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Structure determination of the glycolipid sulfate from the extreme halophile *Halobacterium cutirubrum*

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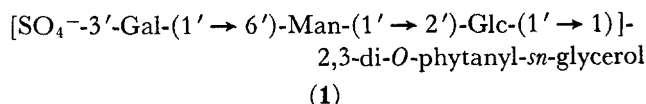
Abstract A sulfur-containing glycolipid, accounting for ca. 25% of the total polar lipids, has been isolated from the extreme halophile *Halobacterium cutirubrum*. The ammonium salt of the lipid was found to have the molecular formula $C_{61}H_{117}O_{21}S \cdot NH_4$, and on strong acid hydrolysis it yielded 2,3-di-*O*-phytanyl-*sn*-glycerol, glucose, mannose, galactose, and sulfate in equimolar proportions. Infrared and NMR spectra indicated the presence of a secondary sulfate group. Solvolysis of the lipid in 0.004 M HCl in tetrahydrofuran resulted in rapid release of inorganic sulfate and formation of galactosyl-mannosyl-glucosyl diphytanyl glycerol ether. With higher acid concentration (0.25 M methanolic HCl), stepwise hydrolysis of monosaccharide units occurred, giving mannosyl-glucosyl glycerol diphytanyl ether and monoglucosyl glycerol diphytanyl ether. The position of attachment of the sugars and of the sulfate group was determined by methylation of the free acid form of the glycolipid sulfate, followed by acid hydrolysis and gas–liquid chromatographic analysis of the partially methylated sugars as the alditol acetates. The configuration of the glycosidic linkages was established both by optical rotation measurements and by specific enzymatic hydrolysis. The results obtained established the structure as 2,3-di-*O*-phytanyl-1-*O*-[β-D-galactopyranosyl-3′-sulfate-(1′→6′)-*O*-α-D-mannopyranosyl-(1′→2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol.

Supplementary key words sulfated triglycosyl glycerol diphytanyl ether · 2,3-di-*O*-phytanyl-*sn*-glycerol · solvolytic desulfation · β-D-galactopyranosyl-(1 → 6)-D-mannopyranose · mono-, di-, and triglycosyl glycerol diphytanyl ethers · thin-layer chromatography · NMR and infrared spectra · halophilic bacteria

Abbreviations: MGD, monoglycosyl glycerol diphytanyl ether; DGD, diglycosyl glycerol diphytanyl ether; TGD, triglycosyl glycerol diphytanyl ether; SL, sulfolipid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance.

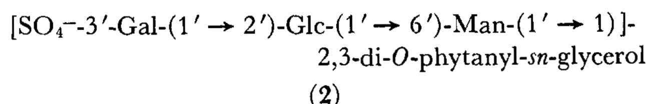
[1] This work forms part of a thesis to be submitted to the Department of Biochemistry, University of Ottawa, for the Ph.D. degree.

When cells of the extreme halophile *Halobacterium cutirubrum* are grown in the presence of $^{35}SO_4^{2-}$, at least three of the organism's lipids become labeled with ^{35}S (1): a glycolipid sulfate (2), a phosphosulfolipid (3), and an unidentified minor glycolipid (1). The glycolipid sulfate is a major component, constituting approximately 25% of the total acetone-insoluble lipids. In a preliminary report, Kates et al. (2) showed that this lipid was a sulfate ester of a triglycosyl diphytanyl glycerol ether. The sugars were found to be glucose, mannose, and galactose, and the linkages between them were determined; the sulfate group was found to be on the terminal sugar, galactose. However, the order of the sugars and the configuration of their linkages were not known. At this stage, two structures were possible:



[SO_4^--3′-Gal-(1′ → 6′)-Man-(1′ → 2′)-Glc-(1′ → 1)]-2,3-di-*O*-phytanyl-*sn*-glycerol

(1)



[SO_4^--3′-Gal-(1′ → 2′)-Glc-(1′ → 6′)-Man-(1′ → 1)]-2,3-di-*O*-phytanyl-*sn*-glycerol

(2)

The present paper describes the complete elucidation of the structure of this glycolipid sulfate and shows it to be structure (**1**).

MATERIALS AND METHODS

Materials

Silica gel H, silica gel G, and kieselguhr G were obtained from Brinkmann Instruments (Canada) Ltd., Rexdale, Ontario. Unisil silicic acid (200–325 mesh) was supplied by Clarkson Chemical Co., Williamsport, Pa. β-Galactosidase (a lactase preparation from *Escherichia*

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coli) was obtained from Worthington Biochemical Corp., Freehold, N.J. All solvents were glass-distilled prior to use.

Analytical methods

Samples were analyzed for phosphorus by the microprocedure of Bartlett (4). Total hexose content of lipids was determined by the phenol–sulfuric acid procedure of Dubois et al. (5).

Elemental analyses were performed by Alfred Bernhardt, Elbach über Engelskirchen, West Germany, and by Chemalytics, Tempe, Ariz. The sodium and potassium ion contents of the glycolipid sulfate salts were determined by flame photometry as described elsewhere (3).

Chromatography

TLC of lipids was carried out on silica gel H plates (20 × 20 cm, 0.5-mm-thick layer) for analytical or preparative separations, or on microslides for monitoring purposes. The plates were developed at room temperature, in jars lined with Whatman 3MM paper, in solvent systems: A, chloroform–90% acetic acid–methanol 30:20:4 (v/v/v); B, chloroform–methanol–water 65:25:4 (v/v/v); or C, chloroform–methanol–concentrated ammonium hydroxide 65:35:5 (v/v/v). Lipids were visualized by spraying the plates with 40% sulfuric acid, followed by charring, or for preparative TLC, by staining with I_2. Lipids were also chromatographed on silicic acid-impregnated Whatman 3MM paper (1) with an ascending solvent system of diisobutylketone–acetic acid–water 40:25:5 (v/v/v) (solvent D) and were detected by staining the chromatograms with rhodamine 6G and viewing under ultraviolet light (388 nm).

Carbohydrates were analyzed by descending chromatography on Whatman no. 1 paper in the solvent system pyridine–ethyl acetate–water 2:5:5 (v/v/v, upper phase) (solvent E) and visualized by the alkaline silver nitrate stain (6), or by TLC on kieselguhr G–silica gel G 4:1 (w/w) in ethyl acetate–methanol–water 68:23:9 (v/v/v) (solvent F) with visualization by spraying the plates with 0.5% α-naphthol and 40% sulfuric acid followed by heating.

Column chromatography was carried out on Unisil silicic acid activated at 120°C for 12 hr using at least a 50:1 weight ratio of silicic acid to material applied.

Gas–liquid partition chromatography of methylated sugar derivatives was carried out on a 4-ft glass column (4 mm I.D.) of 3% ECNSS-M on Gas-Chrom Q at 170°C and 37 psi of helium carrier gas, using a Hewlett-Packard model 402 gas chromatograph with a flame ionization detector.

TABLE 1. R_F values of glycolipid sulfate and glycosyl diethers in various solvent systems

Compound	Solvent System A[a]	B[a]	C[a]	D[b]
Glycolipid sulfate (Na, K, or NH_4 salts)	0.15	0.25	0.11	0.33
Monoglycosyl diether	0.98	0.96	0.86	0.83
Diglycosyl diether	0.77	0.75	0.42	0.56
Triglycosyl diether	0.41	0.46	0.08	0.41

[a] TLC on silica gel H in solvent systems: A, chloroform–90% acetic acid–methanol 30:20:4; B, chloroform–methanol–water 65:25:4; C, chloroform–methanol–concd ammonium hydroxide 65:35:5.

[b] Chromatography on silica-impregnated Whatman 3MM paper in solvent D, diisobutyl ketone–acetic acid–water 40:25:5.

Physical measurements

Infrared spectra were measured on solutions of the substances in chloroform or carbon tetrachloride in 0.5-mm cells using a Beckman IR-20 infrared spectrophotometer. Optical rotations were measured at 22°C at 589 nm in chloroform or chloroform–methanol 95:5 (v/v) solutions of the substances, using a Perkin-Elmer polarimeter, model 141, with digital readout. NMR spectra were measured in [^{2}H]chloroform solutions containing an internal standard of tetramethylsilane, using a Varian T-60 and HA-100 NMR spectrometer. Flame photometric determinations of inorganic ions were carried out using a Unicam SP90A atomic absorption spectrophotometer.

EXPERIMENTAL

Purification of glycolipid sulfate

The acetone-insoluble lipids of *H. cutirubrum* were obtained from the organism as described previously (7). The mixture of polar lipids was separated by TLC (50 mg/plate) in solvent system A. The plates were allowed to dry overnight to remove most of the acetic acid, at which time the glycolipid sulfate could be seen as a white band (R_F 0.15; Table 1), which was eluted as rapidly as possible, at the centrifuge, with chloroform–methanol–0.1 N HCl 1:2:0.8 (v/v/v) (twice with 38 ml of solvent/8 plates). The combined extracts (made up to 76 ml with the eluting solvent, if necessary) were immediately converted to a two-phase system by the addition of 20 ml of chloroform and 20 ml of water. The mixture was centrifuged, the chloroform layer was removed, and the aqueous phase was washed twice with 10 ml of chloroform. The combined chloroform extracts were immediately neutralized with 0.2 N methanolic ammonium hydroxide, diluted with benzene, and taken to dryness under reduced pressure. The residual ammonium salt of the sulfolipid (NH_4-SL) represented ca. 24% by

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TABLE 2. Analytical data for salt forms of glycolipid sulfate

	$C_{61}H_{117}O_{21}S \cdot NH_4$		$C_{61}H_{117}O_{21}S \cdot Na$		$C_{61}H_{117}O_{21}S \cdot K$	
	Calcd	Found	Calcd	Found	Calcd	Found
Mol wt	1236.7		1241.6		1257.8	
C, %	59.24	59.25	59.00	*a*	58.25	58.08
H, %	9.86	9.46	9.49	*a*	9.37	9.08
S, %	2.59	2.56	2.58	2.44	2.54	2.84
Total hexose, %	43.70	45.41	43.53	43.65	42.97	43.57
Diether, %	52.81	51.42	52.60	51.89	51.93	51.28
Hexose/diether, molar ratio	3.00	3.13	3.00	3.01	3.00	3.04
S/diether, molar ratio	1.00	1.00	1.00	1.00	1.00	1.13
Cation, %	1.46	1.43	1.85	2.24	3.11	3.58
Equivalent cation/mole diether	1.00	0.98	1.00	1.21	1.00	1.15
$[\alpha]_D$		+46.79°		+46.16°		+45.15°
$[M]_D$		578.7		573.1		567.9

[a] C and H analyses for the Na salt were consistently low because of incomplete combustion.

weight of the total acetone-insoluble lipids; it showed only one spot on TLC in solvent systems A, B, or C and on chromatography on silicic acid-impregnated paper (Table 1). The ammonium salt was stable in the dry form and in chloroform solution at room temperature for several years. It served as the starting material for the preparation of other derivatives and salt forms. Analytical data and optical rotations for NH_4-SL are given in Table 2.

Preparation of various salt forms of the glycolipid sulfate

A solution of NH_4-SL (177 mg) in 10 ml of chloroform–methanol 1:1 was acidified with 4.5 ml of 0.1 N HCl, and the two-phase system was centrifuged briefly. The chloroform phase containing the free acid form of SL was washed with an equal portion of methanol–water 10:9 and was immediately neutralized (to pH 8) by the addition of 0.2 N methanolic sodium or potassium hydroxide to form the sodium or potassium salt. The chloroform solution was then diluted with benzene and taken to dryness under a stream of nitrogen. A solution of the residual SL salt in chloroform (1 ml), cleared by centrifugation, was diluted with methanol (2 ml) and then with acetone (10 ml). After several hours at 4°C, the SL salt precipitate was centrifuged down and reprecipitated from chloroform solution as described above. The SL salt was finally washed with cold acetone (1 ml) and dried in vacuo at room temperature; before analysis the white powder was further dried under vacuum at 80°C for 12 hr. Analytical data and optical rotations for the various salt forms are given in Table 2; R_F values were the same for all salt forms (Table 1).

Preparation of permethylated glycolipid sulfate

The ammonium salt of the sulfolipid (143 mg) was converted to the free acid form as described above. A solution of the free acid (115 mg; dried over KOH pellets under high vacuum for 1 hr) in 20 ml of freshly distilled methyl iodide was heated under reflux with magnetic stirring in the presence of silver oxide (ca. 250 mg) for 48 hr; more silver oxide and methyl iodide were added at 16-hr intervals. The reaction mixture was diluted with an equal volume of ethyl ether and the silver salts were removed by centrifugation and washed twice with 10 ml of benzene–chloroform 1:1 (v/v). The combined supernates were brought to dryness and the residue was dried under vacuum over KOH pellets; yield, 134 mg of colorless oil.

The crude product, containing the permethylated SL as the major component and three minor partially methylated components, was fractionated on a column of sicilic acid (20 g, made up in benzene), using the following elution sequence: benzene, 100 ml; benzene–ether 90:10, 100 ml; benzene–ether 75:25, 400 ml; benzene–ether 50:50, 200 ml; benzene–ether 25:75, 200 ml; and ethyl ether, 200 ml. The permethylated SL appeared in the benzene–ether 75:25 eluate; it was recovered on evaporation of the solvent and dried under high vacuum; yield, 85 mg of TLC-pure permethylated SL; R_F 0.65 in ethyl ether on silica gel H; $[\alpha]_D$ = +57.4° (1.28 g/dl in chloroform).

Analysis: $C_{71}H_{138}O_{21}S$ (1359.9);
calculated[2]: C, 62.71; H, 10.23; S, 2.36; OCH_3, 25.10
found: C, 62.62; H, 10.28; S, 2.79; OCH_3, 24.38

Determination of glycosidic linkage positions by methylation analysis

The permethylated glycolipid sulfate (17 mg) was methanolyzed in 2.5% methanolic HCl (4.5 ml) under

[2] The value for OCH_3 has been corrected to include the propyl iodide formed from the glycerol moiety of the lipid during the analysis (assuming one equivalent propyl iodide per molecule of lipid).

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TABLE 3. Analytical data for glycolipids

	Monoglycosyl Diether (MGD)		Diglycosyl Diether (DGD)		Triglycosyl Diether (TGD)	
	Calcd	Found	Calcd	Found	Calcd	Found
Mol wt	815.3		977.5		1139.6	
C, %	72.19	71.78	67.58	67.19	64.29	64.14
H, %	12.12	11.93	11.14	10.79	10.44	10.07
Total hexose, %	22.09	21.93	36.86	37.06	47.42	47.62
Diether, %	80.11	79.42	66.82	65.18	57.32	56.71
Hexose/diether, molar ratio	1.00	1.00	2.00	2.06	3.00	3.03
Sugars present[a]		Glucose		Glucose Mannose		Glucose Mannose Galactose
$[\alpha]_D$		+41.36°		+59.43°		+47.02°
$[M]_D$	363[b]	337	517[c]	581	518[d] 899[e]	536

[a] Identified by paper chromatography (see Materials and Methods).
[b] Calculated for α-Glc*p*-diether ($C_{49}H_{98}O_8$).
[c] Calculated for α-Man*p*-α-Glc*p*-diether ($C_{55}H_{108}O_{13}$).
[d] Calculated for β-Gal*p*-α-Man*p*-α-Glc*p*-diether ($C_{61}H_{118}O_{18}$).
[e] Calculated for α-Gal*p*-α-Man*p*-α-Glc*p*-diether.

reflux for 2 hr (8). After the addition of 0.5 ml of water, the hydrolyzate was extracted with low-boiling petroleum ether (3 × 5 ml) to remove the diphytanyl glycerol ether; only traces of methylated methyl glycosides were extracted. The methanol–water phase was taken to dryness and the residue was heated in 1 ml of 1 N aqueous HCl in a boiling water bath for 3 hr. HCl was removed by repeated concentration in vacuo on a rotary evaporator (bath temperature 30°C), and the residual methylated sugars were dissolved in 1 ml of water and reduced to the corresponding alditols with excess sodium borohydride (ca. 10 mg) at room temperature for 1 hr. Glacial acetic acid was added dropwise to destroy the excess borohydride, and the mixture was taken to dryness repeatedly on a rotary evaporator with additions of methanol to remove boric acid as the volatile trimethyl ester. The partially methylated alditols were acetylated with acetic anhydride (2 ml) at 100°C for 3 hr. The acetylation mixture was brought to dryness on a rotary evaporator, and the residual methylated alditol acetates were analyzed by GLC on ECNSS-M (9). Authentic reference standards of alditol acetates (9) of 3,4,6-trimethyl glucose, 2,4,6-trimethyl galactose, and 2,3,4-trimethyl mannose (gift of Dr. G. A. Adams, National Research Council, Ottawa) were used to identify the sugars in the glycolipid sulfate (see Table 4).

Preparation of glycosyl diphytanyl glycerol ethers by partial hydrolysis of the glycolipid sulfate

A solution of 335 mg NH_4-SL in 4.5 ml of chloroform and 6 ml of 0.25 N methanolic HCl (made by bubbling HCl gas into anhydrous methanol) was kept at room temperature. The course of the hydrolysis was monitored by TLC in solvent system A. After 4 days, 1.5 ml of chloroform and 5.4 ml of water were added and the biphasic mixture was centrifuged; the chloroform phase was washed with methanol–water 10:9 and taken to dryness on a rotary evaporator with the addition of benzene to aid in removal of traces of water. The residue (154 mg) was found by TLC in solvent system A, B, or C to contain about equal amounts of mono-, di-, and triglycosyl diphytanyl glycerol ethers. The mixture was separated by preparative TLC using solvent system A. The separated glycosyl glycerol diethers were eluted with chloroform–methanol–water 1:2:0.8 (v/v/v) and the eluates converted to biphasic systems as described for the NH_4-SL. Each of the glycosyl diethers obtained on evaporation of the chloroform phases (MGD, 52 mg; DGD, 46 mg; TGD, 42 mg) was analytically pure and chromatographically homogeneous; R_F values and analytical data are given in Tables 1 and 3, respectively. The infrared spectra of the glycosyl diethers showed OH absorption at 3400 cm^{-1} increasing in intensity with respect to number of sugar moieties; all three spectra showed strong CH_2 and CH_3 absorption at 2900 and 1455 cm^{-1} as well as a pronounced isopropyl absorption (doublet) at 1370 cm^{-1} and a C—O—C ether band at 1110 cm^{-1}.

Isolation of water-soluble products of partial acid hydrolysis

The methanol–water phase of the partial acid hydrolyzate of the glycolipid sulfate, when subjected to TLC on kieselguhr G–silica gel G in solvent F, showed two main spots, corresponding to a disaccharide (R_F 0.28) and its methyl glycoside (R_F 0.44), and minor spots corresponding to the methyl glycosides of the monosaccharides glucose (R_F 0.76), galactose (R_F 0.81), and mannose

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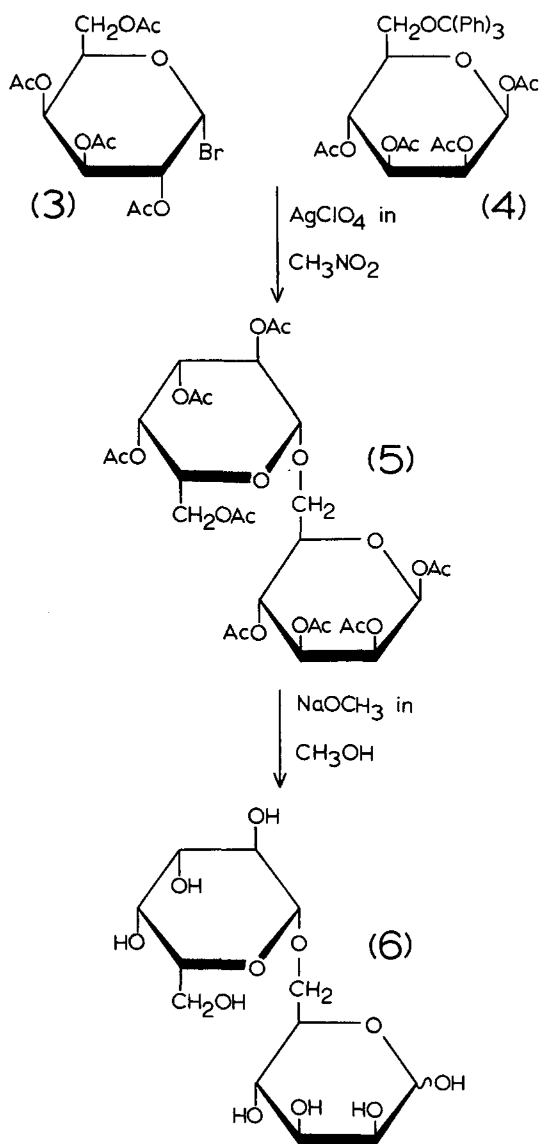

FIG. 1. Scheme for synthesis of β-D-galactopyranosyl-(1 → 6)-D-mannopyranose.

(R_F 0.82). The disaccharide (ca. 2 mg) and its methyl glycoside (ca. 1.5 mg) were isolated as amorphous compounds by preparative TLC on kieselguhr G–silica gel G in solvent F. After acid hydrolysis of the disaccharide or its methyl glycoside, the only sugars detected were galactose and mannose. The configuration of the glycosidic linkage in the disaccharide was determined by enzymatic hydrolysis as described below.

Enzymatic hydrolysis studies on the configuration of the terminal glycosidic linkage

To a solution (1 mg in 0.05 ml of water) of the galactosyl mannose disaccharide or its methyl glycoside derived from SL, or the synthetic disaccharide β-D-galactopyranosyl-(1 → 6)-D-mannopyranose (see below), was added 0.05 ml of an unbuffered aqueous solution of *E. coli* β-galactosidase (1 mg/ml), and the mixture was left at room temperature for 6 hr. The hydrolysis products were then examined by chromatography on Whatman no. 1 paper in solvent E and by TLC on kieselguhr G–silica gel G in solvent F; authentic hexose and methyl hexoside standards were included (see Table 5). The β-galactoside specificity of the enzyme was verified by its positive action on lactose, *o*-nitrophenyl-β-D-galactopyranoside, and the synthetic β-galactosyl mannose, and its inability to hydrolyze cellobiose, maltose, melibiose, or *p*-nitrophenyl-α-D-galactopyranoside.

Complete acid hydrolysis procedure

Hydrolysis of the glycolipid sulfate salts and the three glycosyl glycerol diethers (25–30 mg each) was carried out in side-arm flasks (8) in 2.5% methanolic HCl (4.5 ml) under reflux for 3 hr. After addition of water (0.5 ml), each hydrolyzate was extracted with low-boiling petroleum ether (3 × 5 ml), the extracts were brought to dryness and the residual lipid was weighed to determine the diphytanyl glycerol ether content (Tables 2 and 3). TLC of the pooled glycerol diether residues in chloroform–ether 9:1 showed a single spot corresponding to authentic (7) 2,3-di-*O*-phytanyl-*sn*-glycerol (R_F 0.65). After preparative TLC in the same solvent, the diether had $[\alpha]_D = +8.2°$ [reported (7) for 2,3-di-*O*-(3′*R*,7′*R*,11′*R*,15′-tetramethylhexadecyl)-*sn*-glycerol, $[\alpha]_D = +8.4°$]. The infrared spectrum was identical with that of authentic diphytanyl glycerol ether (10).

The methanol–water phase of each hydrolyzate was taken to dryness under a stream of nitrogen, and the residue was heated in 1 ml of 1 N aqueous HCl at 100°C (boiling water bath) for 3 hr to hydrolyze sugar methyl glycosides. The solution was taken to dryness under nitrogen, and the residue was dried in vacuo over KOH for 24 hr to remove HCl; it was then examined by paper chromatography in solvent E to identify the sugar moieties.

Solvolytic desulfation of glycolipid sulfate

To a solution of NH_4-SL (2.5 mg; 2 μmoles) in anhydrous tetrahydrofuran (1 ml) was added 1 ml of anhydrous 0.008 N HCl in tetrahydrofuran (made by bubbling anhydrous HCl gas into $LiAlH_4$-dried tetrahydrofuran and determining the normality by titration), and the mixture was kept at room temperature. The course of the solvolysis was monitored by TLC in solvent A of samples taken at 10-min intervals. Desulfation was rapid and virtually complete after 90 min. The desulfated product was isolated by preparative TLC in solvent A and was found to have chromatographic mobilities identical with those of the triglycosyl glycerol diether (Table 1).

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Synthesis of β-D-galactopyranosyl-(1 → 6)-D-mannopyranose (see Ref. 11 and Fig. 1)

Acetobromogalactose (12) (**3**; 411 mg, 1 mmole) was added to an ice-cold mixture of silver perchlorate (207 mg, 1 mmole), 1,2,3,4-tetraacetyl-6-trityl-D-mannose (**4**) (577 mg, 1 mmole), and Drierite (1 g) in 20 ml of nitromethane. The mixture was shaken vigorously and allowed to warm up to room temperature. After 5 min the orange precipitate of silver bromide and trityl perchlorate was removed by centrifugation and washed with 10 ml of nitromethane. The combined nitromethane solutions were washed with a cold saturated solution of sodium bicarbonate followed by water. The solution was then cleared by centrifugation, diluted with an equal volume of chloroform, dried over sodium sulfate, and taken to dryness on a rotary evaporator.

The residue was shown by TLC on silica gel H in benzene–methanol 96:4 (v/v) to contain the peracetylated disaccharide (**5**) (R_F 0.21) as the major product together with 2,3,4,6-tetraacetyl galactose (R_F 0.17) and some fast-moving unreacted starting materials. To remove the tetraacetyl galactose the residue was treated with 20 ml of pyridine–acetic anhydride 1:1 for 18 hr to convert the tetraacetyl monosaccharide to the faster-running pentaacetate (R_F 0.46). The peracetylated disaccharide was then readily purified by preparative TLC in the above solvent system; yield, 366 mg (54%) of colorless oil; $[\alpha]_D = +36.6°$ (1.05 g/dl in chloroform).

The peracetylated disaccharide (170 mg, 0.25 mmole) was deacetylated in 10 ml of 25 mM methanolic sodium methoxide for 6 hr at room temperature. The solution was then deionized on a column of Amberlite MB-2 (mixed-bed) ion-exchange resin and taken to dryness on a rotary evaporator. The residual β-D-galactopyranosyl-(1 → 6)-D-mannopyranose (**6**) was dried in vacuo over KOH at 60°C for 24 hr; yield, 82 mg (96%) of colorless gum; $[\alpha]_D = +52.8°$ (0.71 g/dl in H_2O); R_{Glc} values are given in Table 5. The product, although free from other sugars, contained traces of the α-linked disaccharide.

Analysis: $C_{12}H_{22}O_{11} \cdot H_2O$ (360.3);
calculated: C, 40.00; H, 6.71
found: C, 39.49; H, 6.62

RESULTS AND DISCUSSION

The analytical data for the ammonium, sodium, and potassium salt forms of the glycolipid sulfate (Table 2) indicated that there are three sugar residues, one sulfur atom, and one cation equivalent per diphytanyl glycerol ether residue. These data are consistent with the molecular formula $C_{61}H_{118}O_{21}S$ for the free acid. The infrared spectrum of the potassium salt of the glycolipid sulfate

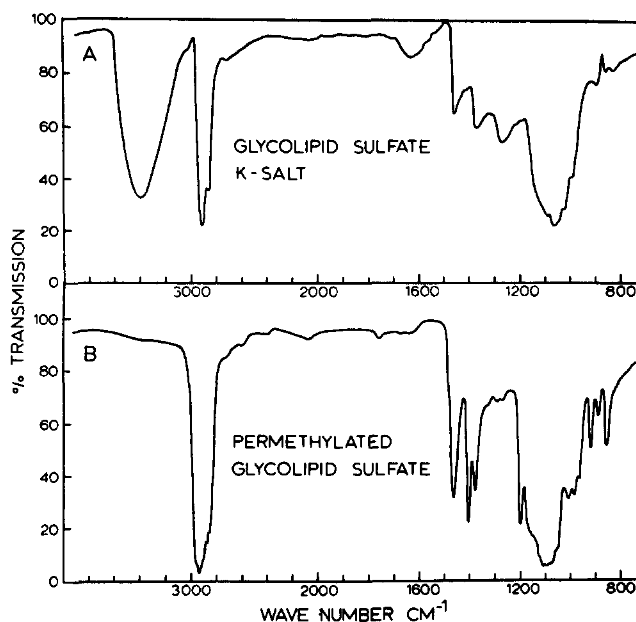

Fig. 2. Infrared spectra of (*A*) the glycolipid sulfate (K salt) in chloroform and (*B*) the permethylated glycolipid sulfate in carbon tetrachloride.

(Fig. 2*A*) showed strong OH absorption at 3400 cm^{-1}, strong CH_2 and CH_3 absorption at 2900 and 1455 cm^{-1}, isopropyl absorption at 1370 cm^{-1} (doublet), a sulfate S=O band at 1265 cm^{-1}, and a typically weak sulfate *S—O*—C band at 830 cm^{-1}. No carboxyl or ester carbonyl bands were present. C—O—C ether absorption may be discerned at ca. 1100 as a shoulder on the strong alcohol C—O band at 1060 cm^{-1}.

The infrared spectrum of the permethylated sulfolipid (Fig. 2*B*) showed no OH absorption, indicating that methylation had been complete. The spectrum showed the same strong CH_2 and CH_3 absorption as in that of SL but exhibited strong and more discrete sulfate bands: —O—SO_2—O— at 1405 and 1195 cm^{-1}, *S—O*—C at 855 cm^{-1}, and secondary sulfate S—*O—C* (13) at 915 cm^{-1}. A C—O—C ether band centered at 1100 cm^{-1} was now prominent, and no alcohol C—O band was present.

The NMR spectrum of the permethylated glycolipid sulfate (Fig. 3) showed the following: sharp methyl signals (phytanyl chains) at δ 0.75–0.95 ppm (30 protons); a broad methylene envelope (phytanyl chains) at 0.95–1.70 ppm (52 protons); several sharp methoxy signals between 3.25 and 3.75 ppm; and a sharp singlet at 4.0 ppm (3 protons) attributable to the secondary S—O—CH_3 group (see Ref. 3). Direct evidence for the presence of a sulfate ester group was obtained by solvolytic desulfation (13) in 4 mM HCl in tetrahydrofuran which resulted in rapid and quantitative cleavage of SL to the triglycosyl diphytanyl glycerol ether and inorganic sulfate.

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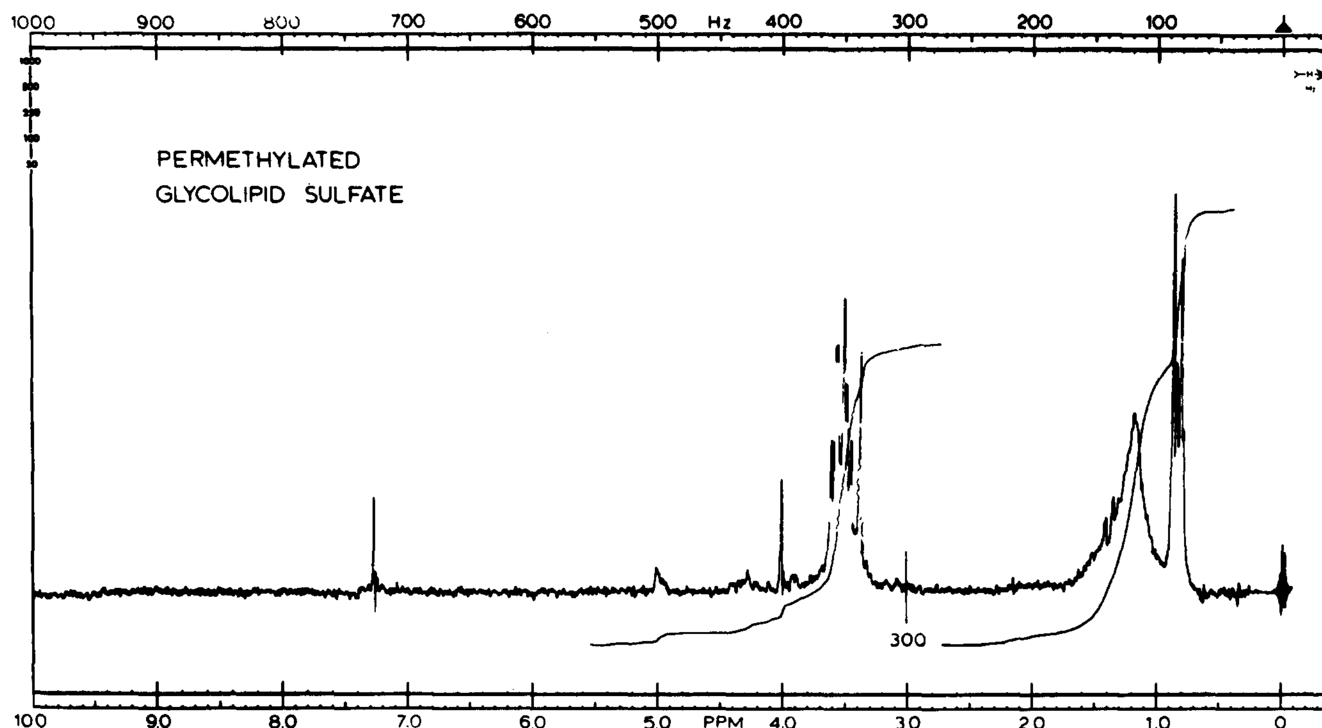
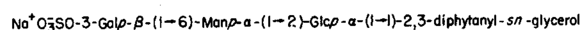


FIG. 3. 100-MHz NMR spectrum of the permethylated glycolipid sulfate in [²H]chloroform with tetramethylsilane as internal standard.

Mild acid hydrolysis of the glycolipid sulfate also resulted in rapid removal of the sulfate group, followed by stepwise cleavage of the monosaccharide units. The chloroform-soluble hydrolysis products consisted of a mixture of mono-, di-, and triglycosyl glycerol diethers (Table 3) together with some diphytanyl glycerol ether. Strong acid hydrolysis of the monoglycosyl glycerol diether produced glucose as the only water-soluble product, indicating that glucose was linked directly to the diphytanyl glycerol ether. The diglycosyl glycerol diether was found to contain mannose and glucose, indicating that mannose was linked to the glucose residue, and the triglycosyl glycerol diether contained galactose, mannose, and glucose, indicating that galactose was the terminal sugar. Hence, the glycolipid sulfate is a sulfated galactosyl mannosyl glucosyl glycerol diether.



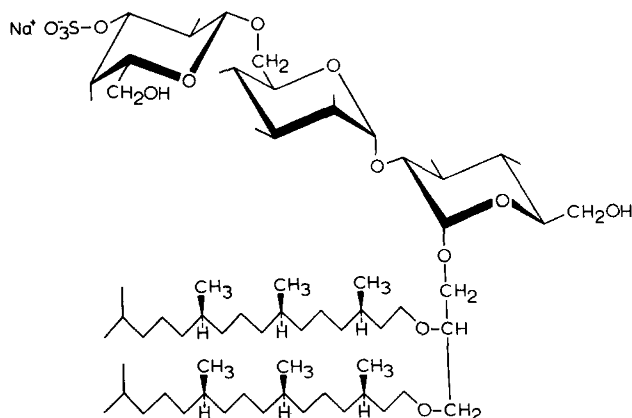


FIG. 4. Structure of glycolipid sulfate of *Halobacterium cutirubrum*.

Glycosidic linkage positions

The linkages between the sugars was established by GLC of the alditol acetates of the partially methylated sugars obtained by acid hydrolysis of the permethylated glycolipid sulfate. The three methylated products found corresponded to 3,4,6-trimethyl glucose, 2,4,6-trimethyl galactose, and 2,3,4-trimethyl mannose in molar proportions 0.6:1:1, respectively (Table 4). The formation of 3,4,6-trimethyl glucose indicated that glucose was linked at position 2, and formation of 2,3,4-trimethyl mannose indicated that mannose was linked at position 6. The presence of 2,4,6-trimethyl galactose showed that

TABLE 4. Hydrolysis products of permethylated glycolipid sulfate

Methylated Sugar[a]	Relative Retention Time[b] Unknown	Relative Retention Time[b] Standard	Molar Ratio
3,4,6-Trimethyl glucose	2.02	2.00	0.6
2,4,6-Trimethyl galactose	2.34	2.33	1.0
2,3,4-Trimethyl mannose	2.56	2.55	1.0

[a] Analyzed by GLC as the partially methylated alditol acetate on 3% ECNSS-M (Gas-Chrom Q) at 170°C.

[b] Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; retention time, 8.5 min.

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TABLE 5. Enzymatic hydrolysis of various glycosides by a specific β-galactosidase[a]

Substrate	R_{Glc}[b]		Hydrolysis Products (R_{Glc} Values)[b]					
			Galactose		Mannose		Methyl Mannoside	
	TLC	Paper	TLC	Paper	TLC	Paper	TLC	Paper
"Natural" Gal-(1 → 6)-Man	0.38	0.34	0.91	0.86	1.06	1.18		
Synthetic Gal-β-(1 → 6)-Man	0.37	0.32	0.91	0.85	1.06	1.17		
"Natural" Gal-(1 → 6)-Man, methyl glycoside	0.66	0.63	0.90	0.85			1.25	ND[c]
Synthetic Gal-β-(1 → 6)-Man, methyl glycoside	0.66	0.64	0.91	0.86			1.26	ND[c]

[a] A lactase preparation from *E. coli*, found to be absolutely specific for β-galactosides.
[b] Separated by (*a*) TLC on kieselguhr G–silica gel G 4:1 in ethyl acetate–methanol–water 68:23:9 (v/v/v) and (*b*) chromatography on Whatman no. 1 paper in pyridine–ethyl acetate–water 2:5:5 (v/v/v, upper phase).
[c] Not detected due to insensitivity of detecting reagent.

the sulfate group must be esterified to position 3 of the terminal galactose. The assignment of these positions is in agreement with those determined previously by GLC of the partially methylated methyl glycosides (2). The low molar proportion of 3,4,6-trimethyl glucose found in the present study may be explained on the basis that the partially methylated glucose is more easily decomposed under the hydrolysis conditions or during the subsequent workup than the other methylated sugars.[3]

Configuration of the glycosidic linkages

On the basis of the high dextrorotatory optical activity of the glycolipid sulfate, Kates et al. (2) concluded that the glycosidic linkages probably had the α configuration. However, comparison of the observed molecular rotations of the three glycosyl glycerol diethers with their theoretical molecular rotations calculated (Table 3) from those of known glycosides and the diphytanyl glycerol ether indicated that, although the mannosidic and glucosidic linkages had the α configuration, the galactosidic linkage was probably in the β configuration.

In order to establish conclusively the configuration of the terminal galactosidic linkage, the small amount of galactosyl mannose produced during the weak acid hydrolysis of the sulfolipid was isolated by TLC and found to have the same R_F values as the synthetic β-D-galactopyranosyl-D-mannopyranose (Table 5). Both the natural and the synthetic disaccharides were readily hydrolyzed by a β-galactosidase preparation known to be completely specific for β-galactosides, thereby establishing the β configuration of the terminal galactosidic linkage (Table 5). The structure of the glycolipid sulfate in *H. cutirubrum* is thus established as 2,3-di-*O*-phytanyl-1-*O*-[β-D-galactopyranosyl-3′-sulfate-(1′ → 6′)-*O*-α-D-mannopyranosyl-(1′ → 2′)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (Fig. 4).

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