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Glycolipids from Sponges. 13.[1] Clarhamnoside, the First Rhamnosylated α-Galactosylceramide from *Agelas clathrodes*. Improving Spectral Strategies for Glycoconjugate Structure Determination

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Reinvestigation of the glycosphingolipid composition of the marine sponge *Agelas clathrodes* revealed the presence of a new tetraglycosylated α-galactoglycosphingolipid (**1a**), containing an unusual L-rhamnose unit in the sugar head. The structure of the new compound was elucidated using extensive 2D NMR studies. Because of the strong overlapping of the signals of the sugar protons in the ^{1}H spectrum, ^{13}C-coupled and ^{13}C-decoupled phase-sensitive HMQC spectra were used to study the multiplicity of the overlapping signals. In addition, the absolute configuration of sugars was determined using a simple and efficient, yet underutilized CD method.

Introduction

Glycosphingolipids (GSLs) are glycolipids composed of a long-chain amino alcohol, known as a sphingoid base, a fatty acid residue linked to its amino group (the resulting amide is called ceramide), and a carbohydrate chain attached to the primary hydroxyl group of the ceramide. The scientific interest in GSLs has recently increased on account of the role they could play as therapeutical immunomodulating agents. Unlike common β-glucosyl- or β-galactosylceramides from higher animals and plants, sponges of the genera *Agelas* and *Axinella*[2−6] produce α-galactoglycosphingolipids (α-Gal-GSLs), unique glycosphingolipids with an α-galactose as the first sugar of the carbohydrate chain. Natural α-Gal-GSLs have been shown to possess interesting immunomodulating activities,[3,6] and the mechanism of action of these compounds and of their synthetic analogues has been studied in detail. They are potent ligands of the CD1d antigen presenting protein, and can specifically activate natural killer T cells (NKT cells) in vivo.[7] In addition, KRN7000, the simplest α-Gal-GSL, is considered as a novel anticancer agent, acting through stimulation of the immune system. The first clinical trial with KRN7000, which included pharmacokinetic studies, has recently been completed.[8]

The GSL composition of the Caribbean sponge *Agelas clathrodes* was investigated by our research group in 1995,[4] and three immunostimulating α-Gal-GSLs (**2**−**4**) were isolated. The availability in our laboratory of new specimens of *A. clathrodes* prompted us to reexamine the GSL content of this sponge, and this led to the identification of a further α-Gal-GSL, clarhamnoside (**1a**), a tetraglycosylceramide which is the first α-Gal-GSL having an L-rhamnose unit in the sugar head.

As usual in the procedure we set up for GSLs, structure elucidation of the new GSL **1a** was achieved by extensive 2D spectroscopic analysis performed on its peracetate **1b**, supplemented by microscale degradation. One key point of our procedure is the analysis of vicinal ^{1}H−^{1}H coupling constants for sugar stereochemical assignments. However, in complex molecules such as **1b**, characterized by extensive signal overlapping in the ^{1}H NMR spectrum, this information can be hardly accessible using standard NMR experiments, particularly if the overlapped signals are coupled to each other. In this paper, we show that this problem can be overcome using a $^1J_{CH}$-resolved spectrum obtained from a ^{13}C-coupled HMQC experiment, and apply this method to the structure elucidation of clarhamnoside.

Results and Discussion

A. clathrodes was extracted, in sequence, 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 a SiO_2 column. The fraction containing glycolip-

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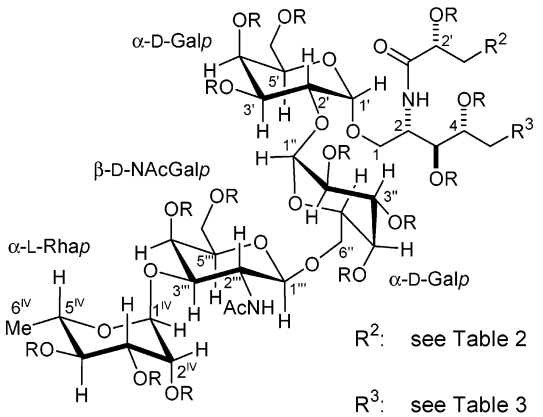
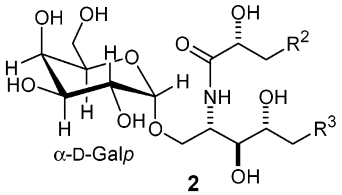


1a R = H
1b R = Ac



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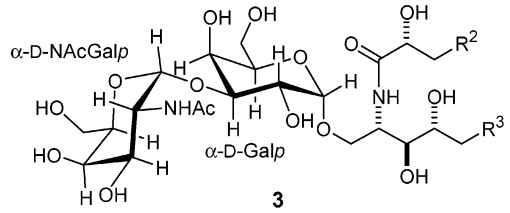

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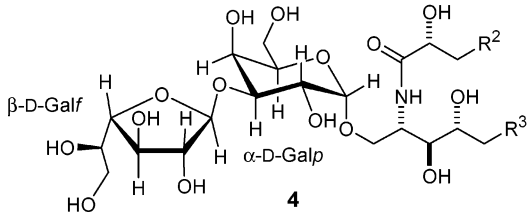


4

R^2 = unbranched C_{17}-C_{21} alkyl chains
R^3 = unbranched and *iso* C_{13}-C_{16} alkyl chains

ids was acetylated, and purified by repeated HPLC on SiO_2 columns, using *n*-hexane/EtOAc and *n*-hexane/*i*-PrOH as eluents, and gave 4.3 mg of clarhamnoside peracetate (**1b**). Native **1a** was obtained by deacetylation of compound **1b** with a 0.02 M solution of MeONa in MeOH.

As usual for GSLs from *Agelas*, compound **1a** is composed of a very complex mixture of homologues, which could not be further separated. Thus, the ESI mass spectrum showed a series of sodiated pseudomolecular ion peaks at *m*/*z* 1393, 1379, 1365, 1351, 1337, and 1323, in accordance with the molecular formula $C_{63}H_{118}N_2O_{23} + nCH_2$ ($n = 0-5$). A high-resolution measurement performed on the most abundant ion at *m*/*z* 1351.8489 confirmed the molecular formula $C_{66}H_{124}N_2NaO_{23}$ for the dominant homologue.

A preliminary ^{1}H analysis of peracetyl derivative **1b** in $CDCl_3$ showed appropriate signals of a glycosphingolipid: the intense aliphatic chain signal at δ 1.24, several signals of oxymethine and oxymethylene groups between δ 5.43 and δ 3.52, and two characteristic amide NH doublets at δ 7.25 and at δ 6.49, suggesting the presence of an additional amide function other than that of ceramide, tentatively that of an aminosugar. In addition, a methyl doublet at δ 1.20 was suggestive of a 6-deoxysugar. The ^{1}H NMR spectrum also showed in the methyl region a triplet at δ 0.88 (ethyl terminus) and a doublet at δ 0.86 (isopropyl terminus), whose intensities were not in an integral ratio with respect to those of other signals in the spectrum. This showed that the alkyl chain mixture differed not only in the length but also in the branching of the alkyl chains.

The ceramide part of the molecule was characterized as composed of a 4-hydroxysphinganine and a 2-hydroxy acid from NMR data. The amide proton of the ceramide, which resonated as a doublet at δ 7.25 in $CDCl_3$, was a useful starting point for the assignment of the sphinganine protons from H_2-1 to H_2-5 using the COSY spectrum, whereas the proton of the hydroxy acid at δ 5.13 showed a correlation peak in the ROESY spectrum with the same amide doublet.

Structure elucidation of the carbohydrate moiety was hindered by the strong overlapping of the signals of the −CH−O protons. In particular, the chemical shifts of several pairs of mutually coupled signals were nearly coincident, so that evaluation of the stereochemistry of the sugars based on coupling constant analysis was impossible. Measurement of ^{1}H NMR spectra in three different deuterated solvents ($CDCl_3$, C_6D_6, and acetone-d_6) was conducted in search of the best separation of signals. Acetone-d_6 was selected as the best solvent for the subsequent experiments, although some overlapped signals were still observed.

The presence of four sugar units was revealed by the four anomeric carbon signals in the ^{13}C NMR spectrum of **1b** at δ 100.4 (C-1‴), 99.8 (C-1^{IV}), 99.4 (C-1′), and 96.1 (C-1″). The heteronuclear chemical shift correlation HMQC NMR spectrum allowed the assignment of the anomeric protons, which are not readily discernible in the ^{1}H NMR spectrum, as four doublets at δ 5.30 (J = 3.5 Hz, H-1″), δ 5.15 (J = 3.5 Hz, H-1′), δ 4.92 (J = 8.4 Hz, H-1‴), and δ 4.88 (J = 1.2 Hz, H-1^{IV}), respectively. Combined use of the COSY, HOHAHA, and HMQC spectra led to the identification of all the protons and carbon atoms of the four sugars, which included one *N*-acetylaminohexose, one 6-deoxyhexose, and two hexoses. Information on the position of the acetoxy groups (the free hydroxyl groups in the native glycolipid) was provided by chemical shifts, because acetoxymethine protons are considerably more deshielded than the other oxymethine protons (typical chemical shift ranges are δ 4.7−5.7 and 3.5−4.5, respectively). Due to overlap, the multiplicity of some signals could only be determined from the relevant cross-peak in the phase-sensitive HMQC spectrum, exploiting the larger chemical shift dispersion of the ^{13}C nucleus. Even at a relatively low digital resolution (0.90 Hz), the large axial−axial couplings were always well visible.

The sugar linked to the ceramide is an α-galactopyranose, as shown by the following evidence: the upfield chemical shifts of H-5′ (δ 4.32) indicated a pyranose sugar, while the upfield chemical shift of H-2′ (δ 4.15) showed that this pyranose is glycosylated at C-2′. The

galacto configuration was evinced by the mutual large axial−axial couplings (J = 12 Hz) for H-2′ and H-3′ (δ 5.25), while the small coupling constants of H-4′ (δ 5.42, dd, J = 3.3 and 1.1 Hz) showed its equatorial orientation. To define the orientation of H-5′, we used an ROESY 2D NMR experiment, in which a correlation peak between H-5′ and H-3′ pointed to a 1−3 diaxial relationship between the two protons. Finally, the α-stereochemistry of the glycosidic linkage was revealed by the small coupling constant of H-1′ (δ 5.17, d, J = 3.7 Hz). The linkage of this sugar directly to the ceramide was shown by the prominent cross-peak of H-1′ with H-1a and H-1b in the ROESY spectrum and by the correlation peak of H-1′ with C-1 in the HMBC spectrum.

The structure of the second sugar, also an α-galactopyranoside, was determined in a similar way. This sugar is linked to the first one, as shown by an ROESY correlation between H-1″ (δ 5.30) and H-2′, and is glycosylated at C-6″, as shown by the upfield chemical shift of the protons at C-6″, both below δ 4.0, as well as by the correlation peak between H-1‴ (δ 4.92) and C-6″ (δ 65.3) in the HMBC spectrum.

The third sugar of the chain is a 2-acetamido-2-deoxy-β-galactopyranoside. The 8.3 Hz coupling constant between H-1‴ (δ 4.92) and H-2‴ (δ 3.66) 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‴ and C-2‴ (δ 54.5), and by the presence of a D_2O exchangeable doublet at δ 7.34, coupled with H-2‴. The large mutual coupling between H-2‴ and H-3‴ (11.1 Hz) showed their axial orientation, while the ROESY correlation of H-3‴ with H-5‴ (δ 4.00) demonstrated the axial nature of H-5‴. Finally, the equatorial orientation of H-4‴ (δ 5.40) was evident from its multiplicity and coupling constants (br d, J = 3.3 Hz). The upfield chemical shift of H-3‴ (δ 4.45) and the HMBC correlation between H-1^{IV} (δ 4.88) and C-3‴ (δ 75.5) evidenced the linkage of this sugar with the last one of the chain.

The remaining spin system belonged to a rhamnose. The doublet at δ 1.12 (H_3-6^{IV}) was coupled with the signal of H-5^{IV} (δ 4.03), which was in turn coupled with that of H-4^{IV} (δ 5.00), which appeared as a well-resolved triplet (J = 11.0 Hz). Therefore, H-3^{IV} (δ 5.09) and H-5^{IV} are both axially oriented. Unfortunately, the nearly coincident chemical shifts of H-2^{IV} (δ 5.10) and H-3^{IV} prevented us from measuring their coupling constants. Even the HMQC spectrum was not useful in this case, because the two strongly coupled protons gave rise to distorted multiplets. This left the orientation of H-2^{IV}, as well as that of H-1^{IV}, undetermined.

To solve this problem, a ^{13}C-coupled HMQC spectrum was obtained (Figure 1). In this experiment, each correlation peak is split into two correlation peaks along the ^{1}H dimension by the large $^1J_{CH}$ coupling constant (about 150 Hz for the oxymethine protons). In particular, the cross-peak of H-3^{IV} was split into two peaks centered at δ 5.22 and 4.91 on our 500 MHz spectrometer, which were completely resolved from all other signals of the sugar. Consequently, the signal of H-3^{IV} appeared as a well-defined first-order multiplet in this spectrum, with only one large axial−axial coupling constant. The orientation of H-2^{IV} was therefore determined to be equatorial, and the 6-deoxysugar was established to be a rhamnose.

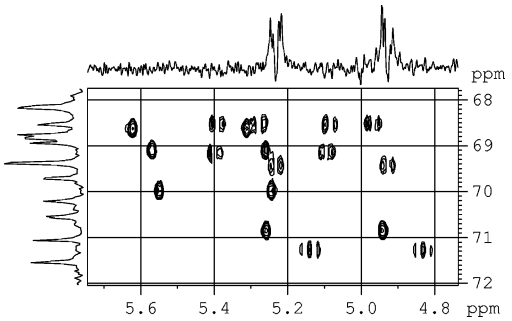


FIGURE 1. Partial plot of the ^{13}C-coupled HMQC spectrum of **1b**. The top trace is the section at δ 69.7 of the spectrum, which was used to measure coupling constants of H-3 ^{IV}.

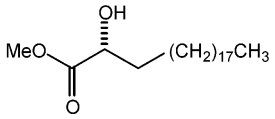
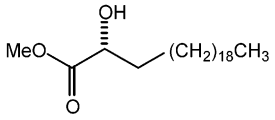
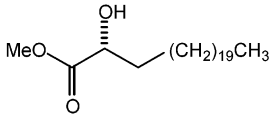
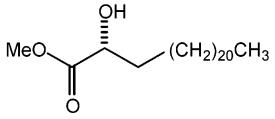
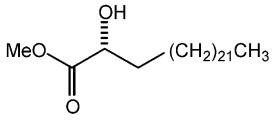
TABLE 1. ^{1}H and ^{13}C NMR Data of 1b (Acetone-d_6)

position		δ_H (mult, J (Hz))	δ_C (mult)
1	a	3.92 (dd, 11.4, 6.2)	70.5 (CH_2)
	b	3.80 (dd, 11.4, 3.9)	
2		4.41 (m)	49.9 (CH)
2-NH		7.66 (d, 9.1)	
3		5.22 (br d, 9)[d]	73.2 (CH)
4		5.02[c]	73.5 (CH)
5		1.83 (m)	32.9 (CH_2)
6		1.41[c]	26.0 (CH_2)
1′		5.15 (d, 3.5)	99.4 (CH)
2′		4.15 (dd, 12, 4)[d]	72.5 (CH)
3′		5.25 (br d, 12)[d]	69.4 (CH)
4′		5.42 (dd, 3.3, 1.2)	69.4 (CH)
5′		4.32 (br t, 6.8)	68.1 (CH)
6′	a	4.15[c]	62.5 (CH_2)
	b	4.06 (dd, 11.4, 6.8)	
1″		5.30 (d, 3.5)	96.1 (CH)
2″		5.15 (dd, 12, 4)[d]	68.8 (CH)
3″		5.24[c]	68.8 (CH)
4″		5.47 (dd, 3.0, 1.1)	68.9 (CH)
5″		4.38[c]	68.5 (CH)
6″	a	3.87 (t, 8.7)	65.5 (CH_2)
	b	3.65[c]	
1‴		4.92 (d, 8.4)	100.4 (CH)
2‴		3.66[c]	54.5 (CH)
2‴-NH		7.34 (d, 8.1)	
3‴		4.45 (dd, 11.1, 3.3)	75.5 (CH)
4‴		5.40 (br d, 3.3)	70.2 (CH)
5‴		4.00[c]	72.3 (CH)
6‴	a	4.14[c]	63.3 (CH_2)
	b	4.00[c]	
1^{IV}		4.88 (d, 1.2)	99.8 (CH)
2^{IV}		5.10 (br d, 3)[e]	71.0 (CH)
3^{IV}		5.09 (dd, 11, 3)[e]	69.7 (CH)
4^{IV}		5.00 (t, 11)[d]	71.5 (CH)
5^{IV}		4.03[c]	68.1 (CH)
6^{IV}		1.12 (d, 6.2)	17.9 (CH_3)
1^{V}			170.8 (C)
2^{V}		4.98[c]	75.1 (CH)
3^{V}	a	1.73 (m)	29.3 (CH_2)
	b	1.62 (m)	

[a] Additional ^{1}H signals: δ 2.152 (3H, Ac), 2.143 (3H, Ac), 2.132 (3H, Ac), 2.129 (3H, Ac), 2.106 (3H, Ac), 2.088 (3H, Ac), 2.081 (3H, Ac), 2.041 (3H, Ac), 2.023(6H, 2 Ac), 2.017 (3H, Ac), 1.996 (3H, Ac), 1.927 (3H, Ac), 1.922 (3H, Ac), 1.916 (3H, Ac), 1.25 (broad band, alkyl chain protons), 0.87 (t, J = 7.0 Hz, *n*-chain Me groups), 0.86 (d, J = 6.5 Hz, isochain Me groups). [b] Additional ^{13}C signals: δ 171.6−170.4 (several C, Ac carbonyl groups), 32.9 (CH_2, ω-2), 26.5 (CH_2, C-4^V), 23.5 (CH_3, NAcGal Ac methyl group), 23.5 (CH_2, ω-1), 23.1 (CH_3, isochain Me groups), 21.8−21.5 (several CH_3, Ac methyl groups), 14.5 (CH_3, ω). [c] Submerged by other signals. [d] Coupling constants were measured from the HMQC NMR experiment. [e] Coupling constants were measured from the ^{13}C-coupled HMQC NMR experiment.

Finally, the anomeric configuration of the rhamnose was determined on the basis of the coupling constant

TABLE 2. Fatty Acyl Composition of 1a

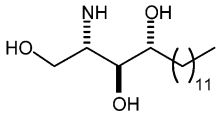
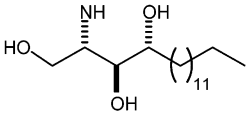
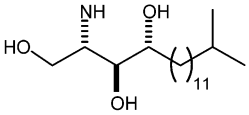
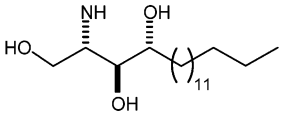
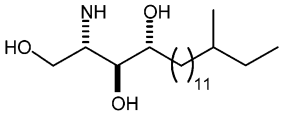
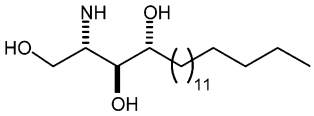
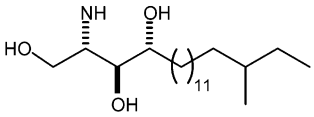
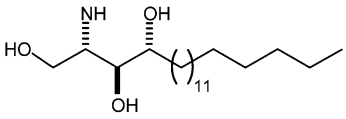
Fatty acid methyl ester	
	16.2 %
	11.2 %
	42.0 %
	18.4 %
	12.2 %

between H-1^{IV} and C-1^{IV}, which was also provided by the ^{13}C-coupled HMQC experiment. It is known that the $^1J_{CH}$ coupling constant of an axial anomeric proton is approximately 160 Hz, while that of an equatorial anomeric proton is ~170 Hz.[7] The measured coupling constant between H-1^{IV} and C-1^{IV} (J = 172 Hz) clearly pointed to the α anomeric configuration of the rhamnose.

Once the structure of the peracetyl derivative **1b** was assessed, the assignment of the ^{1}H and ^{13}C NMR resonances of the natural GSL **1a** became a feasible task. This was performed on the basis of the COSY, HOHAHA, and HMQC NMR spectra, and is reported in Table 1. The ^{13}C assignment was particularly useful to confirm the α anomeric configuration of the rhamnose, because resonances of oxymethine carbon atoms of this sugar were all within 1 ppm from those reported for known α-rhamnopyranosides, while they were significantly different (Δδ up to 3.2 ppm) from those of β-rhamnopyranosides.[9]

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.[4] The sample was subjected to acidic methanolysis, and the products, consisting of methyl glycosides, sphinganines, and fatty acid methyl esters were separated chromatographically. Fatty acid methyl esters were directly analyzed by GC−MS, and were identified as unbranched 2-hydroxy fatty acid by comparison of their retention times and mass spectra with those of authentic samples (Table 2). The absolute configuration at C-2 of the methyl esters was determined as *R* on the basis of the CD spectrum, showing a negative Cotton effect at λ_{max} = 212 nm ($\Delta\epsilon$ = −1.3), which is a general feature of the 2(*R*)-hydroxy fatty acid derivatives.[10]

TABLE 3. Sphinganine Composition of 1a

Sphinganine	
	16.3 %
	13.5 %
	14.8 %
	8.9 %
	7.6 %
	28.1 %
	5.1 %
	5.6 %

The fraction composed of 4-hydroxysphinganines was divided into two portions. One portion was treated with $KMnO_4/NaIO_4$ to convert 4-hydroxysphinganines to carboxylic acids with three less carbon atoms, which were methylated with CH_2N_2 and analyzed by GC−MS. The results are reported in Table 3, in the form of structures of the corresponding sphinganines. The other portion was perbenzoylated with benzoyl chloride in pyridine, and purified by HPLC. The *ribo* relative configuration of the benzoylated sphinganines was determined by recording their ^{1}H NMR spectrum, which was identical (apart from the methyl region) to that of an authentic sample of D-*ribo*-phytosphingosine perbenzoate (**5**). Their absolute configuration was deduced from the CD spectrum in MeCN solution, which matched that of compound **5** recorded in the same solvent.

The methyl glycosides were used to determine the absolute configuration of the four sugars, which resulted to be D for galactose and *N*-acetylgalactosamine and L

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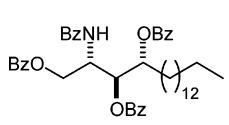
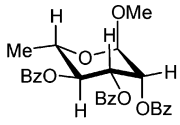
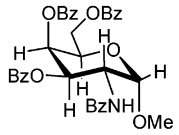


5



6



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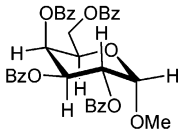

8

for rhamnose, as follows. Authentic samples of D-galactose, *N*-acetyl-D-galactosamine, and L-rhamnose were subjected to acidic methanolysis in the same conditions as compound **1a**, followed by perbenzoylation with benzoyl chloride in pyridine. After HPLC purification, the major reaction products, methyl tri-*O*-benzoyl-α-L-rhamnopyranoside (**6**), methyl tetra-*O*-benzoyl-α-D-galactopyranoside (**7**), and methyl tri-*O*-benzoyl-2-deoxy-2-benzamido-α-D-galactopyranoside (**8**), were identified on the basis of their ^{1}H NMR spectra.

The methyl glycoside mixture from methanolysis of compound **1a** was also subjected to benzoylation, and the reaction mixture was separated on HPLC. The chromatogram contained three peaks, which were collected and identified (apart from their absolute configuration) as compounds **6**−**8** on the basis of their retention times. Because sugar benzoates contain several exciton-coupled chromophores, CD spectroscopy was used to elucidate the absolute configuration of compounds **6**−**8**. The CD spectrum of each glycoside from clarhamnoside **1b** was recorded, and matched that of the corresponding synthetic glycoside, thus showing the absolute stereochemistry of glycosides from **1b** and reference glycosides to be the same.

Conclusion

The novel α-Gal-GSL **1a** is the first example of an α-Gal-GSL containing the unusual rhamnose unit in the sugar head. It is a further proof of the ability of sponges of the genus *Agelas* to synthesize a variety of unique GSLs, characterized by an α-galactose as sugar directly linked to the ceramide.

Structure elucidation of clarhamnoside was made possible by two NMR methods, which improve the standard strategy for the NMR analysis of a carbohydrate chain: ^{13}C-coupled HMQC for $^1J_{CH}$-resolved interpretation of the coupling constant of overlapping protons, and correlation of the one-bond C−H coupling constant of the anomeric proton to the anomeric configuration at the glycosidic linkage of a pyranose sugar with an equatorial proton at C-2.

The absolute stereochemistry of the sugar components was determined by matching CD spectra of the respective benzoates with authentic standards. A similar method was proposed over 10 years ago,[11] but since then it has been used only occasionally, while more sophisticated derivatizations, as well as HPLC or GC separation on chiral columns, have been preferred. In our hand, this method appears to be effective and sensitive, and fits well with the microscale degradation procedure needed for analysis of the alkyl chains of ceramide.

Experimental Section

Collection, Extraction, and Isolation. Specimens of *A. clathrodes* were collected in the summer of 2000 along the coast of Grand Bahamas Island (Sweetings Cay) 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 (160 g of dry weight after extraction) was homogenized and extracted with methanol (3 × 1 L) and then with chloroform (3 × 1 L); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated in vacuo and afforded 34.9 g of a dark brown oil, which was chromatographed on a column packed with RP-18 silica gel. A fraction eluted with $CHCl_3$ (7.8 g) was further chromatographed on a SiO_2 column, giving a fraction [857 mg, eluent EtOAc/MeOH (7:3)] mainly composed of glycolipids. This fraction was peracetylated with Ac_2O in pyridine for 12 h. The acetylated glycolipids were subjected to HPLC separation on a SiO_2 column [eluent *n*-hexane/EtOAc (2:8)], thus affording a mixture (51 mg) containing **1b** and other glycolipids. Further normal-phase HPLC purification [eluent *n*-hexane/*i*-PrOH (85:15)] gave 4.3 mg of clarhamnoside **1b**.

Clarhamnoside Peracetate (1b). The peracetylated derivative **1b**, $[\alpha]_D^{25} = +36$ ($c = 0.43$ in $CHCl_3$), was obtained as a colorless oil. ^{1}H and ^{13}C NMR: Table 1. Composition in fatty acids: Table 2. Composition in sphinganines: Table 2.

Deacetylation of 1b. Compound **1b** (3.0 mg) was dissolved in 950 μL of MeOH, and 50 μL of a 0.4 M solution of MeONa in MeOH was added. The reaction was allowed to proceed for 18 h at 25 °C, and then the reaction mixture was dried under nitrogen and the residue partitioned between water and chloroform. After removal of the solvent, the organic layer gave 2.1 mg of the native glycosphingolipid **1a** (quantitative yield).

Clarhamnoside (1a). White solid. $[\alpha]_D^{25} = +25$ ($c = 0.20$ in MeOH). HRESIMS (positive ion mode, MeOH): *m/z* 1351.8489 ($[M + Na]^+$, $C_{66}H_{124}N_2NaO_{23}$ gives 1351.8442. ESIMS (positive ion mode, MeOH): *m/z* 1393, 1379, 1365, 1351, 1337, 1323 ($[M + Na]^+$ series). ^{1}H NMR (pyridine-d_5): δ 9.04 (1H, d, $J = 8.5$ Hz, NH-2‴), 8.60 (1H, d, $J = 9.8$ Hz, NH-2), 5.78 (1H, br s, H-1^{IV}), 5.63 (1H, d, $J = 3.0$ Hz, H-1′), 5.55 (1H, d, $J = 3.3$ Hz, H-1″), 5.27 (1H, d, $J = 7.8$ Hz, H-1‴), 5.16 (1H, m, H-2), 5.07 (1H, m, H-5″), 4.92 (1H, m, H-2‴), 4.73 (overlapped, H-2′), 4.72 (overlapped, H-4‴), 4.66 (overlapped, H-2^{IV}), 4.64 (overlapped, H-2^{V}), 4.63 (overlapped, H-3‴), 4.61 (overlapped, H-5^{IV}), 4.60 (overlapped, H-2″), 4.58 (overlapped, H-1a), 4.57 (overlapped, H-3′), 4.49 (overlapped, H-4′), 4.48 (overlapped, H-5′), 4.48 (overlapped, H-3″), 4.48 (overlapped, H-3^{IV}), 4.47 (overlapped, H-6″a), 4.42 (overlapped, H-1b), 4.40 (overlapped, H-3), 4.35 (overlapped, H_2-6‴), 4.34 (overlapped, H-6″b), 4.32 (overlapped, H_2-6′), 4.27 (overlapped, H-4), 4.27 (overlapped, H-4″), 4.25 (partially overlapped, t, $J = 9.4$ Hz, H-4^{IV}), 3.95 (1H, m, H-5‴), 2.19 (1H, m, H-3^{V}a), 2.08 (1H, m, H-5a), 2.00 (1H, m, H-3^{V}b), 1.90 (1H, m, H-5b), 1.56 (3H, d, $J = 6.2$ Hz, H-6^{IV}), 1.25 (large band, alkyl chains), 0.86 (*n*-chain and isochain Me groups). ^{13}C NMR (pyridine-d_5): δ 175.3 (C, C-1^{V}), 104.2 (CH, C-1^{IV}), 102.6 (CH, C-1‴), 99.0 (CH, C-1″), 98.1 (CH, C-1′), 79.8 (CH, C-3‴), 76.8 (CH, C-5‴), 76.0 (CH, C-3), 75.6 (CH, C-2′), 74.0 (CH, C-4^{IV}), 73.0 (CH, C-5′), 72.7 (CH, C-4), 72.7 (CH, C-3^{IV}), 72.5 (CH, C-2^{IV}), 72.5 (CH, C-2^{V}), 71.2 (CH, C-4′), 71.2 (CH, C-5″), 71.0 (CH, C-4″), 70.4 (CH, C-2″), 70.4 (CH, C-3″), 70.4 (CH_2, C-6″), 70.4 (CH, C-5^{IV}), 70.1 (CH, C-3′), 69.6 (CH, C-4‴), 68.6 (CH_2, C-1), 62.4 (CH_2, C-6′), 62.1 (CH_2, C-6‴), 53.6 (CH, C-2‴), 51.1 (CH, C-2), 35.8 (CH_2, C-3^{V}), 33.8 (CH_2, C-5), 30.5−29.5 (several CH_2 groups, alkyl chains), 23.4 (CH_3, Ac methyl group), 22.8 (CH_3, isochain Me

(11) Kaluarachchi, K.; Bush, C. A. *Anal. Biochem.* **1989**, *179*, 209−215.

groups), 18.6 (CH_3, C-6^{IV}), 14.3 (CH_3, *n*-chain Me groups). Composition in fatty acids: Table 2. Composition in sphinganines: Table 2.

Methanolysis of 1a. A small amount (300 μg) of **1a** 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, and partitioned between $CHCl_3$ and H_2O/MeOH (8:2). The aqueous layer was concentrated to give a mixture of methyl glycosides (fraction A). The organic layer was concentrated and 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 1% pyridine in $CHCl_3$ gave a mixture of α-hydroxy acid methyl esters (fraction B), and subsequent elution with 1% pyridine in MeOH afforded a mixture of sphinganines (fraction C).

D-*ribo*-Phytophingosine Perbenzoate (5). (2*S*,3*S*,4*R*)-2-Amino-1,3,4-octadecanetriol (D-*ribo*-phytosphingosine, Sigma, 1.0 mg) was benzoylated with benzoyl chloride (50 μL) in pyridine (500 μL) at 25 °C for 16 h. After quenching with MeOH, the reaction mixture was dried and purified by HPLC, giving 1.1 mg of compound **5**. ^{1}H NMR ($CDCl_3$): δ 8.04 (2H, d, J = 7.9 Hz, benzoyl ortho protons), 8.00 (2H, d, J = 7.9 Hz, benzoyl ortho protons), 7.93 (2H, d, J = 7.9 Hz, benzoyl ortho protons), 7.83 (2H, d, J = 7.9 Hz, benzoyl ortho protons), 7.60−7.34 (12 H, overlapping signals, benzoyl protons), 7.07 (1H, d, J = 8.9 Hz, NH-2), 5.76 (1H, dd, J = 6.7 and 3.7 Hz, H-3), 5.55 (1H, ddd, J = 8.6, 4.4, and 3.7 Hz, H-4), 5.12 (1H, m, H-2), 4.69 (1H, dd, J = 11.8 and 3.5 Hz, H-1a), 4.58 (1H, dd, J = 11.8 and 5.8 Hz, H-1b), 1.99 (2H, m, H_2-5), 1.42 (2H, m, H_2-6), 1.34−1.16 (large band, alkyl chain), 0.87 (3H, d, J = 6.9 Hz, H_3-18). CD (MeCN): λ_{max} = 234 nm ($\Delta\epsilon$ = −7.3), 222 nm ($\Delta\epsilon$ = +1.8).

Methyl Tri-*O*-benzoyl-α-L-rhamnopyranoside (6). L-Rhamnose (2.5 mg) was subjected to acidic methanolysis as described above. The resulting methyl glycosides were benzoylated with benzoyl chloride (50 μL) in pyridine (500 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH, and after 30 min was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column Luna SiO_2, 5 μm; eluent *n*-hexane/*i*-PrOH, 99:1; flow 1 mL/min), affording **6** (t_R = 6.0 min) as the major reaction product. ^{1}H NMR ($CDCl_3$): δ 8.10 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.97 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.83 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.62 (1H, t, J = 7.5 Hz, benzoyl para proton), 7.52 (1H, t, J = 7.5 Hz, benzoyl para proton), 7.49 (2H, d, J = 7.7 Hz, benzoyl meta protons), 7.42 (1H, t, J = 7.5 Hz, benzoyl para proton), 7.39 (2H, d, J = 7.7 Hz, benzoyl meta protons), 7.26 (2H, d, J = 7.7 Hz, benzoyl meta protons), 5.82 (1H, dd, J = 10.1 and 3.4 Hz, H-3), 5.67 (1H, t, J = 10.0 Hz, H-4), 5.65 (1H, br s, H-1), 4.91 (1H, br s, H-1), 4.18 (1H, d quartet, J = 9.9 and 6.3 Hz, H-5), 3.51 (3H, s, OMe), 1.38 (3H, d, J = 6.3 Hz, H_3-6). CD (MeCN): λ_{max} = 237 nm ($\Delta\epsilon$ = +91), 222 nm ($\Delta\epsilon$ = −26).

Methyl Tetra-*O*-benzoyl-α-D-galactopyranoside (7). D-Galactose (2.0 mg) was subjected to acidic methanolysis followed by benzoylation as described above. HPLC purification in the same conditions as above afforded **7** (t_R = 7.2 min) as the major reaction product. ^{1}H NMR ($CDCl_3$): δ 8.07 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 8.00 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.96 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.77 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.62−7.36 (12 H, overlapping signals, benzoyl protons), 6.00 (1H, br d, J = 3.2 Hz, H-4), 5.97 (1H, dd, J = 10.8 and 3.2 Hz, H-3), 5.66 (1H, dd, J = 10.8 and 3.5 Hz, H-2), 5.31 (1H, br d, J = 3.5 Hz, H-1), 4.60 (2H, m, H-5 and H-6a), 4.37 (1H, m, J = 7.1 and 5.1 Hz, H-6b), 3.47 (3H, s, OMe). CD (MeCN): λ_{max} = 238 nm ($\Delta\epsilon$ = +85), 223 nm ($\Delta\epsilon$ = −32).

Methyl Tri-*O*-benzoyl-2-benzamido-2-deoxy-α-D-galactopyranoside (8). 2-Acetamido-2-deoxy-D-galactose (3.0 mg) was subjected to acidic methanolysis followed by benzoylation as described above. HPLC purification in the same conditions as above afforded **8** (t_R = 47.2 min) as the major reaction product. ^{1}H NMR ($CDCl_3$): δ 8.16 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 8.03 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.92 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.68 (2H, d, J = 7.8 Hz, benzoyl ortho protons), 7.66−7.35 (12 H, overlapping signals, benzoyl protons), 6.51 (1H, d, J = 9.5 Hz, NH-2), 5.96 (1H, br d, J = 3.1 Hz, H-4), 5.77 (1H, dd, J = 10.8 and 3.2 Hz, H-3), 5.16−5.08 (2H, m, H-1 and H-2), 4.60 (1H, dd, J = 10.8 and 6.9 Hz, H-6a), 4.54 (1H, br dd, J = 6.9 and 5.4 Hz, H-5), 4.43 (1H, dd, J = 10.8 and 5.4 Hz, H-6b), 3.49 (3H, s, OMe). CD (MeCN): λ_{max} = 238 nm ($\Delta\epsilon$ = +46), 221 nm ($\Delta\epsilon$ = −19).

Absolute Stereochemistry of Methyl Glycosides from Compound 1a. Fraction A from methanolysis of compound **1a** was benzoylated with benzoyl chloride (20 μL) in pyridine (200 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH, and after 30 min was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column Luna SiO_2, 5 μm; eluent *n*-hexane/*i*-PrOH, 99:1; flow 1 mL/min). The chromatogram contained three peaks, which were identified as compounds **6**−**8** by a comparison of their retention times and CD spectra with those of the respective synthetic glycosides (see the Results and Discussion for details).

Analysis of Fatty Acid Methyl Esters. Fraction B from methanolysis of compound **1a** was analyzed by GC−MS, and its components were identified by a comparison of their retention times and mass spectra with those of authentic samples. The results are compiled in Table 2. In addition, the CD spectrum (EtOH) of the mixture was recorded: λ_{max} = 212 nm ($\Delta\epsilon$ = −1.3).

Sphinganines: Oxidative Cleavage and GC−MS Analysis. Fraction C from methanolysis of compound **1a** was divided into two portions. One portion was subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described,[6] and the resulting carboxylic acids were methylated with diazomethane. The obtained esters were analyzed by GC−MS, and the results are compiled in Table 3, expressed in terms of original sphinganines.

Sphinganines: Perbenzoylation. The remaining portion of fraction C was benzoylated as described above and purified by HPLC (column Luna SiO_2, 5 μm; eluent *n*-hexane/*i*-PrOH, 99:1; flow 1 mL/min), giving a fraction composed of perbenzoylated sphinganines. ^{1}H NMR ($CDCl_3$): same spectrum as for compound **5**, except for the methyl region, where signals at δ 0.87 (t, J = 7.0, *n*-chain Me groups) and 0.86 (d, J = 6.5, isochain Me groups) were present. CD (MeCN): λ_{max} = 233 nm ($\Delta\epsilon$ = −8.0), 221 nm ($\Delta\epsilon$ = +1.5).

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Supporting Information Available: General experimental procedures, ^{1}H NMR and ESI MS spectra of **1a**, ^{1}H and ^{13}C NMR spectra and COSY, TOCSY, ROESY, HMQC, ^{13}C-coupled HMQC, and HMBC 2D NMR spectra of compound **1b**, ^{1}H NMR and CD spectra of phytosphingosine benzoate **5** and of benzoylated methyl glycosides **6**−**8**, and CD spectrum of 2-hydroxy fatty acid methyl esters from **1b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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