Glycolipids from Sponges, III[◇]

Glycosyl Ceramides from the Marine Sponge *Agelas conifera*

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The glycosphingolipid (GSL) composition of the marine sponge *Agelas conifera* was investigated. In addition to three GSLs previous isolated from *Agelas clathrodes* (**2a**–**4a**), the novel α-glycosylceramide **1a** was isolated as a major component of the GSL mixture, and its structure was elucidated by extensive NMR studies and by chemical analysis.

By virtue of their amphiphilic character conferred by the combination of lipophilic chains with hydrophilic carbohydrate head groups, glycosphingolipids (GSLs) play very important biological roles in cellular interaction and differentiation[1]. GSLs are probably involved in the reproductive processes of sponges, very primitive animals which have peculiar biological features, including the capability of reproducting themselves in an asexual (agamic) way be gemmation. They also possess a surprising power of regeneration, so that any piece of sponge is capable of growing into a new individual, and even a sponge cell suspension can reorganize itself to develop a typical sponge construction[2]. The sugar chains of cell surface glycolipids are supposed to be the reference structures in the cell recognition processes, which are of primary importance in this kind of processes[3]. While the presence of GSLs in sponges was first suggested by chromatographic evidence since 1967[4], chemical proof for their existence was not provided until the mid-seventies, when Schmitz and McDonald isolated a glucosyl ceramide from *Chondrilla nucula*[5]. Subsequent investigations allowed the isolation of several novel mono-, di-, and triglycosylceramides from several *Porifera* species[6–14]; until now, there is no report of ganglosides from sponges. The most distinctive structural feature of sponge GSLs seems to be the α-linkage between the first sugar residue and the aglycon exhibited by most GSLs examined.

In this paper we report on the analysis of the glycosphingolipid fraction of the sponge *Agelas conifera*. Of the four GSLs isolated, three (**2a**, **3a**, and **4a**) were also present in *A. clathrodes*[13], while one (**1a**) is a novel GSL.

Results and Discussion

The sponge *Agelas conifera* was collected in the Caribbean area, along the coast of Little San Salvador Island. It was stored frozen until extraction twice with methanol and subsequently twice with chloroform. The BuOH-soluble part of the extracts was roughly purified by passing it through an RP-18 column, and subsequently through a SiO_2 column, and a fraction mainly composed of GSLs was obtained. Final separation was achieved by HPLC on a DIOL column, and yielded compounds **1a** and **3a** as the major components, together with small amounts of compounds **2a** and **4a**. The four GSLs appeared pure as revealed by TLC and, as far as the sugar "head" is concerned, by ^{1}H-NMR spectroscopy.

Compound **1a** proved to be a mixture of homologs, as evidenced by its negative ion FAB mass spectra, displaying several pseudomolecular ion peaks at *m/z* 1034, 1020, 1006, and 992, in accordance with the molecular formula $C_{53}H_{103}NO_{15} + nCH_2$ ($n = 0-3$). According to our standard procedure, the individual components of the mixture were not separated. Instead, the common polar part of their structures was determined by NMR spectroscopy, while the nature of the alkyl side chains was established by degradation of a small amount of sample. As usual, the NMR studies were performed on the peracetyl derivative **1b** of compound **1a**, in order to take advantage of the better proton dispersion in the ^{1}H-NMR spectrum of this derivative.

The carbohydrate part of the molecule comprises two sugar units as indicated by the ^{13}C-NMR spectrum, which displays the signals of two anomeric carbon atoms at δ = 98.5 and 96.3. The relevant anomeric protons were identified by a two-dimensional heteronuclear chemical-shift correlation (HMQC) NMR experiment as two doublets at δ = 4.84 (1′-H) and 5.12 (1″-H).

^{1}H-NMR data deduced from the homonuclear two-dimensional COSY, HOHAHA, and ROESY NMR experiment allowed a full determination of the nature of the two sugar units and the linkage between them. First of all, all the protons of the two sugar units were assigned (see Table 1) by analysis of the COSY spectrum: one couple of methylene protons and five methine protons were identified for each sugar residue, which are therefore hexoses. Three methine protons, 5′-H (δ = 4.26), 5″-H (δ = 4.1), and 2′-H (δ = 3.92), are considerably shielded with respect to the

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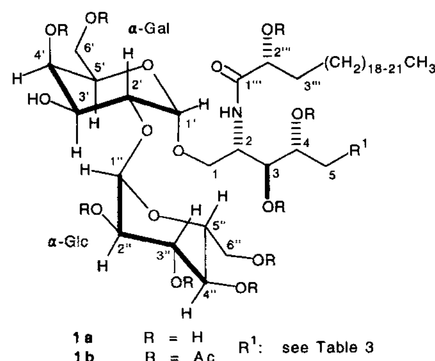

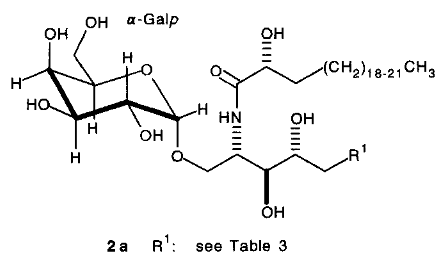

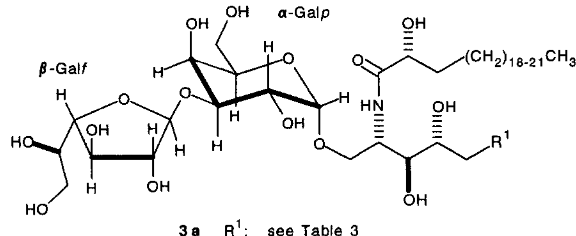

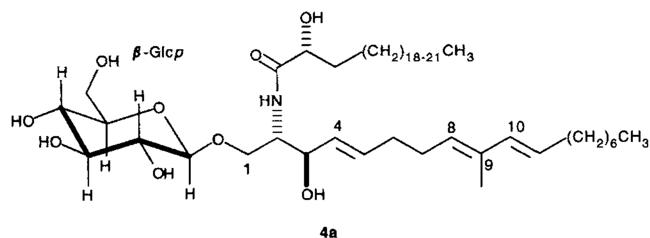

Table 1. ^{1}H- and ^{13}C-NMR data of compound **1b** ($CDCl_3$)

Pos.	δ_H (mult., J [Hz])[a]	δ_C (mult.)[b]
1 a	3.83 (dd, 11.5, 3.4)	69.3 (CH_2)
b	3.69 (dd, 11.5, 3.2)	
2	4.32[c]	48.6 (CH)
2-N*H*	7.13 (d, 9.1)	
3	5.21 (dd, 8.4, 3.4)	71.7 (CH)
4	4.90 (m)	72.8 (CH)
5	1.58[c]	27.4 (CH_2)
1'	4.84(d, 3.7)	98.5 (CH)
2'	3.92 (dd, 10.5, 3.7)	73.9 (CH)
3'	5.26 (dd, 10.5, 3.4)	68.3 (CH)
4'	5.42 (br.s)	68.3 (CH)
5'	4.26 (br.t, 6.7)	67.4 (CH)
6' a	4.11 (d, 6.7)	61.3 (CH_2)
b	4.11 (d, 6.7)	
1"	5.12 (d, 3.7)	96.3 (CH)
2"	4.89 (dd, 10.5,3.7)	70.8 (CH)
3"	5.38 (t, 9.8)	69.9 (CH)
4"	5.07 (t, 9.1)	68.1 (CH)
5"	4.1[c]	68.1 (CH)
6" a	4.23 (dd, 12.5, 3.7)[d]	61.4 (CH_2)
b	4.05 (dd, 12.5, 2.7)[d]	
2'''	5.08[c]	74.3 (CH)
3'''	1.81[c]	29.2(CH_2)
Ac's	1.98-2.22 (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 [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] Overlapped by other signals. – [d] Resonances may be reversed.

other ones, thus indicating that the relevant oxymethine protons are not acetylated. This means that the two sugar units are pyranoses, C-5′ and C-5″ being connected to the respective anomeric carbons by an ether linkage, and that C-2′ is glycosylated.

Finally, the nature of the two sugar units was established by evaluation of the coupling constants. That the terminal hexose is a glucose was demonstrated by the large coupling constants between 2″-H and 3″-H, 3″-H and 4″-H, as well as 4″-H and 5″-H (see Table 1), which evidenced the axial stereochemistry of all these protons. The same reasoning applied to the other sugar showed 2′-H and 3′-H (J = 10.5 Hz) to be axial, but 4′-H to be equatorial due to the small 3′-H/4′-H (J = 3.4 Hz) coupling constant. The 4′-H/5′-H coupling constant was not diagnostic of the stereochemistry of 5′-H, which was determined as axial on account of its 1,3-diaxial relationship with 3′-H, attested by a strong ROESY correlation peak between these two protons. Therefore, this saccharide is a galactose. The linkage between the two sugar units was confirmed by prominent ROESY correlation peaks of 1″-H with 1′-H and 2′-H. Coupling constant analysis was also used for the determination of the stereochemistry of glycosidic linkages, which is α for both sugar units since the small coupling constant of 1′-H and 1″-H (3.7 Hz for each proton) is a clear indication of their equatorial nature. According to these data, the carbohydrate moiety of the molecule is fully defined as a α-glucopyranosyl-(1→2)-α-galactopyranoside.

The nature of the lipidic part of the molecule was established by acid methanolysis of **1b**. The reaction mixture was separated into three fractions containing, respectively, methyl glycosides, sphingoid bases, and long-chain methyl α-hydroxy esters. This last fraction was analyzed by GC/MS and found to be mainly composed of methyl 2-hydroxytetraocosanoate, with small amounts of other homologs (Table 2). All the sphingoid bases were (2*S*,3*S*,4*R*)-4-hydroxysphinganines, as shown by the ^{1}H-NMR spectrum of the mixture after acetylation, which displayed, in the middle field region, signals coincident with those reported for the natural phytosphingosine[15]. The individual sphinganines were identified by oxidation to fatty acid methyl esters, with loss of three carbon atoms, as reported[7]; the resulting mixture of methyl esters was analyzed by GC/MS. The obtained results are listed in Table 3, expressed in terms of original sphinganines. The absolute stereochemistry of fatty acid methyl esters as well as that of sphinganines was established by their respective optical rotations[16,17].

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

Fatty acyl	1a	2a	3a	4a
OH, 18, C, O	5.1 %	5.5 %	7.5 %	6.5 %
OH, 19, C, O	10.6 %	10.1 %	10.5 %	10.9 %
OH, 20, C, O	77.2 %	78.3 %	76.8 %	75.5 %
OH, 21, C, O	7.1 %	6.1 %	5.2 %	7.1 %

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

Sphinganine	1a	2a	3a
HO, NH2, OH, OH, 10	7.8 %	5.2 %	12.0 %
HO, NH2, OH, OH, 10	29.7 %	27.6 %	29.3 %
HO, NH2, OH, OH, 11	12.3 %	16.8 %	19.5 %
HO, NH2, OH, OH, 11	20.8 %	21.4 %	16.2 %
HO, NH2, OH, OH, 12	22.0 %	23.1 %	13.0 %
HO, NH2, OH, OH, 14	7.4 %	5.9 %	10.0%

The methyl glycosides were acetylated and subjected to HPLC separation, giving methyl α-D-galactopyranoside tetraacetate and methyl α-D-glucopyranoside 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.

Compounds **2a, 3a,** and **4a** were identified by a comparison of their NMR spectrum with those of GSLs isolated from *A. clathrodes*[13]. Their composition in fatty acids and sphinganines was established by degradative analysis as described for compound **1**. The results were very similar to those of **1** (Tables 2 and 3), except for the sphingoid base of compound **4a**, which is a pure triunsaturated sphinganine whose length was determined by positive ion FAB/MS.

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Experimental

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; 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[18] 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 $^{1}J_{CH}$ of 142 Hz. During the acquisition time ^{13}C broad-band decoupling was performed by using the GARP sequence[19]. – HPLC: Varian 2510 apparatus equipped with an RI-3 refractive index detector, and with Hibar columns. – GC/MS: Hewlett-Packard 5890 gas chromatography equipped 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 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 conifera* were collected in the summer of 1992 along the coast of Little San Salvador Island (depth of 10 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 (53.5 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 16.7 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 (944 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 (422.4 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 **1a** (124 mg), **2a** (4 mg), **3a** (94 mg), and **4a** (7 mg), homogeneous as far as the polar head was concerned.

(2S,3S,4R)-1-O-[α-D-glucopyranosyl-(1→2)-α-D-galactopyranosyl]-2-[(R)-(2-hydroxyalkanoyl)amino]-1,3,4-alkanetriol

(**1a**): White solid, $[\alpha]_D^{25}$ = +35 (*c* = 0.004 in DMSO). – Negative FAB-MS, *m/z*: 1034, 1020, 1006, and 992, $[M - H]^-$ series. – ^1H NMR (DMSO, 317 K): δ = 7.41 (d, *J* = 9.8 Hz, NH), 5.47 (1H, d, *J* = 4.4 Hz, 2'''-OH), 4.88 (1H, d, *J* = 3.4 Hz, 1'-H), 4.85 (1H, d, *J* = 3.4 Hz, 1''-H), 4.71 (d, *J* = 4.1 Hz, 4''-OH), 4.59 (1H, br. s, 3-OH), 4.53 (1H, d, *J* = 6.1 Hz, 3''-OH), 4.48 (1H, t, *J* = 5.4 Hz, 6'-OH), 4.45 (1H, d, *J* = 4.4 Hz, 4'-OH), 4.38 (1H, d, *J* = 6.7 Hz, 2''-OH), 4.28 (1H, d, *J* = 6.1 Hz, 3'-OH), 4.21 (1H, t, *J* = 5.4, 6''-OH), 4.12 (1H, d, *J* = 6.4 Hz, 4-OH), 4.04 (1H, m, 2-H), 3.87 (1H, 2'''-H), 3.76 (overlapped, 4',5'-H), 3.75 (overlapped, 2'-H), 3.73 (overlapped, 3'-H), 3.70 (overlapped, 1_a-H, 5''-H), 3.69 (overlapped, 3'-H), 3.47 (overlapped, 3-H), 3.39 (overlapped, 4-H), 3.19 (overlapped, 2''-H), 3.14 (overlapped, 4''-H), 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 = 173.4 (CO), 96.9 (CH, C-1''), 96.8 (CH, C-1'), 74.4 (CH, C-2' or C-5'', these signals may be reversed), 74.1 (CH, C-3), 73.2 (CH, C-3''), 72.4 (CH, C-2' or C-5'', these signals may be reversed), 72.1 (CH, C-2''), 71.1 (CH, C-5' and C-2''', overlapped), 70.9 (CH, C-4), 70.2 (CH, C-4''), 69.0 (CH, C-4'), 67.9 (CH, C-3'), 66.9 (CH_2, C-1), 60.8 (CH_2, C-6''), 60.6 (CH_2, C-6'), 49.6 (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)-1-O-[α-D-Glucopyranosyl-(1→2)-α-D-galactopyranosyl]-2-[(R)-(2-hydroxyalkanoyl)amino]-1,3,4-alkanetriol Peracetate (**1b**): An aliquot (10 mg) of compound **1a** was acetylated by using the standard procedure[7]. The peracetylated compound **1b** was obtained as a colorless oil, $[\alpha]_D^{25}$ = +52 (*c* = 0.01 in $CHCl_3$). ^1H and ^{13}C NMR: see Table 1. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-[(R)-(2-hydroxyalkanoyl)amino]-1,3,4-alkanetriol (**2a**) was identified by a comparison of its ^1H-NMR spectrum with that of the GSL isolated from *A. clathrodes*[13]. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(2S,3S,4R)-1-O-[β-D-Galactofuranosyl-(1→3)-α-D-galactopyranosyl]-2-[(R)-(2-hydroxyalkanoyl]amino]-1,3,4-alkanetriol (**3a**) was identified by a comparison of its ^1H-NMR spectrum with that of the GSL isolated from *A. clathrodes*[13]. – Composition in fatty acids: Table 2. – Composition in sphinganines: Table 3.

(4E,8E,10E,2S,3S,4R)-1-O-(β-D-Glucopyranosyl)-2-[(R)-(2-hydroxyalkanoyl)amino]-9-methyl-4,8,10-octadecatriene-1,3,4-triol (**4a**) was identified by a comparison of its ^1H-NMR spectrum with that of the GSL isolated from *A. clathrodes*[13]. – 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 (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 $CHCl_3$), **2a** ($[\alpha]_D^{25}$ = −3, *c* = 0.002 in $CHCl_3$), **3a** ($[\alpha]_D^{25}$ = −3, *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 **1a** ($[\alpha]_D^{25}$ = +8, *c* = 0.001 in $CHCl_3$), **2a** ($[\alpha]_D^{25}$ = +9, *c* = 0.001 in $CHCl_3$), and **3a** ($[\alpha]_D^{25}$ = +9, *c* = 0.001 in $CHCl_3$) were subjected to oxidative cleavage with $KMnO_4/NaIO_4$ as described in ref.[7], 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 length of the triunsaturated sphinganine from **4a**, isolated as a pure compound, was established by its FAB-MS spectrum.

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 α-D-glucopyranoside 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 D-glucose under the same conditions employed for the methanolysis of GSLs.

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