Sulcaceramide, a Novel Triglycosylceramide from the Marine Ascidian *Microcosmus sulcatus*

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A new glycosphingolipid, sulcaceramide (**1a**), has been isolated as its peracetate (**1b**) from the marine ascidian *Microcosmus sulcatus*, and its structure elucidated by spectroscopic and chemical analysis. The unprecedented trisaccharide chain present in **1a** is a rare example of a fucosylated carbohydrate moiety encountered as a constituent of glycosphingolipids isolated from marine invertebrates.

Introduction

Glycolipids have long been recognized as lipid constituents of marine invertebrates but only recently is the interest in these compounds increasing. The chemistry, occurrence, structure, and bioactivity of these compounds have been investigated and a variety of glycolipids have been isolated and characterized, most of them from sponges and echinoderms.[1] However, in spite of the incredible surge of interest in ascidian chemistry in the last ten years, which has shown these organisms to be a rich source of unique and biologically active secondary metabolites, very few studies have been directed towards ascidian glycolipids. In fact, the only report of a glycolipid isolated from a tunicate is a glycosphingolipid (GSL), namely a 1-*O*-β-glucopyranoside of phytosphingosine-type ceramides containing 2-hydroxy fatty acids, obtained from the ascidian *Botrillus leachii*.[2] In this paper we wish to report the isolation and structure determination of sulcaceramide (**1a**; Figure 1), a novel fucose-containing glycosphingolipid from the Mediterranean ascidian *Microcosmus sulcatus*. The unprecedented trisaccharide chain present in **1a** is a rare example of a fucosylated carbohydrate moiety encountered as a constituent of glycosphingolipids isolated from marine invertebrates.

Results and Discussion

Specimens of *M. sulcatus* Harant 1927 collected in the bay of Naples, were extracted at room temperature with methanol and, subsequently, with chloroform. The extracts were concentrated in vacuo and the water-insoluble lipid fraction obtained was treated with *n*BuOH. The *n*BuOH soluble portion was subjected to reversed- and direct-phase

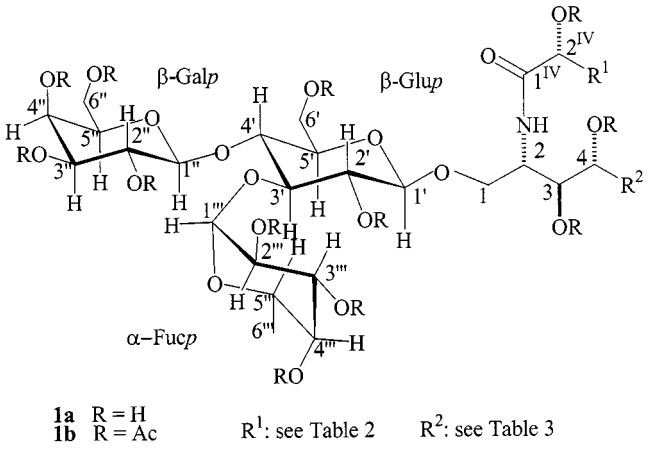

Figure 1. Structures of sulcaceramide (**1a**) and sulcaceramide peracetate (**1b**)

column chromatography to give a crude product, which was recognized as a mixture of GSLs from its ^{1}H NMR spectrum ($CDCl_3$), which contained a large signal at δ = 1.25 and a series of overlapping signals at δ = 3.5−5.5. Since acetylation of glycolipids makes their separation easier and, in addition, structural elucidation can be better performed on peracetylated glycolipids, this fraction was acetylated with acetic anhydride/pyridine and successively separated by HPLC on a SiO_2 column. This afforded the sulcaceramide peracetate **1b**, on which all the experiments devoted to the structure elucidation were performed.

The FAB mass spectrum (negative ion mode) of sulcaceramide showed several molecular ion peaks at *m*/*z* = 1600, 1614, 1628, 1642, and 1656 $[M - H]^-$, indicative of a mixture of homologues, in accordance with the molecular formula $C_{80}H_{131}NO_{31} + nCH_2$ (*n* = 0−4). A detailed analysis of the COSY and ROESY NMR spectra allowed us to identify the ceramide portion of sulcaceramide as being composed of a trihydroxylated, saturated sphinganine and

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a 2-hydroxy fatty acyl group. In fact, starting from the glycosylated oxymethylene protons 1a-H and 1b-H, the COSY spectrum (C_6D_6) allowed us to delineate the spin system from H-1a/1b to H_2-5 (including the amidic proton at C-2 as a doublet at δ = 7.01) in the long-chain base (see Table 1). The presence of a 2-hydroxy fatty acyl chain was deduced from the occurrence of a signal at δ = 5.49 (2^{IV}-H), coupled with the methylene protons (at δ = 2.11) which were, in turn, coupled with a signal included in the large CH_2 signal at δ = 1.35. A correlation peak observed in the ROESY spectrum between the amidic proton (δ = 7.01) and 2^{IV}-H (δ = 5.49) provided the evidence that this fatty acyl moiety was linked to the NH group at C-2. The remaining portions of the ceramide moiety consist of two long-chain alkyl groups at C-5 and 3^{IV-}C whose nature was later established by chemical degradation followed by GC-MS analysis.

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of compound **1b** (C_6D_6)

Pos.	δ_H (mult., *J* [Hz])[a]	δ_C
1a	3.53 (dd 10, 2.9)	67.0
1b	3.77 (bd, 10)	
2	4.60[b]	48.5
2-N*H*	7.01 (d, 9.5)	
3	5.56[b]	72.0
4	5.31 (bd, 11)	73.5
5	1.80[b]	29.1
1′	3.84 (d, 8.1)	101.0
2′	5.11 (dd, 9.5, 8.1)	73.8
3′	3.91 (t, 9.5)	74.2
4′	3.73 (t, 9.5)	74.6
5′	3.07 (m)	73.5
6′a	4.69 (bd, 12)	61.5
6′b	4.05 (dd, 12, 5)	
1′′	4.43 (d, 8.5)	101.5
2′′	5.52[c] (dd, 10, 8.5)	69.2
3′′	5.18 (dd, 10, 2.9)	71.5
4′′	5.66 (bd, 2.9)	66.5
5′′	3.60 (bt, 6.6)	71.2
6′′a	4.75 (dd, 12, 6.6)	60.8
6′′b	4.57[b]	
1′′′	5.69 (d, 3.7)	96.4
2′′′	5.54[c] (dd, 11, 3.7)	69.0
3′′′	5.71[b]	68.5
4′′′	5.81 (bd, 2.9)	72.1
5′′′	5.36 (bq, 7.3)	64.8
6′′′	1.68 (d, 7.3)	16.0
2^{IV}	5.49[b]	74.0
3^{IV}	2.11[b]	31.9
Ac′s	1.62−2.27 (12s)	

[a] Additional ^{1}H signals: δ = 1.50 [m, C*H*$(CH_3)_2$], 1.35 (br., alkyl chain protons), 0.91 (t, *J* = 7.0 CH_2CH_3), 0.92 [d, *J* = 6.5, $CH(CH_3)_2$]. [b] Hidden by other signals. [c] Hidden by other signals; multiplicity and coupling constants are from the spectrum recorded in $CDCl_3$.

The structure of the sugar moiety was established exclusively on the basis of NMR spectroscopic data. Particular attention was devoted to the very crowded mid-field region of the ^{1}H NMR spectrum of **1b**, containing the methylene and methine proton signals of the sugars. This region was characterized by the partial overlap of some signals; thus, the proton spectrum as well as some 2D NMR experiments were recorded both in C_6D_6 and $CDCl_3$ as signals overlapped in one spectrum were often distinct in the other one. The ^{13}C NMR spectrum (C_6D_6) displayed three anomeric methine carbon signals at δ = 96.4, 101.0 and 101.5 which were correlated in the HMQC spectrum with the proton signals at δ = 5.69, 3.84 and 4.43, respectively, thus indicating that three sugar units were present in **1b**. The anomeric protons were used in the analysis of the COSY and HOHAHA spectra as starting points for the sequential assignments of all the proton resonances within each monosaccharide unit.

Analysis of COSY and HOHAHA data (C_6D_6) starting from the anomeric proton at δ = 3.84 allowed us to identify the first sugar unit as a β-glucopyranose. In fact, the large coupling constant between 1′-H and 2′-H, 2′-H and 3′-H, 3′-H and 4′-H and 4′-H and 5′-H indicated the axial position of these protons. The chemical shifts of 3′-H (δ = 3.91) and 4′-H (δ = 3.73) also suggested that the relevant hydroxyl groups were glycosylated rather than acetylated.

The second anomeric proton (signal at δ = 4.43, C_6D_6) was proven to belong to a β-galactopyranose unit in a similar manner. The measurement of the coupling constants of the protons permitted the determination of the axial nature of 1′′-H, 2′′-H and 3′′-H, showing large axial-axial couplings, as well as the equatorial nature of 4′′-H (see Table 1). Coupling constant data did not, however, allow us to determine the stereochemistry of 5′′-H. It was deduced to be axial from a ROESY experiment, which displayed distinct correlation peaks of 5′′-H with 1′′-H and 3′′-H, indicating a 1,3-diaxial relationship with the above protons.

Once the nature of the lipid aglycon and two out of the three components of the sugar chain had been secured, we could reasonably hypothesize that the last sugar was a deoxy-sugar by considering the molecular formula of sulcaceramide. The presence in the ^{1}H NMR spectrum of **1b** of a methyl group resonating as a doublet at δ = 1.68 (C_6D_6, *J* = 7.3 Hz) indicated that it was a 6-deoxysugar that was revealed to be a fucose on the basis of the following arguments. COSY and HOHAHA NMR spectra carried out in C_6D_6 allowed us to determine the chemical shifts of all the protons of this sugar, although partial overlapping of 1′′′-H and 3′′′-H prevented us from measuring the coupling constants. In the spectrum in $CDCl_3$, however, the signals of the deoxysugar were well separated, so the coupling constant analysis was possible. In the same manner as that used for the characterization of the previous sugar units, a large coupling constant was observed between the axial 2′′′-H and 3′′′-H, while 1′′′-H and 4′′′-H, showing only small coupling constants, were deduced to be equatorial. Finally, the observation of a ROESY correlation peak between 3′′′-H and 5′′′-H allowed us to identify the last sugar as an α-fucopyranose unit.

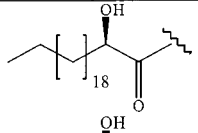
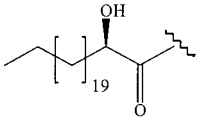
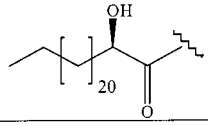
The build-up of the sulcaceramide from the above substructures was inferred from some key peaks appearing in

the HMBC spectrum. A long-range correlation of 1′-H with C-1 indicated the β-glucopyranose to be the first sugar of the carbohydrate chain, while those of 3′-H with C-1‴ and of 4′-H with C-1″ confirmed that the glucopyranose is glycosylated at position 3′ and 4′ and allowed us to identify the sugars linked at each position as indicated in the structure of **1b**.

The nature of the alkyl chains in the ceramide portion of the molecule, as well as their stereochemistry, were established from the following chemical and spectral evidence. In addition to the usual triplet at δ = 0.86 due to the terminal methyl group, a doublet at δ = 0.84, characteristic of an isoalkyl chain[3] was present in the ^{1}H NMR spectrum ($CDCl_3$) of sulcaceramide; the intensity of both signals accounted for a non-integral number of protons. Therefore, the individual homologues seen in the mass spectrum must differ not only in the number of the methylene groups but also in the presence of either an ethyl or an isopropyl as the end group of the chains.

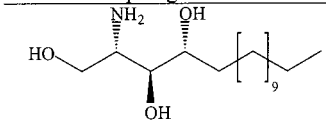
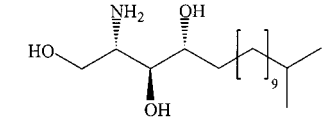
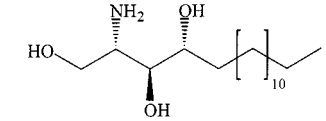
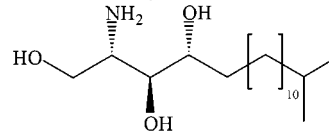
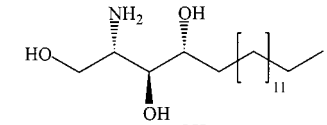
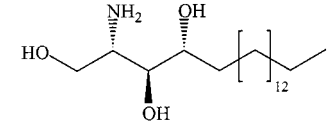
Compound **1b** was subjected to acid methanolysis with 1 M HCl in 90% MeOH and the resulting reaction mixture was separated into fractions of different polarity. The less polar fraction A, as indicated in the ^{1}H NMR spectrum, contained exclusively unbranched long-chain α-hydroxy fatty acid methyl esters, which could be identified and quantified by GC-MS analysis (see Table 2). The configuration at C-2 was assigned as *R* on the basis of the negative $[\alpha]_D$ of the mixture.[4]

Table 2. Fatty acyl composition of compound **1b**

Fatty acyl	Composition
	53.0%
	34.0%
	13.0%

The high-field region of the ^{1}H NMR spectrum indicated the fraction B to be composed of a mixture of 4-hydroxysphinganines with unbranched and isoalkyl chains. An aliquot of this fraction was subjected to acetylation with Ac_2O/py, and its ^{1}H NMR spectrum recorded. In the region of δ = 6.0−4.0 ($CDCl_3$) signals were present whose chemical shift and coupling constants were coincident with those reported for natural (2*S*,3*S*,4*R*)-phytosphingosine (see Exp. Sect.), thus defining the relative stereochemistry of our compound. In order to elucidate the structures of the terminal alkyl chains the sphinganines were converted into their carboxylic acids by permanganate/periodate oxidative cleavage of the C-3/C-4 bond, performed according to the procedure reported in ref.[3] The obtained carboxylic acids were methylated with diazomethane, and the resulting esters were analyzed by GC-MS. The results of the analysis are summarized in Table 3. Finally, the absolute stereochemistry of the phytosphingosines was assigned as reported on the basis of the positive $[\alpha]_D$ of the original fraction B.[5]

Table 3. Sphinganine composition of compound 1b

Sphinganine	Composition
	17.7%
	9.0%
	19.7%
	6.9%
	16.5%
	30.2%

GSLs are widely found as cellular constituents of marine and terrestrial animals and they are believed to possess several interesting biological activities, including modulation of growth and regulation of differentiation. It is also commonly acknowledged that this bioactivity depends essentially on the nature of the functionalized part structure of the molecule including the carbohydrate moiety.[6] Thus, the finding in *M. sulcatus* of sulcaceramide, characterized by a unique sugar portion, suggests that GSLs also play an essential biological role in ascidians. In the near future we expect an increasing amount activity in this field on account of the recent improvement in spectroscopic and isolation techniques; it is now possible to perform conclusive structural determination studies on the very small quantities of pure compounds that are usually obtained from the extracts of most marine ascidians.

Experimental Section

General Methods: FAB MS: VG ZAB mass spectrometer (Xe atoms of energy 2−6 kV). Optical rotation: Perkin−Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm micro-

cell. ^{1}H and ^{13}C NMR: Bruker AMX-500 spectrometer, chemical shifts are referred to the residual solvent signal (C_6D_6: δ_H = 7.15, δ_C = 128.5; $CDCl_3$: δ_H =7.26, δ_C = 77.0); signals of methyl, methylene and methine carbon atoms were distinguished by DEPT experiments; homonuclear ^{1}H connectivities were determined from COSY experiments; the 2D HOHAHA experiment was performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing; the reverse detected multiple quantum heteronuclear correlation (HMQC) spectrum was recorded by using a pulse sequence developed by Bax and Subramanian, with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the interpulse delays were adjusted for an average $^1J_{CH}$ of 142 Hz. Two- and three-bond ^{1}H-^{13}C connectivities were determined by an HMBC experiment optimized for a $^{2,3}J$ of 10 Hz. Medium-pressure liquid chromatography (MPLC) was performed on a Buchi 861 apparatus with RP-18 silica gel (particle size 40−63 μm) and SiO_2 (230−400 mesh) packed columns. High performance liquid chromatography (HPLC) separations were achieved on a Waters 501 apparatus equipped with an RI detector. GC-MS analyses were performed on a Hewlett−Packard 5890 gas chromatograph with a mass-selective detector MSD HP 5970 MS, a split/split-less injector, and 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}. The quantitative determination was based on the area of the GLC peaks.

Extraction and Isolation of Sulcaceramide Peracetate (1b): Specimens of *Microcosmus sulcatus* were collected in the Bay of Naples at a depth of 40 m and identified by Prof. Angelo Tursi (University of Bari). They were frozen immediately after collection and kept frozen until extraction. A reference specimen is deposited at the Dipartimento di Chimica delle Sostanze Naturali, University of Naples. For the extraction, the tunic was removed from the animals and the whole bodies (30 g of dry weight after extraction) were homogenized and extracted (4 × 200 mL) twice with methanol and then twice with chloroform. The combined extracts were partitioned between H_2O and *n*BuOH; the organic layer was concentrated in vacuo and afforded 3.5 g of a dark residue which was chromatographed on a column packed with RP-18 silica gel, eluting with H_2O → MeOH → $CHCl_3$. The fraction eluted with 9:1 MeOH/$CHCl_3$ (800 mg) was further chromatographed on a SiO_2 column. The fraction (90 mg) eluted with MeOH contained mainly glycolipids. A portion of this fraction (50 mg) was dissolved in pyridine (500 μL) and allowed to react with Ac_2O (200 μL) for 12 h. The reaction mixture was concentrated and the residue separated by normal-phase HPLC (eluent: *n*-hexane/EtOAc 1:1), affording pure sulcaceramide peracetate **1b** (4 mg).

Sulcaceramide Peracetate (1b): Colorless oil, $[\alpha]_D^{25} = -36$ (c = 0.003 in $CHCl_3$). Negative FAB-MS: m/z = 1600, 1614, 1628, 1642, and 1656 $[M - H]^-$ series. ^{1}H NMR ($CDCl_3$): δ = 1.21 (overlapped, 3 H, 6‴-H), 1.55 (overlapped, 2 H, 5-H), 1.79 (m, 2 H, 3^{IV}-H), 1.93−2.18 (s, 3 H, Ac's), 3.42 (m, 1 H, 5′-H), 3.52 (dd, J = 10, 2.9 Hz, 1 H, 1a-H), 3.74 (bd, J = 10.0 Hz, 1 H, 1b-H), 4.01 (dd, J = 5, 12 Hz, 1 H, 6′b-H), 4.21 (d, J = 8.1 Hz, 1 H, 1′-H), 4.23 (overlapped, 1 H, 2-H), 4.45 (d, J = 8.5 Hz, 1 H, 1″-H), 4.60 (bd, J = 12.0 Hz, 1 H, 6′a-H), 4.86 (m, 1 H, 4-H), 4.91 (overlapped, 1 H, 5‴-H), 4.92 (overlapped, 1 H, 2′-H), 4.95 (overlapped, 1 H, 3″-H), 5.01 (dd, J = 11, 3.7 Hz, 1 H, 2‴-H), 5.06 (dd, J = 8.5 Hz, 10, 1 H, 2″-H), 5.13 (overlapped, 1 H, 2^{IV}-H), 5.13 (overlapped, 1 H, 3-H), 5.15 (overlapped, 1 H, 3‴-H), 5.27 (d, J = 3. 7 Hz, 1 H, 1‴-H), 5.32 (d, J = 2.9 Hz, 1 H, 4‴-H), 5. 40 (d, J = 2.9 Hz, 1 H, 4″-H), 6.78 (d, J = 9.5 Hz, 1 H, 2-NH).

Methanolysis of Sulcaceramide Peracetate (1b): Sulcaceramide peracetate (**1b**; 3 mg) was dissolved in 500 μL of 1 N HCl in 91% MeOH and the obtained solution was kept for 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 passed through an SiO_2 column (230−400 mesh). Elution with 10 mL of 0.1% pyridine in $CHCl_3$ gave a mixture of α-hydroxy acid methyl esters (fraction A, 0.9 mg), 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), and the organic layer was separated and concentrated to give a mixture of sphinganines (fraction B, 0.8 mg); an aliquot of this fraction was acetylated as reported above.

Analysis of Fatty Acid Methyl Esters: Fraction A ($[\alpha]_D^{25} = -3$, c = 0.001 in $CHCl_3$) was analyzed by GC-MS and its components identified by comparison of their retention times and mass spectra with those of authentic samples. These results are reported in Table 2.

Analysis of Sphinganines: Fraction B ($[\alpha]_D^{25} = +8$, c = 0.001 in $CHCl_3$) was subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described in ref.[3] The resulting carboxylic acids were methylated with diazomethane and the obtained esters analyzed by GC-MS. These results are compiled in Table 3, expressed in terms of the original sphinganines.

Sphinganine Tetraacetates from Sulcaceramide. ^{1}H NMR ($CDCl_3$): δ = 0.84 [d, J = 6.5 Hz, CH(CH_3)$_2$], 0.86 (t, J = 7.0 Hz, CH_2CH_3), 1.24 (broad signal, alkyl-chain protons), 1.69 (m, 2 H, 5-H2), 2.03 (s, 3 H, Ac), 2.06 (s, 6 H, 2Ac), 2.08 (s, 3H, Ac), 4.00 (dd, J = 11.5, 4.5 Hz, 1 H,1a-H), 4.29 (dd, J = 11.5 and 5 Hz, 1 H,1b-H), 4.47 (m, 1 H, 2-H), 4.93 (ddd, J = 9, 3, and 3 Hz, 1 H, 4-H), 5.10 (dd, J = 8.5 and 3 Hz, 1 H, 3-H), 5.92 (d, J = 8.0 Hz, 1 H, 2-NH).

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