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STRUCTURAL IDENTIFICATION OF MINOR GLYCOLIPIDS IN *HALOBACTERIUM CUTIRUBRUM* *

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Three minor glycolipids were isolated from the extreme halophile, *Halobacterium cutirubrum*, and their structures were established by chemical degradation, permethylation analysis and spectrometric procedures as follows: (i) a glycolipid sulfate (GL-1), 2,3-di-*O*-phytanyl-1-*O*-[Gal*p*-(3-SO_4^-)β1 → 6Man*p* (3 ← 1αGal*f*)α1 → 2Glc*p*α]-*sn*-glycerol; (ii) a glycolipid (GL-2), the desulfated product of GL-1, 2,3-di-*O*-phytanyl-1-*O*-[Gal*p*β1 → 6Man*p* (3 ← 1αGal*f*)α1 → 2Glc*p*α]-*sn*-glycerol, and (iii) a glycolipid (GL-3), 2,3-di-*O*-phytanyl-1-*O*[Gal*p*β1 → 6Man*p*α1 → 2Glc*p*α]-*sn*-glycerol.

Introduction

The major polar lipids of extremely halophilic bacteria have been identified [1] as the 2,3-diphytanyl-*sn*-glycerol ether analogues of phosphatidylglycerophosphate, phosphatidylglycerol and phosphatidylglycerosulfate, and a sulfated glycotriaosyl diether, 2,3-di-*O*-phytanyl[Gal*p*-(3-SO_4^-)β1 → 6Man*p*α1 → 2Glc*p*α]-*sn*-glycerol. In addition to these lipids, several minor glycolipids (GL-1, GL-2 and GL-3) have been detected. One of these (GL-1) is a sulfolipid which can be labelled by cells grown in the presence of [^{35}S]sulfate [2] but is much more polar than the major glycolipid sulfate. Another one (GL-2) cochromatographs with the desulfated GL-1 and the third (GL-3) cochromatographs with the desulfated glycolipid sulfate [3]. The present report deals with the structural elucidation of these three glycolipids.

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Materials and Methods

Materials. Silica gel G for thin-layer chromatography was obtained from Brinkman Instruments (Canada) Ltd. Silver oxide was prepared as described in Ref. 4 and dried over P_2O_5. All solvents were glass-distilled before use.

Growth of the organism. Cells of *H. cutirubrum* were grown in the following medium containing per 1: 10.0 g oxoid bacteriological peptone, 2.0 g KCl, 20.0 g $MgSO_4 \cdot 7H_2O$, 250 g NaCl, 3.0 g trisodium citrate and 1 ml of a 1% Fe^{2+} solution, pH 6.7; the peptone was added after autoclaving. Cells were grown at 39°C in the light at a shaking rate of 190 rev./min for 72 h followed by a further period of 48–72 h at 100 rev./min; they were harvested by centrifugation at 10000 × *g* for 10 min and washed twice with 25% NaCl.

Chromatography. Thin-layer chromatography of polar lipids was carried out on silica gel G plates (20 × 20 cm; 0.5 mm thick, analytical; 1.0 mm thick, preparative) developed in the following solvent systems: A, $CHCl_3$/90% CH_3COOH/CH_3OH (30 : 20 : 4, v/v); B, $CHCl_3$/CH_3OH/$(C_2H_5)_2$ NH/H_2O (110 : 50 : 8 : 5, v/v); and C, $CHCl_3$/CH_3OH/28%

NH_4OH (65 : 35 : 5, v/v). Lipid products of acid methanolysis were chromatographed in solvent D, $CHCl_3/(CH_3CH_2)_2O$ (9 : 1, v/v) and permethylated glycolipids in solvent E, $(CH_3CH_2)_2O/CH_3OH$ (99 : 1 v/v).

Lipids were detected by the following spray reagents [5]: 0.5% α-napthol for glycolipids; 50% H_2SO_4/50% ethanol followed by charring, and 0.005% aqueous Rhodamine followed by visualization under ultraviolet light (366 nm).

Sugars were examined by descending paper chromatography on Whatman No. 1 paper in solvent F, pyridine/ethyl acetate/water (2 : 5 : 5, v/v; upper phase), and were detected with the alkaline $AgNO_3$ stain [5].

Trimethylsilyl derivatives of sugar methyl glycosides [6] were analyzed by gas-liquid chromatography on a 180 cm × 4 mm inner diameter glass column of 5% SE-30 on chromosorb G, AW-DMCS 100–120 mesh at 180°C. Alditol acetate derivatives of sugars [7] were analyzed on 3% OV-225 on 80–100 Supelcoport at 200°C; partially methylated alditol acetates were analyzed on the same column at 175°C.

Analytical methods. Total hexose content of glycolipids was determined on the water-soluble methanolysis products by the phenol/sulfuric acid procedure [5,8] using a glucose/mannose/galactose (1 : 1 : 1) mixture as standard. Elemental analyses were performed by Mr. H. Seguin, National Research Council of Canada.

Physical measurements. Infrared spectra of lipid material were measured in KBr pellets or as thin films using a Unicam SP 1 000 infrared spectrophotometer. Optical rotations were obtained at room temperature and 589 nm with a Perkin-Elmer model 141 polarimeter equipped with a digital readout. GC-MS of alditol acetates of partially methylated sugars was carried out on a glass column of 3% OV-225, programmed from 180–220°C at 5°C/min and interfaced with a Finnigan 1020 Automated GC/MS quadrupole mass spectrometer. Positions of the *O*-methyl groups in the sugars were assigned by correlation of the MS fragmentation patterns with those of authentic standards [9].

Extraction and purification of lipids. Lipids were extracted from whole cells by a modification [5] of the method of Bligh and Dyer [10] and subjected to fractionation as described previously [11]. Acetone precipitation of total lipids (1.1 g) yielded polar lipids (975 mg) which were freed from phosphatidylglycerophosphate by Ba^{2+} precipitation [11]; the supernatant 'glycolipid-enriched' fraction contained GL-1, GL-2, the major glycolipid sulfate, GL-3, phosphatidylglycerol and phosphatidylglycerosulfate. This mixture was treated by the 'acid' Bligh and Dyer procedure [5] to convert barium salts to ammonium salt forms and fractionated by preparative thin-layer chromatography in solvent A into a mixture of GL-1, GL-2 and glycolipid sulfate, a mixture of GL-3 and phosphatidylglycerosulfate and a mixture of phosphatidylglycerophosphate and phosphatidylglycerol. All lipids were eluted from the silica gel with $CHCl_3/CH_3OH/H_2O$ (1 : 1 : 0.1, v/v). The mixture of GL-1, GL-2 and glycolipid sulfate was resolved by preparative thin-layer chromatography in solvent B and the mixture of GL-3 and phosphatidylglycerosulfate was separated by chromatography in solvent C. Each purified glycolipid showed a single component on thin-layer chromatography in solvent systems A or B, with mobilities as shown in Fig. 1.

GL-1 and glycolipid sulfate were converted to their ammonium salt forms by passing a solution of 5–10 mg in approx. 1 ml of $CH_3OH/CHCl_3/H_2O$ (1 : 1 : 0.005, v/v) through a column of Rexyn 101 (H^+ form, 1 ml) and eluting with 20 ml of $CH_3OH/CHCl_3$ (1 : 1, v/v). The eluate was diluted with an equal volume of benzene, rapidly concentrated to 10–15 ml on a rotary evaporator below 25°C, neutralized with 0.2 M methanolic NH_4OH and brought to dryness in vacuo. The residual ammonium salt was precipitated from a small volume of chloroform with 10 vol. of acetone in the cold, collected by centrifugation and dried in vacuo.

Lipid degradation procedures. Complete acid methanolysis of lipids was performed as described elsewhere [5] using 2.5% HCl gas in CH_3OH. The petroleum ether-soluble material was identified as 2,3-diphytanyl-*sn*-glycerol by thin-layer chromatography, optical rotation and infrared spectrum [1]. Glycolipids, their CrO_3 oxidation products, and permethylated glycolipids were hydrolyzed in HCl as described previously [12].

Partial acid methanolysis of the glycolipids was carried out at room temperature in a mixture of $CHCl_3/CH_3OH$/2.5% methanolic HCl (9 : 8 : 4, v/v) as described previously [3]. The reactions were

monitored by thin-layer chromatography in solvent A. After about 96 h, sufficient amounts of partial methanolysis products were formed and these were isolated as described elsewhere [3].

Desulfation of GL-1 and glycolipid sulfate. GL-1 and glycolipid sulfate were desulfated [3,13] by treatment in 0.05 M methanolic HCl at room temperature for 5 h. The mixture was neutralized with 0.2 M methanolic NH_4OH, brought to dryness in vacuo and the desulfated glycolipids were extracted by the Blight and Dyer procedure [5], and finally purified by thin-layer chromatography on silica gel G in solvent A.

Preparation of permethylated GL-1. A sample of the ammonium salt of GL-1 (5 mg) was dried in vacuo over P_2O_5 and partially methylated in 5 ml of CH_3I containing 1% CH_3OH under reflux in the presence of Ag_2O (25 mg) for 24 h. The partially methylated glycolipids were extracted with diethyl ether and converted to the free acid form by the 'acid' Bligh and Dyer procedure [5]; the free acid was dried in vacuo over P_2O_5 and remethylated in 5 ml CH_3I under reflux in the presence of Ag_2O (25 mg) for 48 h. The course of the methylations was monitored by thin-layer chromatography in solvent E and the products isolated as described previously [3]. Permethylated GL-1 was purified by chromatography on a 1 g column of Bio-Sil A eluted with: 10 ml benzene; 10 ml benzene/diethyl ether (95 : 5); 15 ml benzene/diethyl ether (90 : 10); 15 ml benzene/diethyl ether (80 : 20); 15 ml benzene/diethyl ether (70 : 30); 10 ml benzene/diethyl ether (50 : 50); 10 ml benzene/diethyl ether (25 : 75); 10 ml methanol. The pure permethylated GL-1 (less than 1 mg) appeared in the benzene/diethyl ether (70 : 30) fraction.

Permethylation of desulfated GL-1, GL-2 and GL-3. A sample of these glycolipids (5–20 mg) was methylated in CH_3I (5 ml) under reflux, in the presence of Ag_2O (25–50 mg) as described elsewhere [14,15] and monitored as above. The permethylated glycolipids were purified by preparative TLC on silica gel G in solvent E and eluted with $CHCl_3$/CH_3OH (1 : 1, v/v).

Chromium trioxide oxidation. The glycolipid sample (approx. 1 mg) was acetylated with 1 ml pyridine/acetic anhydride (1 : 1, v/v), then oxidized with CrO_3 [16] and converted to alditol acetates as described elsewhere [7].

Results

Yields of GL-1, GL-2 and GL-3 obtained by the procedure described Materials and Methods were: 1.5–2.5%, about 0.5% and about 5% of the total polar lipids, respectively. Thin-layer chromatography of GL-1, GL-2 and GL-3 showed that these glycolipids were well resolved in solvents A and B (Fig. 1). Note that GL-2 and GL-3 had the same R_F values as desulfated GL-1 and desulfated major glycolipid sulfate, respectively. (Fig. 1).

Identification of GL-1

The infrared spectrum of the ammonium salt of GL-1 showed strong OH absorption at 3 400 cm^{-1}, CH_2 and CH_3 absorption at 2 920 and 1 450 cm^{-1}, an isopropyl doublet at 1 380 cm^{-1}, C-O-C diethyl ether absorption at 1 100 cm^{-1}, a sulfate S=O band at 1 245 cm^{-1} and a weak S-O-C band at 815 cm^{-1}. This spectrum was similar to that of the major glycolipid sulfate [3].

Acid methanolysis of GL-1 yielded *sn*-2,3-di-*O*-phytanyl glycerol which had an infrared spectrum and optical rotation ($[\alpha]_D$ +8.7°) identical to those of the authentic diether [1]. Elemental analyses and the mole ratio of hexose/diether of 4 : 1 (Table I) indicated that GL-1 was a glycotetraosyl diphytanyl glycerol ether. The mole ratio of sulfur to diether of 1 : 1 indicated that the glycolipid was monosulfated. Gas chromatographic analysis of the alditol acetates obtained after acetolysis of GL-1 showed the presence of galactose, mannose and glucose in the mole ratio 2 : 1 : 1 (Table II).

Partial acid methanolysis of GL-1 resulted in formation of the desulfated glycotetraosyl diether containing galactose, mannose and glucose in the mole ratio 2 : 1 : 1; a glycotriaosyl diether containing equimolar amounts of galactose, mannose and glucose; a glycobiosyl diether containing mannose and glucose; and a monoglycosyl diether which contained only glucose (Table II).

The linkage positions between sugars and also the question whether the sugars were arranged in a linear or branched sequence was then studied by permethylation analysis. GC-MS analysis of the alditol acetates of the partially methylated sugars derived from permethylated GL-1 showed the presence of 2,3,5,6-tetramethyl galactose, 3,4,6-trimethyl glucose,

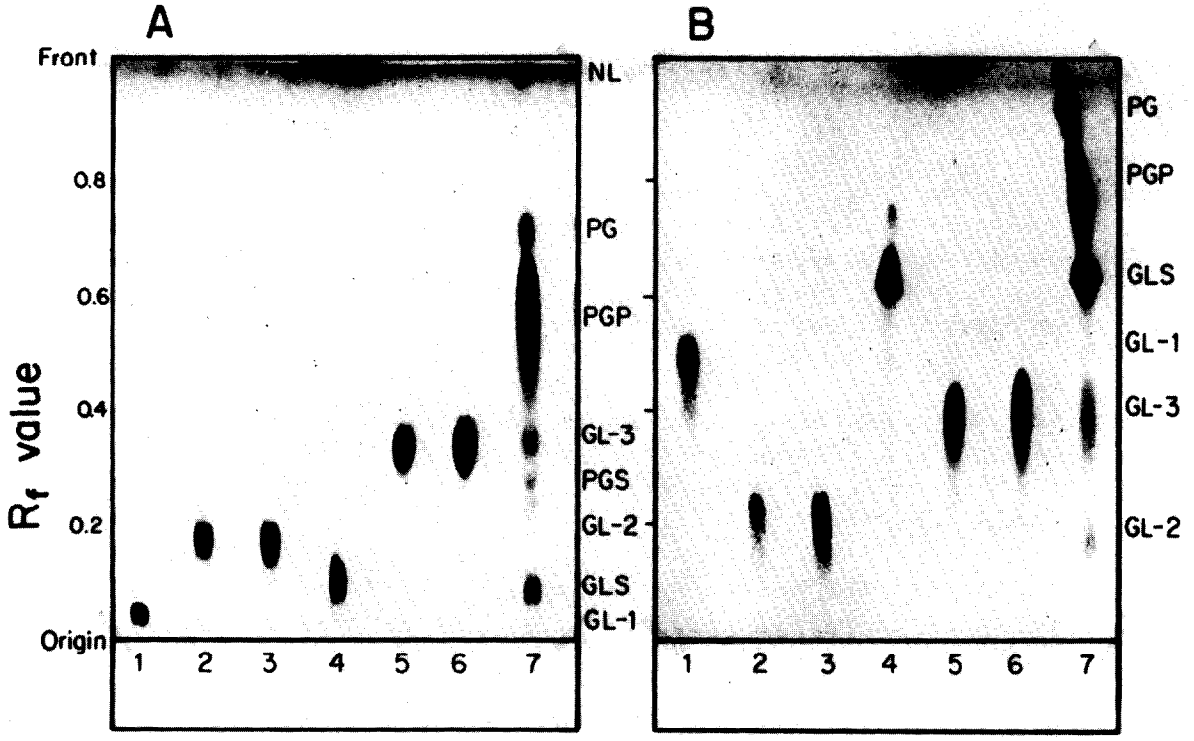

Fig. 1. Thin-layer chromatograms of silica gel G of: 1, GL-1; 2, desulfated GL-1; 3, GL-2; 4, GLS; 5, desulfated GLS; 6, GL-3; 7, total polar lipids. Solvent systems: A, $CHCl_3$/90% CH_3COOH/CH_3OH (30 : 20 : 4, v/v) and B, $CHCl_3$/CH_3OH/$(C_2H_5)_2NH$/H_2O (110 : 50 : 8 : 5, v/v). Abbreviations: PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGS, phosphatidylglycersulfate; GLS, major glycolipid sulfate (sulfated glycotriaosyl diether).

2,4,6-trimethyl galactose and 2,4-dimethyl mannose (Table III). The presence of 2,4-dimethyl mannose showed that the sugar sequence was branched, the mannose being substituted at positions 3 and 6. The presence of 2,3,5,6-tetramethyl galactose indicated that one of the two galactose residues was a terminal furanoside. After permethylation of the desulfated GL-1, the 2,4,6-trimethyl galactose was replaced by 2,3,4,6-tetramethyl galactose, indicating that the sulfate group was on C-3 of a terminal galactopyranose. This also confirmed that GL-1 was monosulfated.

The results so far suggested that one of the following two sequences of sugars (1 or 2) was present in desulfated GL-1:

1, Gal*p*1 → 6Man*p*(3 ← 1Gal*f*)1 → 2 Glc*p* – diether;
2, Gal*p*1 → 3Man*p*(6 ← 1Gal*f*)1 → 2 Glc*p* – diether.

To decide which sequence is correct, the galactofuranose residue was cleaved by partial acid methanolysis as described in Materials and Methods and the isolated glycotriaosyl diether formed was permethylated. Analysis of the alditol acetates of the partially methylated sugars showed the presence of 2,3,4,6-tetramethyl glalactose, indicating that the galactofuranose residue had been cleaved; the other partially methylated sugars were 2,3,4-trimethyl mannose and 3,4,6-trimethyl glucose, identical to those formed from desulfated major glycolipid sulfate which contains the sugar sequence Gal*p*1 → 6Man*p*1 → 2Glc*p*. Therefore, the sequence and linkage positions of sugars in GL-1 must be as shown in structure 1.

Oxidation of desulfated and acetylated GL-1 with CrO_3 resulted in the loss of both galactose residues. Since CrO_3 preferentially oxidizes acetylated hexoses having β-pyranosidic linkages [16], it can be con-

TABLE I

ANALYTICAL DATA FOR GL-1 AND GL-3

	GL-1		GL-3	
	Found	Calculated	Found	Calculated
Mol.wt	–	1 398.8 [a]	–	1 139.6 [b]
C (%)	57.21	57.53	62.28	64.29
H (%)	9.33	9.44	9.75	10.44
S (%)	2.8	2.29	–	–
N (%)	0.89	1.00	–	–
Total hexose (% by wt)	49.1	51.5	47.6	47.4
Diether (% by wt)	44	46.7	54	57.3
Hexose/diether (mole ratio)	4.0	4.0	3.2	3.0
S/diether (mole ratio)	1.3	1.0	–	–
$[\alpha]_D$ (in degrees)	+52.4	–	+46.8	+47.0 [e]
M_D (in degrees) [h]	+733	+720 [c]	+533	+518 [f]
		+301 [d]		+899 [g]

[a] Calculated for $C_{67}H_{127}O_{26}S \cdot NH_4$.
[b] Calculated for $C_{61}H_{118}O_{18}$.
[c] Calculated for Gal$p\beta \rightarrow$ Man$p(\leftarrow$Gal$f\alpha)\alpha \rightarrow$ Glc$p\alpha \rightarrow$ diether.
[d] Calculated for Gal$p\beta \rightarrow$ Man$p(\leftarrow$Gal$f\beta)\alpha \rightarrow$ Glc$p\alpha \rightarrow$ diether.
[e] $[\alpha]_D$ of desulfated glycolipid sulfate [3].
[f] Calculated for Gal$p\beta \rightarrow$ Man$p\alpha \rightarrow$ Glc$p\alpha \rightarrow$ diether.
[g] Calculated for Gal$p\alpha \rightarrow$ Man$p\alpha \rightarrow$ Glc$p\alpha \rightarrow$ diether.
[h] Calculations of M_D values of glycolipids were made according to Hudson's isorotation rules using sugar M_D data reported elsewhere [30,31].

cluded that the galactopyranose residue is β-linked. However, the configuration of the second galactose, which was furanosidically linked, could not be determined by this method since both anomers of furanosides react with CrO_3. The galactofuranose, however, must have the α-configuration since the molecular rotation of GL-1 (Table I) was found to be very close to that calculated for a glycotetraosyl structure con-

TABLE II

CARBOHYDRATE COMPOSITION OF GLYCOLIPIDS DERIVED FROM GL-1, GL-2 AND GL-3 BY PARTIAL METHANOLYSIS

Carbohydrates were determined as alditol acetates except for glycobiosyl diether and monoglycosyl diether in which carbohydrates were determined as trimethylsilyl derivatives.

Glycolipid	Sugar mole ratios								
	GL-1			GL-2			GL-3		
	Glc	Man	Gal	Glc	Man	Gal	Glc	Man	Gal
Sulfated glycotetraosyl diether [a]	1.0	1.0	1.9	–	–	–	–	–	–
Glycotetraosyl diether	1.0	0.8	2.0	1.0	0.8	1.8	–	–	–
Glycotriaosyl diether	1.0	0.8	0.8	1.0	0.8	0.8	1.0	1.0	1.1
Glycobiosyl diether	1.0	0.9	–	1.0	0.8	–	1.0	1.0	–
Monoglycosyl diether	+	–	–	+	–	–	+	–	–

[a] Starting material.

TABLE III

GAS-LIQUID CHROMATOGRAPHY OF PARTIALLY METHYLATED ALDITOL ACETATES FROM PERMETHYLATED GLYCOLIPIDS

2,3,5,6-Tetramethylgalactose was obtained by permethylation of phosphoglycolipid (PGL-II) of *M. hungatei* [24]. (Gal*f*β1 → 6Gal*p*α → tetraether ← glycerol phosphate). All other standards were obtained by permethylation of glycolipid sulfate and its desulfated derivative from *H. cutirubrum*.

Permethylated glycolipid	Peak	Relative retention time	Peak area ratio
GL-1	A	0.62	0.5
	B	1.00	1.0
	C	1.08	0.8
	D [a]	2.29	0.8
Desulfated GL-1	A	0.62	0.6
	E	0.66	1.0
	B	1.00	1.0
	D [a]	2.29	0.8
Glycotriaosyl diether from GL-1	E	0.66	0.8
	B	1.00	1.0
	F	1.18	1.0
GL-2	A	0.62	0.5
	E	0.66	0.8
	B	1.00	1.0
	D [a]	2.29	0.8
GL-3	E	0.66	0.7
	B	1.00	1.0
	F	1.18	1.1
Standards			
2,3,5,6-Tetramethylgalactose	A	0.62	–
2,3,4,6-Tetramethylgalactose	E	0.67	–
3,4,6-Trimethylglucose	B	1.00	–
2,4,6-Trimethylgalactose	C	1.08	–
2,3,4-Trimethylmannose	F	1.17	–

[a] Identified by GC-MS as 2,4-dimethylmannose.

taining α-Glc*p*, α-Man*p*, β-Gal*p* and α-Gal*f*. For a glycoteraosyl structure containing a β-Gal*f* the molecular rotation calculated was less than half the observed value (Table I). The complete structure of GL-1 is therefore:

3, 2,3-di-*O*-phytanyl-1-*O*-[Gal*p*-(3′-SO_4^-)β1 → 6Man*p*-(3 ← 1αGal*f*)α1 → 2Gal*p*α]-*sn*-glycerol (Fig. 2).

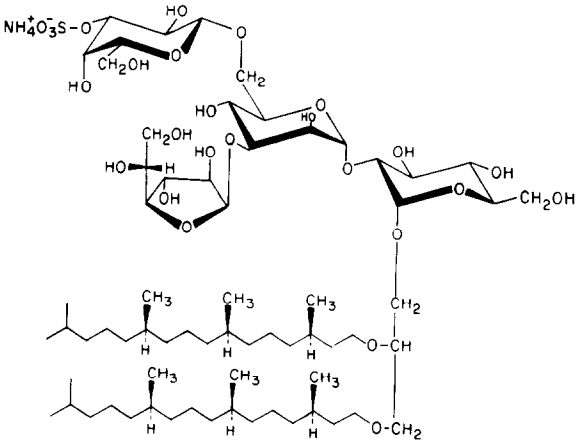

Fig. 2. Structure of the sulfated glycotetraosyl diether [3].

Structure of GL-2

GL-2 had the same R_F values as desulfated GL-1 (Fig. 1) and gave essentially the same partial acid methanolysis products (Table II), CrO_3 oxidation results and partially methylated sugars derived from permethylation (Table III) was were observed for desulfated GL-1. GL-2 is thus the naturally occurring desulfated GL-1, with the following structure:

4, 2,3-di-*O*-phytanyl-1-*O*[Gal*p*β1 → 6Man*p*α(3 ← 1αGal*f*)1 → 2Glc*p*α]-*sn*-glycerol

Structure of GL-3

GL-3 had the same infrared spectrum and R_F values as the desulfated glycolipid sulfate (Fig. 1), and had $[\alpha]_D + 46.8°$, compared to $[\alpha]_D + 47.0°$ reported for the glycotriaosyl diether derived from the major glycolipid sulfate by desulfation [3]. Partial acid methanolysis of GL-3 yielded the same glycolipid products (Table II) as obtained from the glycolipid sulfate [3]. Permethylation analysis of GL-3 showed the presence of 2,3,4,6-tetramethylgalactose, 2,3,4-trimethylmannose and 3,4,6-trimethylglucose (Table III), indicating the sugar sequence Gal*p*1 → 6Man*p*1 → 2Glc*p*. Oxidation of acetylated GL-3 with CrO_3 resulted in the loss of galactose, showing that the mannose and glucose were both α-linked and that the galactose was β-linked. Therefore, GL-3 is identical with the glycosyltriaosyl diether derived from the

major glycolipid sulfate [3] and has the following structure:

5, 2,3-di-*O*-phytanyl-1-*O*-[Gal*p*β1 → 6Man*p*α1 → 2Glc*p*α]-*sn*-glycerol

Discussion

The results presented show that GL-1 (compound 3) essentially has the structure of the major glycolipid sulfate [3] with the addition of a α-galactofuranose to the 3-position of the mannose residue. Because of this structural relationship it is probable that GL-1 is derived biosynthetically from the major glycolipid sulfate by transfer of a galactofuranosyl group to the mannose residue.

The sulfated glycotetraosyl diether (GL-1; compound 3) and its desulfated derivative (GL-2; compound 4) are unusual in having a non-linear sugar sequence. Glycolipids having four sugars do occur in some bacteria, for example, in the extreme thermophile, *Thermus thermophilus* [17], in *Lactobacillus acidophilum* [18] and in *Lactobacillus casei* [19], but these all have linear sequences. In contrast, branched sugar sequences are common in glycolipids of mammalian cells, e.g. gangliosides, some fucolipids, blood group substances, etc. The presence of a branched sugar sequence in glycolipids of an extreme halophile would suggest that this may be another characteristic held in common with eukaryotes, in addition to the properties previously noted [20,21]. The galactofuranose residue found in GL-1 and GL-2 is not unusual since it has been found on glycolipids of several bacteria, namely, *Mycoplasma mycoides* [22], *Bifidobacterium bifidum* [23], *Thermus thermophilus* [17] and *Methanospirillum hungatei* [24].

The naturally occurring glycotriaosyl diether (GL-3; compound 5) found in *H. cutirubrum* was present in small and variable amounts and was identical to desulfated glycolipid sulfate [3]. GL-3 is likely to be an intermediate in glycolipid sulfate metabolism, as was shown in preliminary pulse-labelled studies [25]. Alternatively, it could be an integral component of the membrane, as was found for the glycotriaosyl diether in *Halobacterium marismorui* [26]. The latter glycolipid occurred in much larger amounts (11%) and had a somewhat different structure in which the terminal sugar was glucose instead of galactose. *H. marismortui* does not contain any glycolipid sulfate, and it appears that the lack of a sulfated glycolipid is compensated for by higher contents of phosphatidylglycerosulfate and glycotriaosyl diether [26].

Glycolipid sulfate has been shown to be essential for the formation of stable bilayers of *H. cutirubrum* polar lipids [27]; it is possible that the minor glycolipids, GL-1, GL-2 and GL-3, may also participate in this membrane-stabilizing effect. It is also possible that GL-1, being sulfated, participates in ion transport, as has been suggested for the glycolipid sulfate [28,29].

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