during the summer of 1992. This organism contained large amounts (7.3% of organic extract) of a diglycosylated GSL, longiside (**2c**), possessing the uncommon β-galactofuranose, which exhibited a nearly homogeneous composition of the ceramide portion. This result appeared quite unusual, if we consider that GSLs are generally composed of complex mixtures of homologs varying in the length and branching of the alkyl chains.

We have now investigated the glycolipidic composition of another specimen of *Agelas longissima* (*long. 2*), collected in the same period but in a different place of the Caribbean area. In the present paper we report on the results obtained by complete analysis of the GSL fraction obtained from the methanolic extract.

This study revealed that the composition of *long. 2* was not identical with that previously reported for *long 1*. In fact, the organism under investigation contained a GSL (**2a**) with the same sugar head moiety as that of longiside (**2c**)[2], but with a great variety in the length both of the sphingosine and α-hydroxyacyl portions, as determined by degradative analysis. Furthermore, from the same organism (*long. 2*) we isolated comparable amounts of a novel α-digalactopyranosyl sphingolipid (**1a**), whose complete structural characterization is reported in this paper. Finally, smaller amounts of another known GSL (**3a**) were also found. Stimulated by these results, we re-examined the extract from *long. 1* and found that, in addition to the reported longiside (**2c**)[2], which is by far the most abundant component of the sphingolipidic fraction, minute amounts of GSLs with the same sugar moieties as those of **1a** and **3a** were also present. The quantity of these two compounds was insufficient for their complete structural determination, but since we had the spectra of **1a** and **3a** the identification of their sugar moieties was easy.

All these data show that, as previously observed by a number of authors, the biochemistry of marine sponges is significantly influenced by the environmental conditions. However, it should be noted that both the examined specimens (*long. 1* and *long. 2*) were able to elaborate GSLs with the same functional moiety, even though in different amounts and with different alkyl chains. This result also confirms that the length of alkyl chains in the ceramide moiety plays a minor part in the biological activity of GSLs, which realistically use them only as an anchor to the cellular membrane. Furthermore, this study strengthens the observation that all the GSLs of the same organism show almost the same percentage composition of the alkyl chain length. In this regard, it should be also pointed out that in the present discussion, like in our previous papers of the series, a simple

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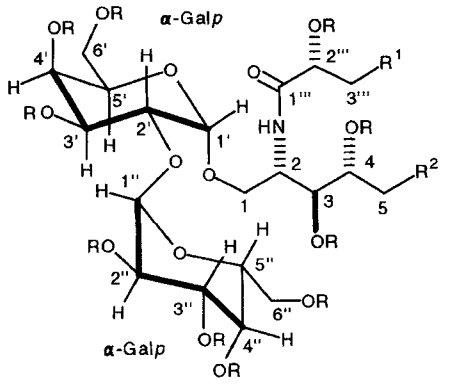
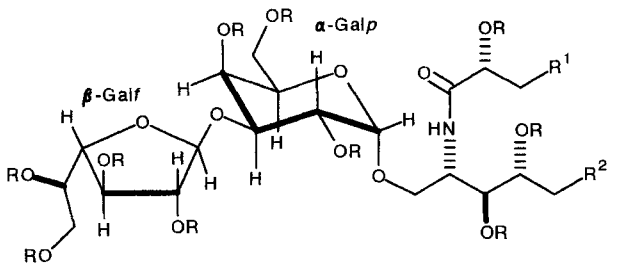


1a R = H R[1]: see Table 2 R[2]: see Table 3

1b R = Ac R[1]: see Table 2 R[2]: see Table 3

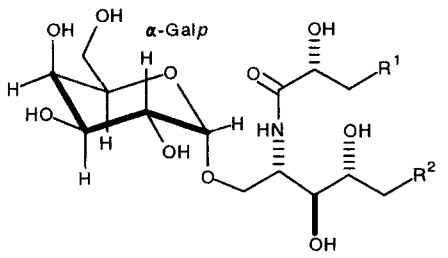


2a R = H R[1]: see Table 2 R[2]: see Table 3

2b R = Ac R[1]: see Table 2 R[2]: see Table 3

2c R = H R[1] = $(CH_2)_{20}CH_3$ R[2] = $(CH_2)_{10}CH_3$

2d R = Ac R[1] = $(CH_2)_{20}CH_3$ R[2] = $(CH_2)_{10}CH_3$



3a R[1]: see Table 2 R[2]: see Table 3

mixture of homologs will be considered as a single compound as far as the structural determination of the sugar head is concerned.

The BuOH partition material obtained from exhaustive MeOH extraction of frozen specimens of *Agelas longissima* (*long. 2*) afforded 4.0 g of a brown-colored viscous oil. This crude extract was initially fractionated by using a silica gel chromatographic column, eluted with solvents of increasing polarity. Various glycolipidic fractions were obtained, and they were subjected to HPLC purifications, affording compounds **1a**–**3a**. Each of them appeared to be homogeneous as for chromatographic behavior (silica-gel TLC and direct-phase HPLC) and ^{1}H-NMR features.

Compound **1a** was isolated as a colorless, amorphous solid, hardly soluble in all organic solvents except for DMSO and pyridine. Its glycosphingolipidic nature was initially inferred from the IR spectrum (neat, $\tilde{\nu}_{max}$ = 1690 cm^{-1}, amide carbonyl group) and from ^{1}H-NMR data ([D$_6$]DMSO), the typical alkyl chain signals at δ = 0.86 and 1.24, the characteristic NH doublet at δ = 7.45, a series of overlapped multiplets between δ = 3.3 and 3.9, and two anomeric proton signals at δ = 4.89 and 4.85. In order to improve the solubility and to achieve a better resolution of the signals in the ^{1}H-NMR spectrum, compound **1a** was acetylated under the usual conditions (Ac_2O/py, room temperature). The peracetylated derivative **1b** was obtained, which was used for all the spectroscopic studies.

The negative ion FAB-MS spectrum of compound **1b** shows a series of pseudomolecular ion peaks at *m/z* = 1370, 1398, 1426, 1440, 1454, and 1468, suggesting the presence of a mixture of homologs, in accordance with the molecular formula $C_{70}H_{117}NO_{25}$ + nCH$_2$ (n = 0, 2, 4–7). The ^{1}H-NMR spectrum ($CDCl_3$) shows the partially overlapped multiplets located in the mid-field region between δ = 3.7 and 5.5, which were assigned with the aid of 2D COSY- and HOHAHA-NMR spectra. This analysis allowed us to assign all the ^{1}H signals to four spin systems (the sphingoid and acyl chains and two monosaccharides) and to elucidate the proton sequence within each spin system. All the carbon atom resonances observed in the ^{13}C-NMR spectrum are unambiguously associated with directly attached proton signals using the 2D ^{1}H detected heteronuclear multiple-quantum coherence (HMQC) experiment (assignment shown in Table 1).

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]
1a-1b	3.68 (m)	63.5 (CH_2)
2	4.32 (m)	48.4(CH)
2-N*H*	7.38 (d, 8.9)	
3	5.37 (dd, 9.4, 3.1)	68.9 (CH)
4	4.85 (m)	73.2 (CH)
5	1.55[c]	26.3 (CH_2)
1'	4.87(d, 3.4)	97.9 (CH)
2'	3.88 (dd, 10.6, 3.4)	73.9 (CH)
3'	5.30 (dd, 10.6, 3.4)	69.3 (CH)
4'	5.40 (dd, 3.4, 1.1)	68.7 (CH)
5'	4.18 (dt, 6.8, 1.1)	67.3 (CH)
6' a	4.10[c]	61.6 (CH_2)
b	4.12[c]	
1"	5.16 (d, 3.4)	96.8 (CH)
2"	5.12[c]	74.1 (CH)
3"	5.32 (dd, 9.4, 3.4)	61.8 (CH)
4"	5.45 (dd, 3.4, 1.1)	68.7 (CH)
5"	4.41(dt, 6.8, 1.1)	67.3 (CH)
6" a	4.00(m)	61.7 (CH_2)
b	4.10[c]	
2'''	5.10 (m)	72.5 (CH)
3'''	1.80[c]	29.3 (CH_2)
Ac's	1.99-2.22 (10 singlets)	

[a] Additional ^{1}H signals: δ = 1.50 [m, C*H*(CH_3)$_2$], 1.25 [broad band, alkyl chain protons], 0.86 [–CH_2CH_3], 0.84 [–$CH(CH_3)_2$]. – [b] Additional ^{13}C signals: δ = 173.0–160.0 (several C), 39.4 (CH), 32.0 (CH_2), 29.7–29.4 (several CH_2), 28.0 (CH), 25.5 (CH_2), 22.5 (CH_3), 22.1 (CH_2), 22.0–20.0 (several CH_3), 14.1 (CH_3). – [c] Overlapped by other signals.

In particular, the anchorage site for the first spin system is the multiplet at δ = 4.32 (2-H), coupled with a signal at δ = 3.68 (1a-H/1b-H), with the D_2O exchangeable doublet at δ = 7.38 (2-NH) and with a double doublet at δ = 5.37

(3-H), which in turn shows a COSY correlation with 4-H (δ = 4.85). A comparison of this sequence and of the respective ^{13}C-NMR resonances with the previously reported GSL spectral features[2,3] allowed an easy assignment of this spin system to a trihydroxylated sphinganine (phytosphingosine). In the same manner, starting from the multiplet at δ = 5.10 (2‴-H), it was possible to assign the second spin system to a 2-hydroxy fatty acyl chain. The two above-mentioned substructures were connected through an amidic linkage on the basis of IR spectral data and of the 2D NMR ROESY peak between δ = 7.38 (2-NH) and 5.10 (2‴-H). In contrast, only a detailed analysis of COSY-, HMQC-, HOHAHA-, and ROESY-NMR data obtained from **1b** permitted the identification of the sugar head portion of the molecule. The signals of the anomeric carbon-atoms at δ = 97.9 (C-1′) and 96.8 (C-1″) in the ^{13}C-NMR spectrum show a correlation peak with proton doublet signals at δ = 4.87 and 5.16, respectively, in the HMQC spectrum. Starting from these two signals, we could identify on the basis of COSY and HOHAHA data the sequences of two hexose units, both sequences extending to an oxymethylene residue via four oxymethine protons. Considering the relatively high-field resonances of 5′-H (δ = 4.18) and 5″-H (δ = 4.41), indicative of a glycosidic linkage rather than an ester bond at C-5′ and C-5″, we concluded that both the two monosaccharide units must exist in the pyranose form. The relative stereochemistry of the monosaccharide units was elucidated by the analysis of the coupling constants in the ^{1}H-NMR spectrum. When this measurement was not possible, some crucial correlation peaks in the ROESY experiment gave us decisive indications. In particular, both sugar units evidenced the same patterns of coupling constants, characterized by a large axial-axial coupling between 2′-H and 3′-H (and between 2″-H and 3″-H), while the axial-equatorial relations 1′-H/2′-H, 1″-H/2″-H, 3′-H/4′-H, and 3″-H/4″-H were deduced. The axial positions of 5′-H and 5″-H are indicated by the strong ROESY correlation peaks with 3′-H and 3″H, respectively. Because of this evidence, the two monosaccharide units in **1b** were identified as α-galactopyranoses. In addition, the relatively high-field chemical shift of 2′-H (δ = 3.88) and its ROESY correlation peak with 1″-H clearly prove the position 2′ to be the glycosidic linkage site. Finally, the gross structure of **1b** was unambiguously determined on the basis of the ROESY correlation peak of 1′-H with the methylene protons at C-1, indicating the glycosidic bond of the first α-galactopyranose unit with the ceramide moiety.

In order to characterize the length and nature of the alkyl chains, 4.0 mg of compound **1b** was subjected to acid methanolysis with 1 N HCl in 85% MeOH. The reaction mixture so obtained, dried under nitrogen, was chromatographed on silica gel (see experimental section[4]), to afford three distinct fractions (α, β, and γ).

The ^{1}H-NMR spectrum of fraction α shows a methyl singlet at δ = 3.68 (OCH_3), a triplet at δ = 0.86 (terminal CH_3) and two multiplets at δ = 4.20 (α-CHOH) and δ = 1.82 (β-CH_2), in addition to the methylene chain signal at δ = 1.24. These data suggest the presence of an unbranched long-chain α-hydroxy acyl methyl ester, as confirmed by the quantitative GC-MS analysis, whose results are compiled in Table 2. In addition, the negative $[\alpha]_D$ value of the mixture suggests the (*R*) configuration at the chiral center[5]. Fraction β was composed of a mixture of (2*S*,3*S*,4*R*)-phytosphingosine with different alkyl chains, as proven by a comparison of the optical rotation ($[\alpha]_D^{25}$ = +5) and of the ^{1}H-NMR data obtained from the tetraacetate derivative with those previously reported for the natural compounds[6]. This fraction was analyzed by oxidative cleavage of the C3–C4 linkage with permanganate/periodate, followed by methylation of the resulting mixture of carboxylic acids with diazomethane. A GC-MS analysis of the mixture furnished the results compiled in Table 3 (comparison of the GLC retention time and MS spectra with those of "*library*" compounds). The structure of **1b** was thus fully established. Since the ^{1}H-NMR spectrum ([D_6]DMSO) of compound **1a**, shows no singlets in the region δ = 1.5–2.5, the existence of any acetyl group before the acetylation reaction can be excluded. Therefore, structure **1a** can be assigned to the natural GSL. Compound **2a** was acetylated and the ^{1}H-NMR spectrum of the peracetylated derivative **2b** was found to be identical with that of longiside decaacetate (**2d**)[2]. However, the methanolysis/GC-MS analysis of **2b** evidenced a very different alkyl chain composition and the GSL, previously reported as the sole compound in *long. 1*, was completely absent in the mixture of fatty acid and sphinganine homologs (see Tables 2 and 3) found in the new specimen (*long. 2*).

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

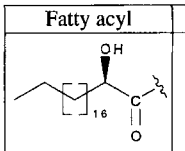
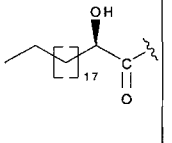
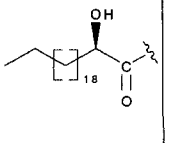
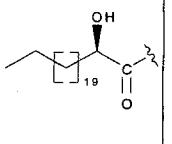
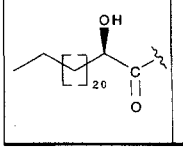
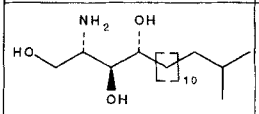
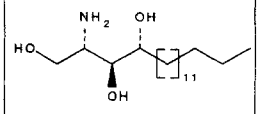
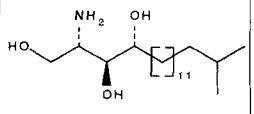
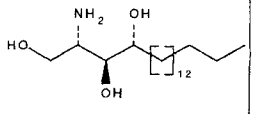
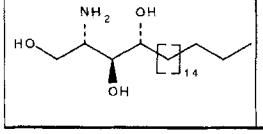
Fatty acyl	1a	2a	3a
	47.4 %	42.7 %	47.8 %
	1.9 %	1.5 %	3.3 %
	32.7 %	28.5 %	27.6 %
	2.3 %	3.9 %	8.6 %
	15.7 %	18.3 %	17.8 %

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

Sphinganine	**1a**	**2a**	**3a**
	25.2 %	18.6 %	19.1 %
	30.3 %	23.6 %	27.7 %
	17.2 %	11.9 %	15.2 %
	18.8 %	17.5 %	16.6 %
	15.5 %	28.4 %	21.4 %

Compound **3a** was identified by a comparison of its ^{1}H- and ^{13}C-NMR spectra with those reported by Natori et al. for agelasphin-1[7]. Tables 2 and 3 show the composition of the alkyl chains, determined by methanolysis/GC MS, as previously reported for **1b** and **2b**.

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Experimental

General Methods: FAB MS (glycerol matrix): VG ZAB mass spectrometer (Xe atoms of energy of 2–6 kV). – Optical rotations: Perkin-Elmer 192 polarimeter equipped with a sodium lamp (λ = 589 nm) and a 10-cm microcell. – IR: Bruker IFS-48 spectrophotometer. – ^{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). Signals of methyl, methylene and methine carbon-atoms were distinguished by DEPT experiments. Homonuclear ^{1}H connectivities were determined by using COSY experiments; 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing[8]; the HMQC spectrum was recorded by using the Bax-Subramanian sequence[9] with a BIRD pulse 0.50 before each scan in order to suppress the signals originating from protons not directly bound to ^{13}C ($^1J_{CH}$ of 142 Hz for the interpulse delay). During the acquisition time ^{13}C broad-band decoupling was performed by using the GARP sequence[10]. – Medium-pressure liquid chromatography (MPLC): Büchi 861 apparatus with an SiO_2 column (230–400 mesh). – HPLC separations: Varian 2510 apparatus equipped with an RI-3 refractive index detector and with Hibar columns. – GC-MS experiments: Hewlett-Packard 5890 gas chromatography with a mass-selective detector MSD HP 5790 MS. A 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}. – Quantification was based on the area of the GLC peaks.

Extraction and Isolation of GSLs from Long. 2: Specimens of *Agelas longissima* were collected in the summer of 1992 along the coasts of Little San Salvador Island at a depth of 24 m and identified by Prof. M. Pansini (University of Genoa). They were frozen immediately after collection and kept frozen until extraction. The sponge (50 g of dry weight after extraction) was homogenized and extracted (5 × 500 ml) twice with methanol and twice with chloroform. The combined extracts were initially partitioned between H_2O and diethyl ether and then the polar extract was partitioned between H_2O and *n*BuOH. The *n*BuOH extract was concentrated in vacuo affording 4 g of a brown-colored viscous oil. This was subjected to MPLC (SiO_2 column; solvent gradient system from EtOAc to MeOH). The fraction eluted with EtOAc/MeOH (9:1), rechromatographed by HPLC (eluant EtOAc/MeOH, 93:7), afforded pure compound **3a** (8 mg) while the fraction eluted with EtOAc/MeOH (8:2) contained pure compound **2a** (45 mg). The fraction eluted with EtOAc/MeOH (7:3) was purified by using HPLC column with a mobile phase EtOAc/MeOH (85:15) affording pure compound **1a** (35 mg).

GSLs from Long. 1: For isolation and physical data of **2c** see the previous paper[2]. ^{1}H-NMR spectra of MPLC fractions eluted with $CHCl_3$/MeOH (95:5) and $CHCl_3$/MeOH (6:4) allowed us to detect the presence of GSLs with the same sugar head moiety of **3a** and **1a**, respectively. Since the amount of these two compounds appeared too small to perform a structural analysis, they were not further purified.

(2S,3S,4R)-O-(α-D-Galactopyranosyl)-(1→2)-(α-D-galactopyranosyl)-(1→1)-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol (**1a**): White solid, $[\alpha]_D^{25}$ = +21 (*c* = 0.002 in DMSO). – Fatty acid composition: Table 2. – Sphinganine composition: Table 3.

(2S,3S,4R)-O-(α-D-Galactopyranosyl)-(1→2)-(α-D-galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol Peracetate (**1b**): Colorless oil, $[\alpha]_D^{25}$ = +11 (*c* = 0.0011 in $CHCl_3$). – Negative FAB MS: *m/z* = 1370, 1398, 1426, 1440, 1454, and 1468 ([M – H]$^-$ series). – ^{1}H and ^{13}C NMR: see Table 1. – Fatty acid composition: Table 2. – Sphinganine composition: Table 3.

(2S,3S,4R)-O-(α-D-Galactofuranosyl)-(1→3)-(α-D-galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol (**2a**): White solid, $[\alpha]_D^{25}$ = +28 (*c* = 0.002 in DMSO). – ^{1}H NMR ([D_6]DMSO, 317 K): identical with that reported in the previous paper[11]. – Composition of fatty acids: Table 2. – Composition of 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 Peracetate (**2b**): An aliquot (10 mg) of compound **2a** was acetylated according to the standard procedure. The peracetylated compound **2b** shows spectroscopic properties identical with those of an authentic sample of longiside decaacetate (**2d**). – Composition of fatty acids: Table 2. – Composition of sphinganines: Table 3.

(2S,3S,4R)-O-(α-D-Galactopyranosyl)-(1→1)-2-[(R)-1-hydroxyalkylcarbonylamino]-1,3,4-alkanetriol (**3a**): Compound **3a**

was identified by a comparison of its ^{1}H- and ^{13}C-NMR spectra with those reported in ref.[7]. – Composition of fatty acids: Table 2. – Composition of sphinganines: Table 3.

Methanolysis of GSLs: A small amount (2–5 mg) of GSL was dissolved in 1 ml of 1 N HCl in 85% MeOH and the obtained solution was kept for about 17 h at 70°C in a sealed tube. The reaction mixture was dried under nitrogen, dissolved in a small amount of $CHCl_3$ and the solutionj was passed through a SiO_2 column (230–400 mesh). Elution with 10–15 ml of 0.1% pyridine in $CHCl_3$ gave a mixture of α-hydroxy acid methyl esters (fraction α) and elution with 10–15 ml of 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 aqueos layers were separated and concentrated to give a mixture of sphinganines (fraction β) and methyl glycosides (fraction γ), respectively.

Analysis of Fatty Acid Methyl Esters: Fractions α from compounds **1b**, $[\alpha]_D^{25} = -1.5$ ($c = 0.0013$ in $CHCl_3$); **2b**, $[\alpha]_D^{25} = -1.6$ ($c = 0.003$ in $CHCl_3$); **3a**, $[\alpha]_D^{25} = -1.5$ ($c = 0.001$ 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: To a solution of an aliquot (0.6–1.0 mg) of fractions β from compounds **1b**, $[\alpha]_D^{25} = +5$, ($c = 0.001$ in $CHCl_3$); **2b**, $[\alpha]_D^{25} = +6$ ($c = 0.003$ in $CHCl_3$); **3a**, $[\alpha]_D^{25} = +5$ ($c = 0.001$ in $CHCl_3$) in 1.5 ml of *t*BuOH were added 0.3 ml of a 0.05 M solution of K_2CO_3, and 1 ml of an aqueous solution 0.025 M in $KMnO_4$ and 0.10 M in $NaIO_4$. The reaction was allowed to proceed at 37°C for 16 h. After acidification with 5 N H_2SO_4, the solution was decolorized with a saturated solution of Na_2So_3 and extracted twice with 5 ml of Et_2O. The combined extracts were concentrated to a volume of 0.5 ml, and the mixture of carboxylic acids was methylated with CH_2N_2 and analyzed by GC MS. The results referring to the original sphinganines, are compiled in Table 3.

Sphinganine Tetraacetates: ^{1}H NMR ($CDCl_3$): See ref.[3].

Analysis of Methyl Glycosides Obtained from Compound **1b**: Fraction γ from compound **1b** was acetylated and subjected to HPLC purification (column RP-18, 250 × 4 mm; eluant H_2O/MeOH, 6:4), giving pure methyl α-D-galactopyranoside tetraacetate. It was identified by a comparison of its ^{1}H-NMR spectrum and optical rotation with those of an authentic sample prepared from D-galactose under the same conditions employed for the methanolysis of GSLs.

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