Ectyoceramide, the First Natural Hexofuranosylceramide from the Marine Sponge *Ectyoplasia ferox*[‡]

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Ectyoceramide (**1a**), a unique monoglycosylceramide with a galactose in the furanose form, was isolated from *Ectyoplasia ferox*, and its structure was determined by NMR spectroscopy, multiple tandem mass spectrometry, and chemical degradation. Ectyoceramide is the first example of a monohexofuranosylceramide and, more generally, the first natural glycosphingolipid with its first sugar in the furanose form.

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Introduction

Glycosphingolipids (GSLs) are characteristic membrane components of eukaryotic cells. They each consist of an aglycon, the ceramide, composed of a long-chain amino alcohol (sphingosine) amide-linked to a fatty acid, and a carbohydrate chain of between one and over 30 sugars, which protrudes from the membrane surface. Over 250 different GSLs have been isolated from natural sources.[1,2] They show a remarkable structural variety: glucose, galactose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, arabinose, and sialic acid (the GSL is called ganglioside when this last amino sugar is present) are only some of the sugars that have been found in GSLs, while the sugar chain may be either linear or branched, and methylation, acetylation, phosphorylation, and sulfation of sugar hydroxy groups have often been observed. However, all the natural glycosphingolipids so far isolated share the same core structure: a ceramide glycosylated with a hexose in the pyranose form. The hexose can be either a β-glucopyranose (most commonly) or (less frequently) a β-galactopyranose, and only a few exceptions have been reported. Among these, worthy of note are α-Gal-GSLs, a series of glycosphingolipids with an α-galactopyranose moiety as the sugar directly linked to the ceramide, present in some species of marine sponges.[3]

As a part of our continuing study on glycolipids from sponges,[4] we now wish to report the isolation from the marine sponge *Ectyoplasia ferox* and structural elucidation of ectyoceramide (**1a**), a unique galactofuranosylceramide, the first example of a monohexofuranosylceramide and, more generally, the first natural GSL with its first sugar in the furanose form.

Results and Discussion

Ectyoplasia ferox was collected in September 2000 along the coasts of Rum Cay (Bahamas). The specimens were extracted, in sequence, with methanol and chloroform, and the extract was partitioned between water and *n*BuOH. Glycolipids were separated from other metabolites by subjecting of the organic extract to subsequent reversed-phase and normal-phase column chromatography. The glycolipid fraction was acetylated with Ac_2O/Py and subjected to repeated normal-phase HPLC purification, yielding 1.8 mg of a mixture of homologous peracetylated glycolipids. Reversed-phase HPLC separation of this mixture showed it to be composed mainly of ectyoceramide peracetate (**1b**, 1.5 mg), plus small amounts of homologues that were not further analyzed. A small portion of the crude glycolipid fraction was acetylated with trideuterioacetic anhydride instead of acetic anhydride,[5] and after purification gave the derivative **1c**, the ^{1}H NMR spectrum of which is identical to that of **1b** except for the absence of the six acetyl methyl singlets, showing that none of the acetyl groups in **1b** had been present in the natural product **1a** before the acetylation reaction. Finally, **1b** was deacetylated with MeOH/Et_3N (8:2), giving 1.1 mg of the non-acetylated ectyoceramide (**1a**, Scheme 1).

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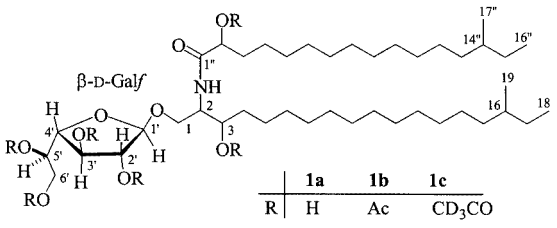


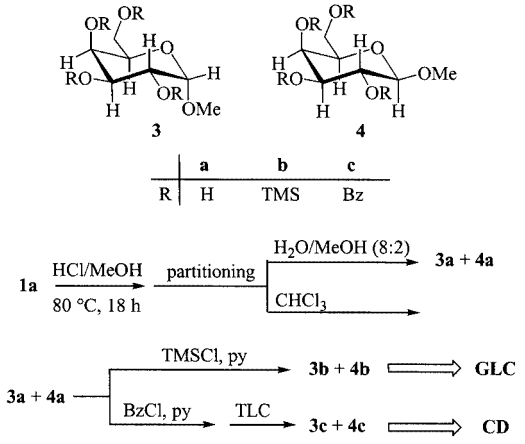
Scheme 1

The high-resolution ESI mass spectrum of ectyoceramide (**1a**) showed a prominent pseudomolecular ion peak $[M + H]^+$ at $m/z = 746.6171$, corresponding to the molecular formula $C_{42}H_{83}NO_9$. A preliminary ^1H NMR analysis of compound **1a** suggested it to be a monoglycosylceramide, as shown by the aliphatic chain signal at δ = 1.24 ppm, the characteristic amide NH doublet at δ = 8.45 ppm, several signals of oxymethine and oxymethylene groups between δ = 4.99 and 4.12 ppm, and one anomeric proton with an unusually small coupling constant (1.7 Hz) at δ = 5.56 ppm.

All the subsequent NMR experiments directed to the structure elucidation were performed on ectyoceramide hexaacetate (**1b**), to take advantage of the better signal dispersion in the ^1H NMR spectrum of this derivative and of the possibility of distinguishing between alkoxymethine and acetoxymethine protons on the basis of their chemical shifts.[2] The nature of the sugar unit was determined as follows. The anomeric proton 1′-H (δ = 4.95 ppm) was identified from its correlation peak with the relevant carbon atom C-1′ (δ = 106.3 ppm) in the HMQC spectrum. This was used in the analysis of the COSY spectrum as a starting point for the sequential assignment of the proton resonances in the monosaccharide unit, which turned out to be a hexose. Couplings between 1′-H and 2′-H, 3′-H and 4′-H, 4′-H and 5′-H, and between 5′-H and both protons at C-6 were readily identified. However, the coupling between 2′-H and 3′-H could not be established from the COSY spectrum, because of the overlapping of these protons, and so was demonstrated indirectly from a correlation peak of 1′-H with 4′-H in the HOHAHA spectrum.

That the hexose was in the furanose form was suggested by the coupling constants of the ring protons, which appeared inappropriate for protons in a six-membered ring, and was definitely demonstrated by evaluation of their chemical shifts. In fact, it is well known[2] that protons of acylated oxymethine groups are significantly more deshielded than protons of alkylated oxymethine groups (typical chemical shift ranges are δ = 4.7−5.7 and 3.5−4.5 ppm, respectively). Consequently, the chemical shift of 4′-H (δ = 4.29 ppm) is a clear indication that the hydroxy group at C-4′ is participating in the acetal function, while that of 5′-H (δ = 5.36 ppm) is consistent with the hydroxy group at C-5′ being acetylated in **1b**, and therefore free in **1a**.

The identity of the hexofuranose could not be established either by coupling constant analysis (unfeasible in a five-membered ring) or by NOE experiments, because the chemical shifts of the key protons 2′-H and 3′-H were very close not only in $CDCl_3$, but also in C_6D_6 and $[D_6]$acetone. We therefore used degradation analysis. A small amount (100 μg) of ectyoceramide was subjected to acidic methanolysis, giving a mixture of the methyl glycosides **3a** and **4a** after partitioning (Scheme 2). A proportion of the methyl glycoside fraction was silylated and analyzed by gas chromatography. Two peaks were detected, and were found to have the same retention times and relative intensities as the silylated methyl α- (**3b**) and β-galactopyranoside (**4b**) obtained by the same procedure from an authentic sample of galactose. The remaining portion of methyl glycosides was perbenzoylated with benzoyl chloride in pyridine, and purified by TLC (Scheme 2). The CD spectrum of the mixture of **3c** and **4c** was recorded (we used the mixture for the measurements because the CD spectra of methyl α- and β-galactopyranoside are almost identical), and proved to be identical with that of perbenzoylated methyl glycosides **3c** and **4c** prepared from D-galactose by the same procedure, thus showing the absolute configuration of the sugar in ectyoceramide **1a** to be D as well.



Scheme 2. Sugar analysis of ectyoceramide **1a**

At this point, only the stereochemistry of the glycosidic linkage, which had been destroyed in the degradation procedure, remained to be established. It was assigned as β on the basis of the ^{13}C NMR chemical shifts of the sugar carbon atoms, which all proved to be within 1 ppm of those reported for the β-galactofuranoside unit present in longiside [β-galactofuranosyl-(1→3)-α-galactopyranosyl-(1→1)-ceramide] peracetate[6] (Table 1).

With regard to the aglycon part of the molecule, the long-chain base of ectyoceramide was shown to be a saturated dihydroxylated sphinganine (2-amino-1,3-alkanediol). In fact, the COSY spectrum showed the amide methine 2-H (δ = 4.26 ppm) to be coupled with the NH doublet at δ = 6.66 ppm, the oxymethylene signals at δ= 3.66 and 3.60 ppm (1-H_2), and the oxymethine signal at δ = 4.91 ppm (3-H). This last proton was in turn coupled with the methylene signals at δ = 1.59 ppm (4-H_2). The COSY and ROESY spectra also allowed identification of the fatty acid residue as an α-hydroxy acid. The signal at δ = 5.15 ppm (2′′-H) showed a correlation peak with the doub-

Table 1. NMR spectroscopic data for ectyoceramide (**1a**) and ectyoceramide peracetate

		1a ([D_5]pyridine)		**1b** ($CDCl_3$)		Longiside peracetate
Pos.[a]		δH [mult, *J* (Hz)]	δC (mult)	δH [mult, *J* (Hz)]	δC (mult)	
1	a	4.40 (dd, 10.3, 5.0)	68.4 (CH_2)	3.66 (dd, 10.7, 4.6)	66.9 (CH_2)	
	b	4.33 (dd, 10.3, 3.4)		3.60 (dd, 10.7, 4.6)		
2		4.71 (m)	54.2 (CH)	4.26 (m)	50.7 (CH)	
2-N*H*		8.45 (d, 9.5)	–	6.66 (d, 9.0)	–	
3		4.17 (m)	71.4 (CH)	4.91 (q, 6.2)	73.6 (CH)	
4		1.85 (m)	35.0 (CH_2)	1.59 (m)	31.5 (CH_2)	
5		1.52 (m)	26.4 (CH_2)	1.27[b]	25.3 (CH_2)	
6–13, 5′′–11′′		1.25[b]		1.25[b]	30.1–29.3 (CH_2)	
14, 12′′		1.25[b]	27.3 (CH_2)	1.25[b]	27.2 (CH_2)	
15, 13′′[c]	a	1.26[b]	37.0 (CH_2)	1.28[b]	36.6 (CH_2)	
	b	1.05 (m)		1.06 (m)		
16, 14′′		1.25[b]	34.7 (CH_2)	1.27[b]	34.4 (CH)	
17, 15′′[c]	a	1.27[b]	29.7 (CH_2)	1.28[b]	29.5 (CH_2)	
	b	1.07 (m)		1.10 (m)		
18, 16′′		0.81[b]	11.6 (CH_3)	0.85 (t, 7.2)	11.4 (CH_3)	
19, 17′′		0.81[b]	19.3 (CH_3)	0.84 (d, 6.5)	19.2 (CH_3)	
1′		5.56 (d, 1.7)	110.1 (CH)	4.95 (br. s)	106.3 (CH)	107.2 (CH)
2′		4.85 (m)	83.0 (CH)	4.99[b]	81.2 (CH)	81.2 (CH)
3′		4.97[b]	78.8 (CH)	5.01[b]	76.2 (CH)	76.5 (CH)
4′		4.97[b]	85.1 (CH)	4.29 (dd, 5.7, 4.1)	80.1 (CH)	80.6 (CH)
5′		4.49 (m)	72.7 (CH)	5.36 (ddd, 7.0, 4.4, 4.1)	69.3 (CH)	69.3 (CH)
6′	a	4.35[b]	54.4 (CH_2)	4.34 (dd, 11.8, 4.4)	62.5 (CH_2)	62.3 (CH)
	b	4.35[b]		4.22 (dd, 11.8, 7.0)		
1′′		–	174.9 (C)	–	169.7 (C)	
2′′		4.62 (m)	72.4 (CH)	5.13 (dd, 7.4, 4.8)	74.2 (CH)	
3′′	a	2.19 (m)	35.7 (CH_2)	1.83 (m)	31.7 (CH_2)	
	b	2.06 (m)				
4′′		1.73 (m)	24.8 (CH_2)	1.32[b]	24.9 (CH_2)	
Ac		–	–	2.17, 2.13, 2.11, 2.10, 2.08, 2.06 (s)	170.4–169.8 (C), 21.0–20.7 (CH_3)	

[a] Diastereotopic protons on the same carbon atom are marked with the letters a and b. [b] Overlapping signal. [c] Two coincident methylene groups, each bearing a pair of diastereotopic protons.

let signal for 2-NH in the ROESY spectrum, and a scalar coupling with 3′′-H_2 (δ = 1.83 ppm) in the COSY spectrum.

In the methyl region, the ^{1}H NMR spectrum of compound **1b** showed a 6 H triplet at δ = 0.85 ppm and a 6 H doublet at δ = 0.84 ppm, while the HMQC spectrum allowed us to assign the relevant carbon signals at δ = 11.4 and 19.2 ppm, respectively. These data are in accordance with both the fatty acid and the sphingosine part of the molecule being of the *anteiso* type.[7]

At this point, all that remained to be established for the complete elucidation of structure **1a** was the length of the alkyl chains in the ceramide. Although mass spectrum in fact indicated that the ceramide contained 36 carbon atoms, it did not provide any information on how these were distributed between the sphingosine and the fatty acid.

This was achieved by use of multiple tandem MS data obtained from a ion-trap instrument. All the experiments were carried out by dissolving the sample in MeOH with 1 mM LiCl, because it has been reported[8] that the lithiated adduct produced under these conditions gives rise to very informative fragments. The MS/MS spectrum of **1a** was recorded by use of the $[M + Li]^+$ pseudomolecular ion at *m*/*z* = 752 as the precursor ion. It contained two intense peaks at *m*/*z* = 590 $[M + Li - 162]^+$ and 484 $[M + Li - 268]^+$. The former peak was due to loss of the dehydrated sugar molecule; more significantly, the latter peak could be accounted for by the loss of a C_{17} 2-hydroxy fatty acyl group (with transfer of one H atom to the sphingosine). This was confirmed by two MS/MS/MS experiments, performed to analyze further fragmentation of the two ions at *m*/*z* = 590 and 484, respectively: each spectrum contained an intense fragment peak at *m*/*z* = 322, accounted for by a lithiated C_{19} sphingosine and originating from the parent fragments by loss of the acyl group and the sugar unit, respectively. The same, although very weak, fragment peak at *m*/*z* = 322 was also observed among the fragments of the pseudomolecular ion $[M + Li]^+$. The complete fragmentation pattern of compound **1a**, the first reported for a glycosphingolipid with a saturated sphingosine, is shown in Figure 1.

Conclusion

We have shown that ectyoceramide from *E. ferox* is a unique monogalactosylceramide with the galactose in the furanose form. Most marine glycolipids are present in the or-

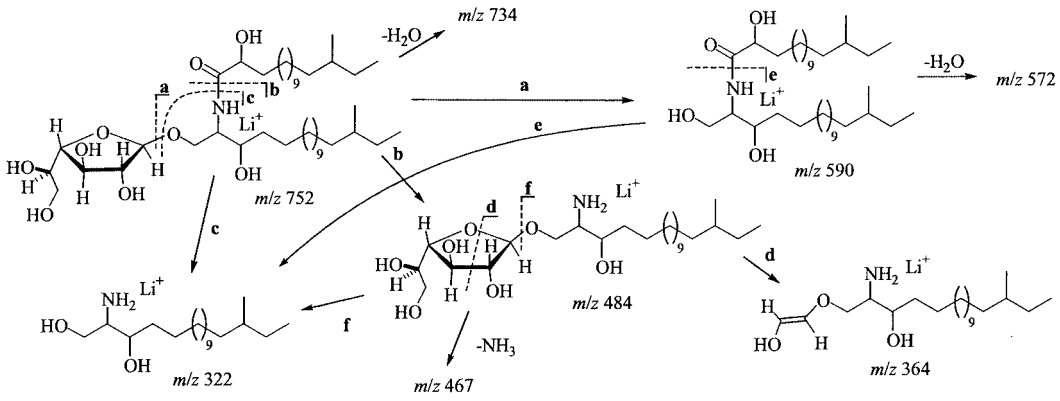


Figure 1. Major fragmentation pathways of the lithiated adduct of ectyoceramide (**1a**) as found by ESI MS/MS experiments

ganisms producing them as complex, inseparable mixtures of homologues; in contrast, a single homologue of ectyoceramide is predominant in *E. ferox*, so compound **1a** could be obtained in pure form. The structure of the ceramide was determined in an innovative way by use of tandem mass spectrometry; in future work this method should allow destructive degradation analysis to be avoided in many cases.

The presence of ectyoceramide in *E. ferox* further confirms that marine sponges are an extremely rich source of new glycolipid structures. Even though only a small number of species have so far been analyzed, some general trends can be outlined. First of all, all the species of sponges we have studied contained glycolipids, but their amounts were very different from species to species. In contrast, high reproducibility was observed for the kind and relative amounts of glycolipids isolated from different specimens of the same species. In addition, fatty acids found in glycolipids of sponges (and sphingosines, which are biosynthesized from fatty acids, found in spongal GSLs) are characteristic in that frequent occurrence of methyl-branched chains, as well as of odd-numbered carbon (both linear and branched) chains, is observed. However, the physiological role of such a variety of unusual glycolipids in sponges is still unclear.

Recent work has demonstrated that a change in the stereochemistry of the glycosidic linkage in monogalactopyanosyl GSLs from the usual β to the α configuration can significantly affect their biological activity, and α-galactosyl GSLs are immunostimulating and antitumor compounds.[9] Likewise, the activity of a galactofuranosylceramide such as ectyoceramide is certainly of interest, but could not be examined thoroughly because of the very small amount of ectyoceramide present in *E. ferox*. Preparation of synthetic analogues of ectyoceramide in order to overcome this problem is in progress in our laboratories.

Experimental Section

General Remarks: High-resolution ESI-MS spectra were performed with a Micromass QTOF Micro mass spectrometer, the sample being dissolved in MeCN/H_2O (1:1) with 0.1% TFA. ESI MS/MS experiments were performed with a Finnigan LCQ ion-trap mass spectrometer. The spectra were recorded by infusion into the ESI source with MeOH/$CHCl_3$ (4:1) containing 1 mM LiCl as the solvent. Optical rotations were measured at 589 nm with a Perkin-Elmer 192 polarimeter in a 10-cm microcell. ^1H and ^{13}C NMR spectra were determined with a Bruker AMX 500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δH = 7.26 ppm, δC = 77.0 ppm; [D_5]pyridine: δH = 8.71, 7.56, and 7.19 ppm, δC = 149.8, 135.3, and 123.4 ppm). For accurate measurement of the coupling constants, the one-dimensional ^1H NMR spectra were transformed at 64 K points (digital resolution: 0.09 Hz). Homonuclear ^1H connectivities were determined by COSY experiments. Through-space ^1H connectivities were evidenced by means of an ROESY experiment with a mixing time of 500 ms. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by use of a pulse sequence 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 $^1J_{C,H}$ of 142 Hz. GLC analysis was performed with a Carlo Erba Fractovap 4160 fitted with a 25-m SPB-1 capillary column, with helium (5 mL/min) as carrier. High-performance liquid chromatography (HPLC) was carried out with a Varian 2510 apparatus fitted with a Varian Star 9040 refractive index detector.

Collection, Extraction, and Isolation: Specimens of *E. ferox* were collected in September 2000 near the coast of the island of Rum Cay (Bahamas), and identified by Prof. M. Pansini (University of Genoa, Italy). They were frozen immediately after collection and kept frozen until extraction. Reference specimens (ref. no. 98-55) have been deposited at the Istituto di Zoologia, University of Genoa, Italy. The sponge (69.4 g dry weight after extraction) was homogenized and extracted with methanol (3 × 1 L) and then with chloroform (2 × 1 L); the combined extracts were then partitioned between H_2O and *n*BuOH. The organic layer was concentrated in vacuo and afforded 11.5 g of a dark red solid, which was chromatographed on a column packed with RP-18 silica gel. A fraction eluted with $CHCl_3$ (2.44 g) was further chromatographed on an SiO_2 column, giving a fraction [126 mg, eluent EtOAc/MeOH (7:3)] mainly composed of glycolipids. The main portion (110 mg) of this fraction was peracetylated with Ac_2O in pyridine for 12 h. The acetylated glycolipids were subjected to HPLC separation on an SiO_2 column [eluent: *n*-hexane/EtOAc (6:4)], thus affording a mixture (4.0 mg) containing ectyoceramide and other glycolipids. Further normal-phase HPLC purification [eluent: *n*-hexane/*i*PrOH (93:7)] gave 1.8 mg of ectyoceramide peracetate (**2b**), plus minor amounts of its homologues. Pure compound **1b** (1.5 mg, 0.013% of the extract) was obtained by reversed-phase HPLC separation, with MeOH as eluent. The natural ectyoceramide (**1a**, 1.1 mg) could be

obtained in quantitative yield by deacetylation of **1b** with 1 mL of MeOH/Et_3N (8:2) at 65 °C for 18 h. The remaining 15 mg of the glycolipid fraction was acetylated with $[D_6]Ac_2O$, and the same separation procedure gave 0.2 mg of ectyoceramide pertrideuterioacetate (**1c**).

Ectyoceramide (1a): Amorphous solid, $[\alpha]_D^{25} = -25$ ($c = 0.11$, MeOH). HRESIMS (positive ions): $m/z = 746.6171([M + H]^+$; calcd. for $C_{42}H_{84}NO_9$ 752.6146. ^1H and ^{13}C NMR: Table 1.

Ectyoceramide Peracetate (1b): Amorphous solid, $[\alpha]_D^{25} = +6.5$ ($c = 0.15$, $CHCl_3$). ^1H and ^{13}C NMR: Table 1.

Sugar Analysis: A small amount (200 μg) of ectyoceramide (**1a**) was dissolved in HCl (1 N, 0.5 mL) in 91% MeOH and the obtained solution was kept in a sealed tube at 80 °C for about 12 h. The reaction mixture was dried under nitrogen, the residue was partitioned between $CHCl_3$ and H_2O/MeOH (8:2), and the aqueous layer, containing methyl glycosides **3a** and **4a**, was taken to dryness. A portion of the mixture of methyl glycosides was silylated with (trimethylsilyl)imidazole in pyridine and analyzed by gas chromatography (oven temperature 150 °C). The chromatogram shows two peaks, the retention times (26.4 min and 30.4 min for **3b** and **4b**, respectively) and relative integration (2.6:1) of which are the same as observed in the chromatogram of a sample of silylated methyl glycosides prepared from galactose by the same procedure. The remaining portion of methyl glycosides was dissolved in pyridine (200 μL) and treated with benzoyl chloride (50 μL). After 12 h at 20 °C, the reaction was quenched with methanol, the reaction mixture was dried under nitrogen, and methyl benzoate was removed by keeping the residue under vacuum with an oil pump for 24 h. The residue was purified by preparative TLC (*n*-hexane/EtOAc, 7:3), and the two partially overlapped bands of the perbenzoylated α- and β-glycosides **3c** and **4c** ($R_f = 0.54$ and 0.51, respectively) were removed together. The CD spectrum (MeCN) of the mixture shows a positive split CD at 237 nm ($\Delta\varepsilon = +21.1$) and 221 ($\Delta\varepsilon = -5.7$), practically identical to the spectrum of a mixture of perbenzoylated methyl glycosides prepared from D-galactose by the same procedure.

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