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Glycolipids from Sponges. IV.[1] Immunomodulating Glycosyl Ceramides from the Marine Sponge *Agelas dispar*.

Valeria Costantino, Ernesto Fattorusso,* Alfonso Mangoni,

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

Massimo Di Rosa, Angela Ianaro, Pasquale Maffia

Dipartimento di Farmacologia Sperimentale, via D. Montesano 49, 80131 Napoli, Italy

Abstract: The GSL composition of the marine sponge *Agelas dispar* was investigated. In addition to four GSLs previously isolated from *Agelas clathrodes* (**2** and **4**), *Agelas conifera* (**3**), and *Agelas longissima* (**5**), the novel triglycosylceramide **1a** was isolated as a major component of the GSL mixture. All the isolated GSLs were tested using the MLR assay, and only some of them were shown to be immunoactivating agents, suggesting a possible structure-activity relationship.

The scientific interest of the immunological properties of glycosphingolipids (GSLs) is recently increased on account of the role that they could play as therapeutical agents. An interesting aspect of these properties is the immunomodulating activity reported for a number of GSLs. Gangliosides have shown to possess immunosuppressive activity[2-4] and their potential role as physiological modulators of the immune response has been hypothesized. More recently, agelasphins, galactosylceramides isolated from the marine sponge *Agelas mauritianus*, exhibited immunostimulatory activity, which was suggested to be related to the interesting in vivo antitumoral properties of these compounds through an activation of the immune system.[5,6]

In our continuing studies on glycolipids from marine organisms we have now examined the extract of the marine sponge *Agelas dispar*, collected along the coast of San Salvador Island (Bahamas). Of the four GLSs which were isolated and identified, four (**2-5**) were also present in others *Agelas* species, namely *A. clathrodes*[7], *Agelas conifera*[1], and *A. longissima*,[8,9] while one (**1a**) is a novel compound, whose structural determination is here reported, along with an examination of the immunostimulating activity of all the isolated GSLs.

A. dispar was extracted successively with MeOH and $CHCl_3$, and the extract was partitioned between water and *n*-BuOH. The organic layer was subjected to chromatography through an RP-18 column and then through

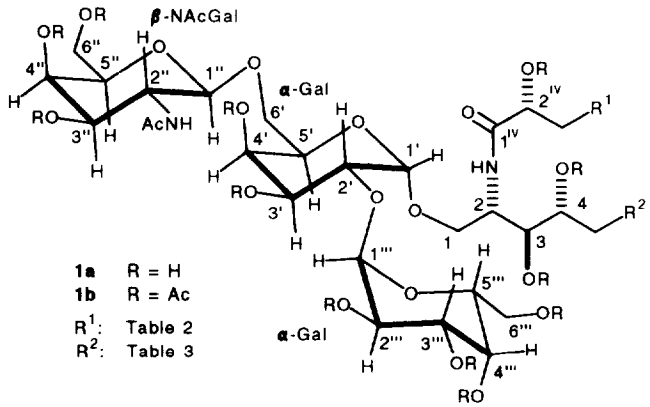


Table 1. NMR Data of Compound **1b** ($CDCl_3$).

Pos.	δ_H (mult, *J* [Hz])[a]	δ_C[b]
1 a	3.78 (dd, 11.3, 4.1)	68.5 (CH_2)
b	3.67[c]	
2	4.36[c]	48.5 (CH)
2-N*H*	7.20 (d, 9.3)	
3	5.20 (dd, 8.3, 4.1)	71.6 (CH)
4	4.95 (ddd, 8.3, 4.1, 4.1)	72.8 (CH)
5	1.58[c]	27.4 (CH_2)
1'	4.85(d, 3.4)	98.3 (CH)
2'	3.87(dd, 10.2, 3.4)	74.8 (CH)
3'	5.27 (dd, 10.2, 3.4)	68.3 (CH)
4'	5.39 (bd 3.4)	68.2 (CH)
5'	4.26[c]	67.3 (CH)
6' a	3.74[c]	64.7 (CH_2)
b	3.62 (dd, 10.3, 5.7)	
1"	5.12[c]	97.4 (CH)
2"	5.11[c]	68.4 (CH)
3"	5.23 (dd, 10.2, 3.4)	67.5 (CH)
4"	5.44 (bd, 3.4)	67.8 (CH)
5"	4.25[c]	67.4 (CH)
6" a-b	4.13[c]	61.3 (CH_2)
1"'	4.87 (d, 8.5)	98.9 (CH)
2"'	3.71[c]	51.8 (CH)
2"'-NH	6.04 (d, 8.3)	
3"'	5.42 (dd, 10.9, 3.4)	69.2 (CH)
4"'	5.35 (bd, 3.4)	66.9 (CH)
5"'	3.95 (t, 6.49	70.6 (CH)
6"' a	4.12[c]	61.3 (CH_2)
2^{IV}	5.13[c]	74.3 (CH)
3^{IV}	1.81[c]	29.2 (CH_2)
Ac's	1.98-2.22 (13 singlets)	

[a] Additional ^1H 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 171.1–169.1 (several C), 39.1 (CH), 31.9 (CH_2), 30.0-29.2 (several CH_2), 28.0 (CH), 25.5 (CH_2), 25.1(CH_2), 22.8 (CH_3), 22.7 (CH_2), 21.0-20.6 (several CH_3), 14.1 (CH_3). [c] Submerged by other signals.

Table 2. Fatty Acyl Composition of Compounds **1a-4**.

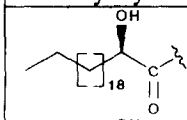
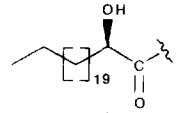
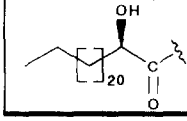
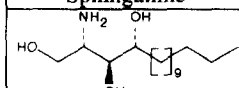
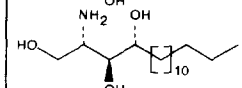
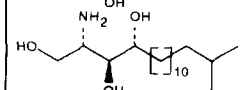
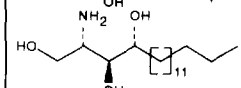
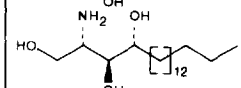
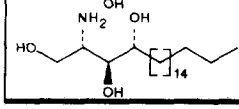
Fatty acyl	**1a**	**2**	**3**	**4**
	24.4%	25.2 %	24.0 %	25.1 %
	22.4%	20.1%	21.8 %	23.4 %
	53.5%	54.7%	54.2 %	51.5 %

Table 3. Sphinganine Composition of Compounds **1a-4**.

Sphinganine	**1a**	**2**	**3**	**4**
	20.7 %	21.5 %	21.7 %	19.8 %
	16.4 %	13.9 %	15.4 %	17.1 %
	12.0 %	13.1 %	13.7 %	11.5 %
	6.7 %	5.7 %	7.8 %	7.7 %
	34.8 %	33.2 %	29.8 %	33.5 %
	9.4 %	12.6%	11.6 %	10.4 %

an SiO_2 column. A fraction containing GSLs was purified by HPLC on a DIOL column, and gave compounds **2-4**, while HPLC separation on the same column of a more polar fraction, which also contained GSLs, yielded the novel compound **1a**. All the GSLs were pure by TLC and, as far as the sugar chain is concerned, by ^1H NMR.

As usual for GSL from *Agelas*, compound **1a** is composed of a mixture of homologs, which differs by the length and the terminus (either ethyl or iso-propyl) of the alkyl chains of the ceramide part of the molecule. Therefore, the negative ion FAB mass spectrum (performed on the peracetyl derivative **1b**) showed a series of molecular ion peaks at *m/z* 1727, 1713, 1699 and 1685, in accordance with the molecular formula $C_{84}H_{138}N_2O_{32}$ + n CH_2 (n = 0-3), and the ^1H NMR spectrum showed in the methyl region a triplet at δ 0.88 (ethyl terminus) and a doublet at δ 0.85 (iso-propyl terminus), whose intensities were not in an integral ratio with respect to those of other signals in the spectrum. We did not separate such a complex mixture of homologs, but performed the structure determination of the common part of the homologs working on the mixture; the composition in sphingoid bases and fatty acids was then established by degradation of a small amount of sample. The complexity of the middle field region of the ^1H NMR spectrum of **1a**, which displayed signals for 19

oxymethine and oxymethylene protons within only 0.35 ppm, forced us to perform NMR studies on its peracetyl derivative **1b**, whose signals in the proton spectrum were spread over a much wider region.

The ceramide part of the molecule was characterized as composed of a 4-hydroxysphinganine and a 2-hydroxyacid from NMR data. The amidic proton of the ceramide, which resonates as a doublet at δ 7.20, was an useful starting point for the assignment of the sphinganine protons from H_2-1 to H_2-5 using the COSY and HOHAHA 2D NMR spectra, whereas the α proton of the hydroxyacid was recognized from its correlation peak in the ROESY spectrum with the same amidic doublet. As a further confirm, all the resonances were very similar with those reported for similar GSLs.[1,6]

Evidence for the presence of three sugar residues in **1a** came from the ^{13}C NMR spectrum, which displayed three anomeric carbon signals at δ 98.3 (C-1'), 97.4 (C-1''), and 98.9 (C-1'''). The heteronuclear chemical shift correlation HMQC NMR spectrum allowed the assignment of the anomeric protons, which are not readily discernible in the ^1H NMR spectrum of a peracetylated glycoside, as two doublets at δ 4.85 (J = 3.4 Hz, H-1') and δ 4.87 (J = 8.5 Hz, H-1'''), and a non-first-order multiplet (due to superimposition to H-2'') at δ 5.12 (H-1''). Combined use of the COSY and HOHAHA spectra led to the identification of four methine protons and one couple of methylene protons for each of the three sugar units, which are therefore hexoses. In addition, the upfield chemical shift of H-5 of each saccharide, showing those positions not to be acetylated, was a clear indication that the three saccharides are in a pyranose form. The nature of each sugar residue, and the linkages between them were established from analysis of ^1H-^1H coupling constants and ROESY data. The inner sugar residue is an α-galactopyranoside: H-2' and H-3' are axial, as indicated by their mutual large axial-axial coupling, and H-1' and H-4', which experience only small couplings, are equatorial. As for H-5', its axial nature was demonstrated by a ROESY correlation peak pointing to a 1-3 diaxial relationship with H-3'. This saccharide is directly linked to the ceramide on the basis of the prominent cross peaks of H-1' with H-1a and H-1b in the ROESY spectrum, and is glycosylated at positions 2 and 6 because the relevant protons displayed a upfield chemical shift (δ 3.87, H-2'; δ 3.74 and 3.62, H-6'a and H-6'b), thus indicating a branched sugar chain.

Also the second saccharide is an α-galactopyranoside, and its structure was determined in a similar way, except that we were unable to establish the stereochemistry at C-1'' from NMR data of **1b**, due to the coincidence of the chemical shifts of H-1'' and H-2''. Therefore, the glycosidic linkage was determined as α oriented by examination of the spectrum of the non-acetylated compound **1a**, which revealed the presence of two anomeric protons of α glycosides and one of a β glycoside, which however is to be attributed to a β-

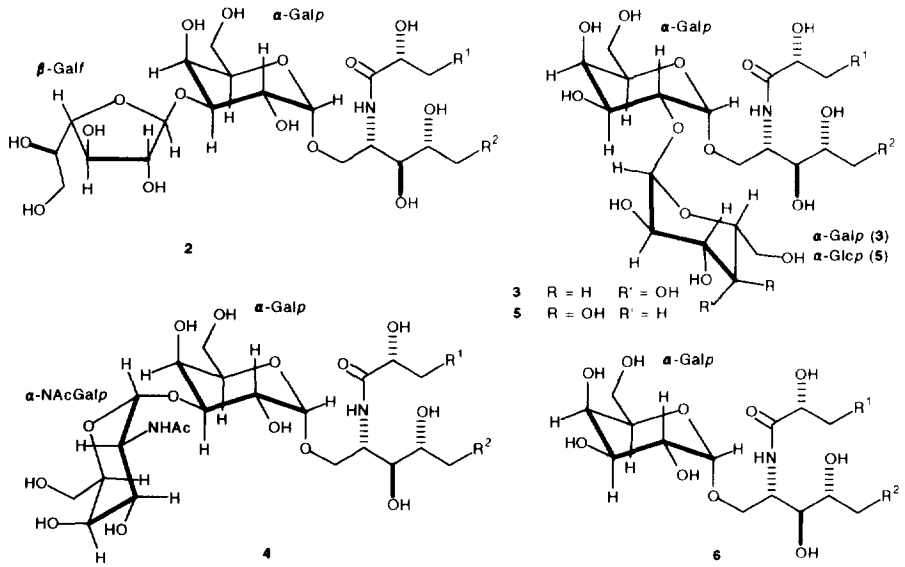


Table 4. Lymphocyte proliferation stimulatory effect of compounds **2**, **4**, and **6**.[a]

Compd	[^{3}H]-thymidine cpm ± s.e.m.			
.	0.01 μg/ml	0.1 μg/ml	1 μg/ml	10 μg/ml
2	628 ±16	1674 ±45[b]	1148 ±42[b]	959 ±45[c]
4	753 ±24	2173 ±75[b]	1926 ±42[b]	1060 ±45
6	933 ±11	2505 ±54[b]	2164 ±99[b]	1017 ±18

[a] Data are expressed as mean total cpm±s.e.m. mean, n=3. Mean total cpm of unsimulated cells (control) was 796±75 cpm, n=5. [b] $p < 0.05$ vs control [c] $p < 0.01$ vs control.

galactosamine (see below). In addition, ROESY cross peaks of H-1'' (and/or the coincident H-2'') with H-1' and H-2' demonstrated the glycosidic linkage of this saccharide to C-2'.

The last sugar residue was a 2-amino-2-deoxy-β-galactopyranoside. The 8.5 Hz coupling between H-1''' and H-2''' indicated the axial nature of these protons. The presence of an acetamido rather than an acetoxy group at position 2''' was suggested by the upfield chemical shifts of H-2''' (δ 3.71) and C-2''' (δ 51.8) and by the presence of a D_2O exchangeable doublet at δ 6.04 coupled with H-2'''. Signal overlapping prevented us from measuring the H-2'''/H-3''' coupling constant, but H-3''', as well as H-5''', could be shown to be axial from their dipolar coupling with H-1''' evidenced by the ROESY spectrum. Finally, the equatorial nature of H-4''' was evident from its multiplicity (br. d, J = 3.4 Hz). The only possible linkage of this sugar to C-6' was confirmed by the dipolar coupling of H-1''' with H-6'a and H-6'b.

With the structure of the peracetyl derivative **1a** in our hands, the assignment of the ^{1}H and ^{13}C NMR spectra of the natural GSL **1a** became a feasible task. This was performed on the basis of the COSY, HOHAHA, ROESY and HMQC NMR spectra, and is reported in the Experimental.

The composition in fatty acids and sphingoid bases of the ceramide part of compound **1a** was established by degradation of a small amount of sample as previously reported.[10] The sample was subjected to acidic methanolysis and methyl glycosides, sphinganines, and fatty acid methyl esters were separated. Fatty acids methyl esters were directly analyzed by GC-MS, whereas the 4-hydroxysphinganines were converted with $KMnO_4/NaIO_4$ to carboxylic acids with three less carbon atoms, with were methylated and analyzed by GC-MS. The results are reported in Tables 2 and 3. The absolute stereochemistry of the ceramide was determined by comparison of the optical rotation of the mixtures of α-hydroxyacid methyl esters and sphinganines with literature data.[11,12]

As a confirm of the determined structure, the mixture of glycosides was acetylated and separated by HPLC, yielding 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.

Compound **2-4** were identical to the GSLs isolated from *A. clathrodes*[7] and *A. longissima*,[9] except for their composition in fatty acids and sphingoid bases, which was determined in the same way as for **1a**, and is reported in Tables 2 and 3.

The sponges of the genus *Agelas* confirm their ability to synthesize a variety of unique GSLs, characterized by an α-galactosyl as the sugar directly linked to the ceramide. Until now, the only living organisms which have been found to contain GSLs with this structural feature belong to the phylum Porifera.[1,5,7-10,13] The novel compound **1a** is the most complex element of this series, the first triglycosylceramide from an *Agelas* species, and the second example of triglycosylceramides from a sponge after the Axiceramide-A and -B from *Axinella* sp.[10]

Compounds **1a** and **2-4** were tested for immunostimulatory activity using the mixed lymphocyte reaction (MLR) assay,[14] along with the diglycosylceramide **5** and the monogalactosylceramide **6**, from *A. conifera*,[1] which were not found in *A. dispar* (the latter, whose immunostimulating activity is known,[5] was included as a reference). As shown in Table 4, compounds **2**, **4**, and **6** exhibited a stimulatory effect on lymphocyte proliferation. The most effective compound resulted **6**, since it significantly stimulated lymphocyte proliferation at concentrations between 0.01 and 10 μg/ml. In contrast, compounds **1a**, **3**, and **5** did not exhibit any

stimulatory activity (data not shown). These results suggest that the immunostimulating activity is affected by a specific structural feature of the GSLs, namely glycosylation of the inner sugar at position 2. In fact, all compounds **2**, **4**, and **6** possess a free 2-OH on the sugar directly linked to the ceramide moiety, while in compounds **1a**, **3**, and **5** this position is glycosylated either by an α-galactosyl (**1a** and **3**) or by an α-glucosyl (**5**).

EXPERIMENTAL

General methods. FAB-MS spectra were performed in a glycerol matrix on a VG Prospec-Autospec (Fisons) mass spectrometer. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell. ^1H and ^{13}C NMR spectra were performed on a 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 ^1H 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,[15] with a BIRD pulse 0.5 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.

High performance liquid chromatographies (HPLC) were achieved on a Varian 2510 apparatus equipped with an RI-3 refractive index detector, and with Hibar columns. GC-MS spectra were performed on a Hewlett-Packard 5890 gas chromatograph with a mass selective detector MSD HP 5970 MS, a split/splitless injector, and a fused-silica column, 25 m × 0.20 mm HP-5 (cross-linked 25 % Ph Me silicone, 0.33-mm film thickness); the temperature of the column was varied, after a delay of 5 min from the injection, from 150 °C to 300 °C with a slope of 5 °C min^{-1}; quantitative determination was based on the area of the GLC peaks.

Collection, extraction and isolation. Specimens of *Agelas dispar* were collected in the summer of 1992 along the coast of San Salvador Island and identified by Prof. M. Pansini (University of Genoa). They were frozen immediately after collection and kept frozen until extraction. Reference specimens were deposited at the Istituto di Zoologia, University of Genoa, Italy. The sponge (41.6 g of dry weight after extraction) was homogenized and extracted with methanol (2 × 500 ml) and then with chloroform (2 × 500 ml); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated *in vacuo* and afforded 12.4 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 (2.8 g), 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 (302 mg) was mainly composed of a mixture of glycosphingolipids, which were separated by HPLC on a DIOL column [eluent: *n*-hexane/*i*PrOH/H_2O (55:43:2)], thus affording compounds **2** (102 mg), **3** (77 mg), and **4** (12 mg), which were identified by comparison of their spectroscopic properties (and/or those of their peracetylated derivatives) with those of GLSs previously isolated from *Agelas* sponges.[1,7-9] Fraction B3 (183 mg), also containing glycolipids, was purified by HPLC using a DIOL column and *n*-hexane/*i*PrOH/H_2O (55:41:4), yielding compound **1a** (35.3 mg). All the GSL isolated were pure by TLC and, as far as the polar head is concerned, by ^1H-NMR.

*Compound **1a**.* White solid, $[\alpha]_D^{25}$ = +39.6 (*c* = 0.01 in DMSO). – ^1H NMR (DMSO, 317 K): δ 7.57 (d, *J* = 9.2 Hz, NH-2'''), 7.40 (d, *J* = 9.2 Hz, NH-2), 5.43 (1H, br. d, *J* = 4.0 Hz, OH-2^{IV}), 4.89 (1H, d, *J* = 2.7 Hz, H-1'), 4.83 (1H, d, *J* = 3.4 Hz, H-1''), 4.70 (d, *J* = 4.4 Hz, OH-3'), 4.54 (submerged, OH-3), 4.52 (submerged, OH-6'''), 4.48 (1H, br. s, OH-6''), 4.43 (1H, br. s, OH-3'''), 4.37 (1H, d, *J* = 3.7 Hz, OH-4'''), 4.34 (submerged, OH-3''), 4.33 (partly overlapped, d, *J* = 8.7 Hz, H-1'''), 4.30 (submerged, OH-4'), 4.30 (submerged, OH-4''), 4.12 (partly overlapped, br. d, *J* = 6.5 Hz, OH-4), 4.09 (partly overlapped, t, *J* = 6.5 Hz, H-5'), 4.06 (submerged, H-2), 4.06 (submerged, OH-2''). 3.88 (1H, m, H-2^{IV}), 3.80 (submerged, H-3'), 3.79 (submerged, H-6'a), 3.73 (submerged, H-2'), 3.72 (submerged, H-2'''), 3.72 (submerged, H-4'), 3.72 (submerged, H-1a), 3.68 (submerged, H-4''), 3.67 (submerged, H-4'''), 3.66 (submerged, H-5''), 3.62 (submerged, H-3''), 3.59 (submerged, H-2''), 3.57 (submerged, H-1b), 3.57 (submerged, H-6'''a), 3.54 (submerged, H-6''a), 3.52 (submerged, H-6'''b), 3.51 (submerged, H-6'b), 3.50 (submerged, H-3'''), 3.48 (submerged, H-6''b), 3.47 (submerged, H-3), 3.38 (1H, m, H-4), 3.33 (1H, t, *J* = 6.1 Hz, H-5'''), 1.85 (3H, s, Ac-2'''), 1.62 (1H, m, H-3^{IV}_a), 1.58 (overlapped, H-5_a), 1.51 (overlapped, H-3^{IV}_b), 1.42 (overlapped, H-5_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, $-CH(CH_3)_2$]. – ^{13}C NMR (DMSO): δ = 173.4 (C, C-1^{IV}), 170.3 (C, CO-2'''), 101.4 (CH, C-1'''), 97.2 (CH, C-1''), 96.6 (CH, C-1'), 75.2 (CH, C-5'''), 74.2 (CH, C-3), 73.9 (CH_2, C-4'), 71.2 (CH, C-5''), 71.2 (CH, C-3'''), 71.1 (CH, C-2^{IV}), 70.9 (CH, C-4), 69.6 (CH, C-5'), 69.4 (CH, C-2''), 69.3 (CH, C-3'), 69.2 (CH, C-4''), 68.5 (CH, C-3''), 68.2 (CH, C-2'), 67.9 (CH_2, C-6'), 67.2 (CH, C-4'''), 66.9 (CH_2, C-1), 60.6 (CH_2, C-6'''), 60.3 (CH_2, C-6''), 52.4 (CH, C-2'''), 49.6 (CH, C-2), 34.3 (CH_2, C-3^{IV}), 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.

*Compound **1b**.* An aliquot (10 mg) of compound **1a** was acetylated by using the standard procedure.[10] The peracetylated derivative **1b** was obtained as a colorless oil. – ^1H- and ^{13}C-NMR: Table 1. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

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 (70-230 Mesh) column. 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 **1a** ($[\alpha]_D^{25}$ = –3, *c* = 0.002 in $CHCl_3$), **2** ($[\alpha]_D^{25}$ = –3, *c* = 0.002 in $CHCl_3$), **3** ($[\alpha]_D^{25}$ = –3, *c* = 0.002 in $CHCl_3$), and **4** ($[\alpha]_D^{25}$ = –4, *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. Fractions B from compounds **1a** ($[\alpha]_D^{25}$ = +8, *c* = 0.002 in $CHCl_3$), **2** ($[\alpha]_D^{25}$ = +8, *c* = 0.002 in $CHCl_3$), **3** ($[\alpha]_D^{25}$ = +7, *c* = 0.002 in $CHCl_3$), and **4** ($[\alpha]_D^{25}$ = +9, *c* = 0.001 in $CHCl_3$) were subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described,[10] and the resulting carboxylic acids were methylated with diazomethane and the obtained esters analyzed by GC-MS. The results are compiled in Table2, expressed in terms of original sphinganines.

Analysis of Methyl Glycosides from Compound 1a. Fraction C from compound **1a** 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, ^1H 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.

MLR Assays. Male Swiss mice, 6-8 weeks old, obtained from Nossan (Italy), were housed in temperature-controlled rooms (22±1°C) and received food and water *ad libitum*. Single limph node cell suspension was obtained from popliteus limph nodes removed from mices killed with CO_2. Cells were suspended (2.5×10^6 cills/ml) in the RPMI-1640 culture medium containing 10% foetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomicin (100 μg/ml) and 2-mercaptoethanol (50 μM). The cell suspension was dispensed at 100 μl/well in 96-well flat-bottomed plates (Nunk, Roskilde, Denmark), and incubated for 24 h at 37°C in an atmosphere of 5% CO_2 and 95% O_2 in the prensence or in the absence of the test compounds. Cultures, in triplicates, were pulsed with 1 μCi/well [^{3}H]-thymidine (47 Ci/mmol, Amersham Intl., Amersham, UK) for the final 6 h of incubaation, then harvested and counted in a β-scintillation counter. Data are expressed as the mean ± s.e.mean; statisctical analysis of the data was preformed using a Pharm/PCS compouter program. Means were compared by Student's test for umpaired data.

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