Structure determination of the phosphatidylglycerosulfate (diether analog) from *Halobacterium cutirubrum*

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Abstract A novel phosphosulfolipid has been isolated as the potassium salt from the extremely halophilic bacterium *Halobacterium cutirubrum;* its molecular formula was established as $C_{46}H_{93}PSK_2$. It accounts for 3–4% by weight of the polar lipids and about 6% of the total lipid phosphorus. The phosphosulfolipid yielded inorganic sulfate and 1-*sn*-phosphatidyl-3′-*sn*-glycerol (diphytanyl ether analog) in equimolecular proportions after solvolysis in 0.005 N HCl in tetrahydrofuran, and it was thus a sulfate ester of phosphatidylglycerol. The position of the sulfate group was determined by comparison of the infrared and NMR spectra of the salt and the methyl ester of natural phosphosulfolipid with those of synthetic 1- and 2-sulfate esters of phosphatidylglycerol. The results obtained established the structure of the bacterial phosphosulfolipid as 2,3-di-*O*-(3′*R*,7′*R*,11′*R*,15′-tetramethylhexadecyl)-*sn*-glycero-1-phosphoryl-3′′-*sn*-glycero-1′′-sulfate.

Supplementary key words phosphosulfolipid · halophilic bacteria · 1-*sn*-phosphatidyl-3′-*sn*-glycero-1′-sulfate · 1-*sn*-phosphatidyl-1′-*sn*-glycero-2′-sulfate · 1′-*sn*-phosphatidyl-3′-*sn*-glycerol · 1-*sn*-phosphatidyl-2′-glycerol · 2,3-*O*-phytanyl-*sn*-glycerol · 1,3-di-*O*-phytanyl-*sn*-glycerol · NMR spectra · infrared spectra · thin-layer chromatography · cation analysis

THE MAJOR polar lipids identified in *Halobacterium cutirubrum* and other extreme halophiles are the diphytanyl ether analogs of phosphatidylglycerophosphate and phosphatidylglycerol and a glycolipid sulfate ester (1, 2). A minor unidentified polar component, containing both phosphorus and sulfur, has also been detected (1, 3). Preliminary studies (4) have shown that this lipid is a sulfate ester of phosphatidylglycerol (diphytanyl ether analog), but the position of the sulfate group was not established. We report here structural studies leading to the unambiguous identification of this phosphosulfolipid as 2,3-di-*O*-phytanyl-*sn*-1-glycerophosphoryl-*sn*-3′-glycero-1′-sulfate.

MATERIALS AND METHODS

Physical measurements

Infrared spectra were measured in CCl_4 with a Beckman IR-20 double-beam spectrophotometer. 100-MHz NMR spectra were recorded for dilute solutions in [^{2}H]-chloroform with a Varian HA-100 NMR spectrometer equipped with a ^{31}P–^{1}H spin decoupler. Optical rotations were measured in chloroform solution at 22°C in a Perkin-Elmer polarimeter, model 141, with digital readout.

Chromatography of lipids

Thin-layer chromatography was carried out on silica gel H (plain) layers spread on 20 × 20 cm glass plates (silica thickness: 0.25 mm for analytical plates, 1 mm for preparative plates). The coated plates were washed once by ascending chromatography in chloroform–methanol 1:1 (v/v), air-dried, and activated at 110°C for 12 hr. For preparative chromatography, the freshly activated plates were streaked with a solution of the lipids (ca. 40 mg/ml in chloroform–methanol 95:5 [v/v] using a TLC sample streaker from Applied Science Laboratories, State College, Pa.) and developed twice in chloroform–

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Abbreviations: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGS, phosphatidylglycerosulfate; SL, glycolipid sulfate; Me_2-PGS, phosphatidylglycerosulfate dimethyl ester.

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methanol–concentrated NH_4OH 65:35:5 (v/v/v). The separated lipids were eluted from the silica with chloroform–methanol–diethyl ether 1:1:1 (v/v/v) after visualization with rhodamine 6G under ultraviolet light (366 nm).

Lipids on analytical plates were detected by spraying the plates with 40% H_2SO_4 followed by charring, or by spraying with the Dittmer and Lester phosphate-detecting reagent (5). Lipids were also chromatographed on silicic acid-impregnated Whatman 3MM paper (3) in diisobutylketone–acetic acid–water 40:25:5 (v/v/v).

Chromatography of water-soluble hydrolysis products

Water-soluble products were examined by ascending chromatography on Whatman no. 1 paper in: (*a*) *n*-butanol–acetic acid–water 5:3:1 (v/v/v); (*b*) saturated phenol–water (107 g of phenol in 38 ml of water); and (*c*) ethanol–1 M ammonium acetate 70:30 (v/v). The developed chromatograms were stained with the sulfosalicylic acid–ferric chloride reagent (6) for phosphate esters and the sodium periodate–tolidine reagent (7) for vicinal hydroxyl-containing components.

Analytical methods

Phosphorus was determined by the method of Allen (8) or by the micromethod of Bartlett (9). Glycerol was analyzed (10) after drastic hydrolysis of lipid samples by the procedure of Renkonen (11). For determination of ester sulfate, the compound was first solvolyzed by the method of Goren (12), as described below, and the liberated inorganic sulfate was assayed by the barium chloranilate procedure of Spencer (13).

Cation analysis was carried out by flame photometry using a Pye Unicam atomic absorption spectrophotometer as follows. A solution of 1.0–1.5 μmoles of lipid in 2 ml of chloroform was diluted with methanol (2 ml) and 1.8 ml of 0.5 N HCl. After centrifugation of the mixture, the aqueous phase containing the cations was removed, and the chloroform phase was washed three times with 2-ml portions of methanol–water 10:9. The combined aqueous phases were diluted to 10 ml with methanol–water 10:9, a 2-ml aliquot was taken to dryness under a stream of nitrogen, and the residue was dried in vacuo for 1 hr to ensure removal of all chloroform which interferes with the flame photometric measurements. A solution of the residue in 2 ml of methanol–water 10:9 was analyzed directly by flame photometry, using standard solutions of potassium chloride (0.05–0.2 mM) and sodium chloride (0.1–0.6 mM) in methanol–water 10:9 for calibration.

Cell culture conditions and lipid extraction

Cells of *H. cutirubrum* were grown aerobically at 37°C in 1.5-l batches of the standard complex medium for halophiles, in 4-l shake flasks, as described previously (2, 14, 15). The cells were harvested, after 4 days growth, by centrifugation at 7000 *g* for 20 min and washed twice with 4 M NaCl solution by centrifugation, and lipids were extracted by the method of Bligh and Dyer (16), modified as described elsewhere (2, 17). The total lipids were fractionated by acetone precipitation to remove the pigments and neutral lipids (14, 15). The tan-colored precipitate of polar lipids was washed several times with small portions of cold acetone, dried in vacuo, and dissolved in chloroform to a known concentration.

TABLE 1. Chromatographic mobilities of polar lipids of *H. cutirubrum*

Lipid Component	R_F Value in Solvent System: 1[a]	2[a]	3[a]	4[b]
Glycolipid sulfate	0.46	0.15	0.13	0.22
Phosphatidylglycerophosphate	0.70	0.58	0.23	0.55
Phosphatidylglycerosulfate	0.62	0.38	0.37	0.43
Phosphatidylglycerol	0.67	0.68	0.66	0.68

[a] TLC on silica gel H in solvent systems: 1, chloroform–methanol–water 65:35:5 (v/v/v); 2, chloroform–methanol–90% acetic acid 30:4:20 (v/v/v); 3, chloroform–methanol–concd ammonium hydroxide 65:35:5 (v/v/v).

[b] Chromatography on silicic acid-impregnated paper in solvent system 4, diisobutylketone–acetic acid–water 40:25:5 (v/v/v).

EXPERIMENTAL

Isolation of phosphatidylglycerosulfate

The total polar lipids were fractionated by preparative TLC essentially as described previously (18) but modified as follows: 480 mg of polar lipid was fractionated on 12 preparative TLC plates by double development in chloroform–methanol–concentrated ammonium hydroxide, as described above. The bands corresponding to PGP (R_F 0.38), PGS (R_F 0.52), PG (R_F 0.78), and SL (R_F 0.24) were eluted with chloroform–methanol–diethyl ether 1:1:1 (v/v/v), using 300 ml of solvent for each component per 12 plates. Each extract was diluted with benzene and brought to dryness under reduced pressure; a solution of the residue in about 5 ml of chloroform–benzene 1:1 (v/v) was centrifuged free from silica and concentrated to dryness in a nitrogen stream. The PGP, PGS, PG, and SL obtained were finally purified by preparative TLC in the same solvent system followed by repeated acetone precipitation to remove any rhodamine. The four major components were recovered in the following yields (percentage by weight of total polar lipids): PGP, 63%; SL, 23%; PG, 4%; and PGS, 3–4% (total recovery was 93 ± 2%). The TLC R_F values of the lipids in various solvents are given in Table 1.

TABLE 2. Specific rotations[a] of various salt forms of bacterial phosphatidylglycerosulfate

Salt Form	Concentration	589 nm	578 nm	546 nm	436 nm	365 nm
	g/dl					
Mixed salt[b]	1.42	−1.41°	−0.35°	+0.14°	+0.70°	+0.99°
Na_2 salt	0.98	−1.75°	−1.63°	−0.75°	−1.63°	−3.75°
K_2 salt	1.60	+2.02°	+2.14°	+2.29°	+4.14°	+6.29°
$(NH_4)_2$ salt	1.03	+1.55°	+1.65°	+2.14°	+3.50°	+5.15°
Mg salt	1.21	+0.58°	+0.66°	+1.00°	+1.67°	+2.50°

[a] In chloroform solution at 22°C.
[b] Mixture of Na, NH_4, and Mg salts, in decreasing proportions, obtained by preparative TLC in chloroform–methanol–concd NH_4OH 65:35:5 (v/v/v).

The PGS obtained was a chromatographically pure colorless amorphous powder consisting of Na, NH_4, and Mg salts. The specific rotations of the PGS salt mixture at various wavelengths are given in Table 2, and analytical data are presented in Table 3.

Preparation of salts of PGS

The potassium salt was prepared as follows. A solution of 45 mg (49 μmoles) of the purified mixed salt of PGS in 10 ml of chloroform–methanol 1:1 (v/v) was diluted with 4.5 ml of 0.5 N aqueous HCl. The biphasic system was briefly centrifuged, and the chloroform phase was washed with methanol–water 10:9 (v/v) and brought to dryness in a stream of nitrogen. The residual PGS free acid was dried in vacuo (40.0 mg; 90 μeq acid) and titrated in chloroform solution with 0.1 N methanolic KOH to the phenophthalein end point (87.6 μeq base consumed). The solution was diluted with benzene, concentrated to a small volume in a nitrogen stream, cleared by centrifugation, and diluted with 10 vol of acetone. After several hours at 0°C, the precipitate was centrifuged down, dissolved in a minimum volume of chloroform, reprecipitated with cold acetone, and finally dried in vacuo; the yield of potassium salt (white hygroscopic powder) was 38 mg (85% based on free acid). Analytical data for the potassium salt of PGS are given in Table 3, and its infrared spectrum in CCl_4 is shown in Fig. 1.

Ammonium and sodium salts were prepared from the "natural" salt mixture as described for the potassium salt, except that the acid form of PGS was neutralized with 0.2 N methanolic ammonium or sodium hydroxide, respectively. Elemental analyses of these salts were not performed because of their limited supply; they were chromatographically pure on TLC, and gave the expected values for phosphorus content (found: 3.15% P and 3.22% P; calculated: 3.29% P and 3.26% P for monohydrated diammonium and disodium salts, respectively).

The magnesium salt was prepared by treating the acid form of PGS (ca. 15 mg) in 2 ml of chloroform with 2 ml of 10% methanolic magnesium acetate solution, followed by 1.8 ml of water. The biphasic system was centrifuged, and the chloroform phase was washed with methanol–water 10:9 (v/v) and brought to dryness in the presence of benzene. The magnesium salt of PGS was purified by acetone precipitation as described for the potassium salt; it had 3.33% P (calculated for the anhydrous mono-Mg salt, 3.40% P).

The optical rotations of the various salt forms are given in Table 2.

Dimethyl ester of PGS (Me_2-PGS)

Bacterial PGS (potassium salt, 8 mg) was converted to the free acid form as described above, and the chloro-

TABLE 3. Analytical data for bacterial phosphatidylglycerosulfate

	Mixed Salt		K Salt		K Salt (Hydrate)	
	Found	Calcd	Found	Calcd[a]	Found	Calcd[b]
C, %	57.54		57.72	57.34	56.24	56.29
H, %	10.20		10.08	9.73	9.51	9.76
P, %	3.40		3.35	3.22	3.06	3.16
S, %	3.70		3.45	3.33		
K, %			8.84	8.12		
S/P, atomic ratio	1.05	1.00	1.00	1.00		
K/P+S, atomic ratio			1.05	1.00		
Diether:P:glycerol, mole ratio			1.0:1.0:0.97	1.0:1.0:1.0		
Equivalent weight			457 ± 10	443.6		

[a] Calculated for $C_{46}H_{93}O_{11}PSK_2$ (963.45); analytical sample dried in vacuo at 65°C.
[b] Calculated for $C_{46}H_{93}O_{11}PSK_2 \cdot H_2O$ (981.47); analytical samples dried in vacuo at room temperature.

form solution was treated with ethereal diazomethane until a permanent yellow color was obtained. The solution was evaporated in a nitrogen stream in the presence of benzene, and the oily residue was dried in vacuo. The infrared (Fig. 1*B*) and NMR (Fig. 2*A*) spectra of the dimethyl ester were recorded with minimum delay. The dimethyl ester of PGS, like the methyl esters of PG and PGP (19), was unstable in solution and decomposed within several hours to diphytanyl glycerol ether, among other decomposition products. The Me_2-PGS had R_F 0.68 in chloroform–methanol–water 90:10:1 (v/v/v).

Degradative procedures

Drastic hydrolysis of PGS (10–20 mg; 325–650 μg of phosphorus) was effected by heating the compound in 0.9 ml of 2 N aqueous HCl for 24 hr at 120°C in a sealed tube. The hydrolysis mixture was diluted with 1.0 ml each of methanol and chloroform, and chloroform-soluble and aqueous methanol-soluble hydrolysis products were then examined chromatographically (see above). Samples of PGS were also hydrolyzed under reflux conditions for 3 hr in (*a*) 4.5 ml of 0.8 N NaOH in 90% methanol or (*b*) 4.5 ml of 2.5% methanolic HCl. The hydrolyzates were diluted with water and chloroform to give biphasic systems (16), and the products were analyzed by chromatography as described above.

Identification of phosphatidylglycerol after solvolytic desulfation of PGS

PGS (potassium salt, 16.5 mg, 16.8 μmoles) was desulfated by solvolysis (12) in 5 ml of 0.005 N HCl in anhydrous tetrahydrofuran at room temperature (Fig. 3). After 1 hr, the solution was neutralized with 0.2 N methanolic ammonium hydroxide and brought to dryness in a nitrogen stream. A solution of the residue in 10 ml of chloroform–methanol 1:1 (v/v) was diluted with 4.5 ml of 0.1 N aqueous HCl, and the biphasic system was centrifuged. The chloroform phase was washed three times with 1 ml of methanol–water 10:9 (v/v) and then neutralized with 0.2 N methanolic NaOH. The sodium salt of PG (14.0 mg, 16.5 μmoles, 98%) was precipitated by acetone from its concentrated solution in chloroform and was characterized by its chromatographic mobility (R_F 0.68 in chloroform–methanol–90% acetic acid 30:4:20), its positive reaction with periodate–Schiff reagent, and its optical rotation. Found: $[\alpha]_D^{22} = +3.15°$ (1.40 g/dl in chloroform); reported (18) for the sodium salt of bacterial PG (*sn*-1-phosphatidyl-*sn*-3′-glycerol): $[\alpha]_D^{22} = +3.43°$ (2.36 g/dl in chloroform).

Analysis: $C_{46}H_{94}O_8PNa \cdot H_2O$ (847.6);
calculated: C, 65.30; H, 11.71; P, 3.66
found: C, 65.31; H, 11.52; P, 3.48

The methanol–water phase plus washings from the Bligh-Dyer (16) partition was analyzed for sulfate ion; calculated release of sulfate ion, 16.8 μmoles; found, 16.0 μmoles (97% recovery of sulfate).

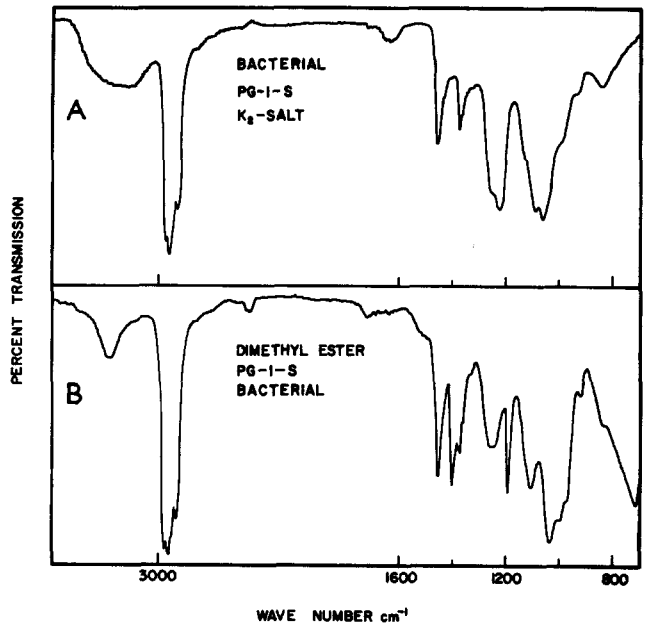

FIG. 1. Infrared spectra in CCl_4 of bacterial phosphatidylglycerosulfate: *A*, dipotassium salt; *B*, dimethyl ester.

Synthesis of reference compounds PG mono- and disulfates

The synthesis and characterization of mono- and disulfates of phosphatidylglycerol (diphytanyl ether analog) and their corresponding methyl esters are described in detail in the accompanying paper (20).

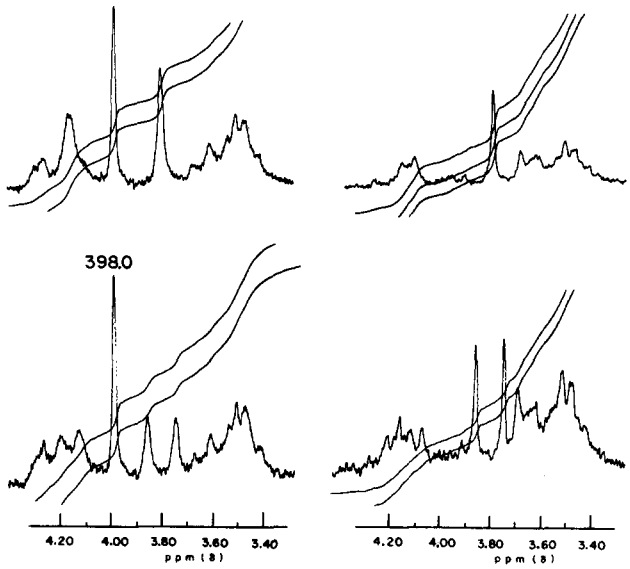

FIG. 2. NMR spectra in [^{2}H]chloroform of *left*, bacterial phosphatidylglycerosulfate (dimethyl ester) and *right*, bacterial phosphatidylglycerol (monomethyl ester). Top spectra are ^{31}P–^1H decoupled; bottom spectra are ^{31}P–^1H coupled.

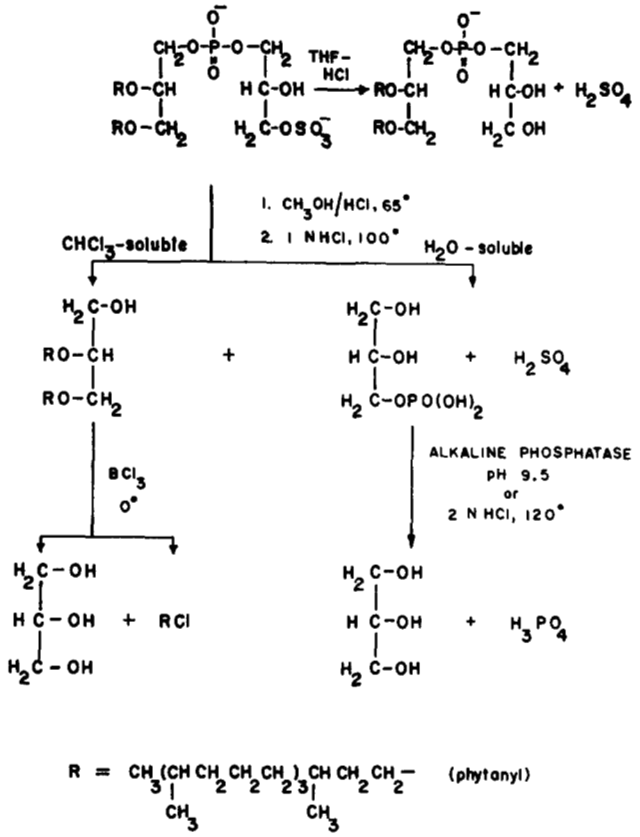

FIG. 3. Scheme for hydrolytic degradation of phosphatidylglycerosulfate.

Monomethyl ester of bacterial phosphatidylglycerol (Me-α-PG)

The ester was prepared by direct methylation of the free acid form of PG (10 mg) as described for PGS. The product slowly decomposed to give phytanyl diether (among other products) on standing at 5°C both in chloroform solution and in the dry state. $[\alpha]_D^{22}$ = +4.12° (1.02 g/dl in chloroform).

Analysis: $C_{47}H_{97}O_8P$ (821.2); calculated: P, 3.77
found: P, 3.89

The NMR spectrum of Me-PG is shown in Fig. 2B.

Synthesis of 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-2′-*sn*-glycerol (β-PG)

A solution of 2,3-di-*O*-phytanyl-*sn*-glycerophosphoric acid (21) (18 mg, 25 μmoles) and 1,3-di-*O*-benzylglycerol (22) (20 mg, 73 μmoles) in 5 ml of anhydrous pyridine was stirred at room temperature for 24 hr in the presence of triisopropylbenzenesulfonyl chloride (35 mg, 116 μmoles). The reaction mixture was cooled on ice, stirred with 0.5 ml of water for 30 min, and evaporated to dryness under reduced pressure. The residual oil was triturated with cold ethyl ether, and the precipitated triisopropylbenzenesulfonic acid was removed by centrifugation and washed twice with ethyl ether. The combined ethereal supernates were washed with water, 0.5 N hydrochloric acid, and again with water and were brought to dryness under reduced pressure after addition of benzene to aid in removal of water.

TLC of the residual oil revealed two phosphate-positive spots (R_F 0.26 and 0.92 in chloroform–methanol–water 90:10:1 (v/v/v). No phosphatidic acid (R_F 0.0 in the above solvent) was detected. The two phosphorus-containing products were separated by preparative TLC in chloroform–methanol–water 90:10:1 (v/v/v); they were isolated as ammonium salts. The less polar product proved to be the pyrophosphate dimer of phosphatidic acid and was not further studied. The more polar product, dibenzyl-β-PG (18 mg, as ammonium salt), was hydrogenolyzed in 5 ml of ethanol–chloroform (9:1, v/v) at room temperature and pressure over palladium–charcoal catalyst for 1 hr. The debenzylated product (10 mg) was converted to the sodium salt and precipitated with acetone as described above. The yield of Na-β-PG was 8 mg (38% based on phosphatidic acid); $[\alpha]_D^{22}$ = +1.37° (0.8 g/dl in chloroform).

Analysis: $C_{46}H_{94}O_8PNa \cdot H_2O$ (847.6);
calculated: P, 3.66
found: P, 3.60

The β-PG was periodate–Schiff negative and had chromatographic mobilities appreciably higher than the α isomer: R_F 0.63 and 0.73 for the β isomer and 0.54 and 0.68 for the α isomer in chloroform–methanol–concentrated NH_4OH 65:35:5 and chloroform–methanol–90% acetic acid 30:4:20, respectively. The β-PG was readily isomerized to a mixture of α and β isomers by incubation for 15 min at 37°C in anhydrous 0.025 N HCl in chloroform–methanol 1:1.

Synthesis of 1,3-di-*O*-phytanylglycerol (1,3-glycerol diether)

A stirred mixture of 2-*O*-benzyl-3-*O*-phytanyl-*sn*-glycerol[3] (22 mg, 5 μmoles) in 10 ml of anhydrous benzene was refluxed for 16 hr with DDD-phytanyl bromide (17) (25 mg, 7 μmoles) in the presence of 2.0 g of powdered KOH. The water formed was removed in a Soxhlet apparatus containing calcium carbide. The cooled reaction mixture was diluted with 20 ml of ice water and neutralized with 10% H_2SO_4. The aqueous phase was extracted with three 10-ml portions of benzene, and the combined benzene extracts were evaporated under reduced pressure. The residual colorless oil showed one major spot on TLC (R_F 0.45 in benzene), corresponding to the 2-*O*-benzyl diphytanyl glycerol ether. The prod-

[3] This compound was prepared from 3-*O*-phytanyl-*sn*-glycerol (17) via the 1-*O*-trityl intermediate (Kates, M., and A. J. Hancock. Manuscript in preparation).

uct was hydrogenolyzed in 5 ml of chloroform–methanol 1:1 (v/v) using 50 mg of palladium–charcoal catalyst at room temperature and pressure for 30 min. The catalyst was removed by centrifugation and washed with chloroform–methanol 1:1 (v/v). Evaporation of the combined supernates gave an oil (36 mg) that yielded on preparative TLC in chloroform–ethyl ether 9:1 (v/v) 18 mg (52%) of chromatographically pure 1,3-diphytanyl glycerol ether. The 1,3 and 2,3 isomeric diethers were only marginally separated (R_F 0.70 and 0.65, respectively) on TLC in chloroform–ethyl ether 9:1 (v/v) but were well resolved in ethyl acetate–carbon tetrachloride 1:5 (v/v) (R_F 0.78 and 0.62, respectively). The 1,3 isomer had $[\alpha]_D^{22} = +1.1°$ (1.2 g/dl in chloroform) compared with $[\alpha]_D^{22} = +8.4°$ (17) for the 2,3 isomer.

Analysis: $C_{43}H_{88}O_3$ (653.13);
calculated: C, 79.07; H, 13.58
found: C, 79.37; H, 13.47

RESULTS AND DISCUSSION

The PGS isolated by preparative TLC as described in the Methods section was chromatographically pure and had analytical data (Table 3) consistent with the molecular formula $C_{46}H_{95}O_{11}PS$ for PGS free acid; the presence of both P and S in the molecule, in the atomic ratio 1:1, is thus established. After drastic acid hydrolysis of PGS, diphytanyl glycerol ether, inorganic phosphate, and glycerol were liberated in a mole ratio close to 1:1:1 (Table 3), indicating that the lipid was most likely a derivative of the diphytanyl glycerol ether analog of phosphatidylglycerol (analysis for sulfate was performed in subsequent experiments; see below). The free acid form of PGS consumed two equivalents of KOH, suggesting the presence of two acidic groups per molecule, and the elemental analytical data of the pure potassium salt obtained were consistent with those calculated for the monohydrated dipotassium salt (Table 3).

Methanolysis of the phosphosulfolipid in 2.5% methanolic HCl under reflux for 3 hr (Fig. 3) yielded a chloroform-soluble, phosphorus-free product accounting for 66% of the lipid weight. This product was identified as 2,3-di-*O*-(3′*R*,7′*R*,11′*R*,15′-tetramethylhexadecyl)-*sn*-glycerol by its chromatographic behavior (R_F 0.65 in chloroform–ether 9:1, v/v), its optical rotation ($[\alpha]_D^{22} = +8.4°$ [17]), and the identification of phytanyl chloride and glycerol after boron trichloride cleavage (3). The same diether was also liberated by alkaline hydrolysis of PGS in 0.8 N methanolic NaOH. The isomeric diether, 1,3-di-*O*-phytanyl-*sn*-glycerol, was shown to have different chromatographic mobility and optical rotation from the bacterial 2,3-diether. The water-soluble fraction of the hydrolyzate (Fig. 3) contained an organic phosphate that accounted for all the lipid phosphorus and was identified as α-glycerophosphate (4). These results indicated that the unknown lipid was a derivative of phosphatidylglycerol, and the relatively lower chromatographic mobility of the unknown compound (Table 1) suggested the presence of an additional polar group, probably a sulfate group.

The infrared spectrum of the potassium salt (Fig. 1*A*) was very similar to that of the diphytanyl ether analog of phosphatidylglycerol (18), showing a strong OH band (3300 cm^{-1}), —CH_2 and —CH_3 bands (2950, 2920, 2860, and 1455 cm^{-1}), isopropyl absorption bands (1365–1375 cm^{-1}), and phosphate absorption bands (1260–1210, 1090, and 1060 cm^{-1}). Absorption bands for sulfate S=O groups could not be detected because of overlap with the broad, strong P=O band at 1260–1210 cm^{-1}, but an *S*—*O*—C band at 840 cm^{-1} was present. Ester or carboxyl carbonyl bands were absent.

The presence of a sulfate group was revealed more clearly by the infrared spectrum of the dimethyl ester of PGS (Fig. 1*B*). Sharp sulfate ester absorption bands (—O—SO_2—O—) at 1198 and 1408 cm^{-1} and a weak absorption band (*S*—*O*—C) at 845 cm^{-1} were evident; little or no secondary sulfate absorption at 935 cm^{-1} (*C*—*O*—S) was apparent.

The 100-MHz NMR spectrum (Fig. 2*A*) of the dimethyl ester of PGS showed three methoxy (—OCH_3) signals (3.74, 3.86, 3.98δ), two of which (3.74, 3.86δ, J_{PH} = 11.8 Hz) collapsed to a single peak (3.80δ) when ^{31}P–^1H spin decoupled, which allowed their assignment to P—OCH_3 groups (19). The third signal (3.98δ) remained unchanged during spin decoupling and was assigned to S—OCH_3 groups; integration of the P—OCH_3 and S—OCH_3 signals indicated the presence of one P—OCH_3 and one S—OCH_3 group per molecule of phosphosulfolipid. These signal assignments were supported by the fact that the NMR spectrum of the monomethyl ester of PG (Fig. 2*B*) also showed ^{31}P–^1H spin decoupling to a single peak (3.79δ); no signal at 3.98δ was apparent.

Direct chemical evidence for the presence of a sulfate ester was obtained by subjecting the potassium salt of the phosphosulfolipid to mild acid-catalyzed solvolysis in anhydrous tetrahydrofuran–HCl (0.005 M) at room temperature (Fig. 3). In accord with the reported acid lability of sulfate esters in the presence of oxonium ion-forming anhydrous solvents (12, 23, 24), the phosphosulfolipid (K salt) was quantitatively desulfated within a few minutes. The rate of desulfation was dependent on the nature of the cation form of PGS, the "natural" salt mixture (Mg, Na, NH_4 salts) requiring longer times (up to 2 hr) than the potassium salt for complete desulfation. The acid-catalyzed solvolysis was fully inhibited in the presence of water (ca. 10%) but not in the presence of methanol (ca. 10%). The lipid product of solvolysis was identical with the bacterial α-PG (2,3-di-*O*-phytanyl-

TABLE 4. NMR chemical shifts of P—OCH_3 and S—OCH_3 groups in methyl esters of phosphatidylglycerol and phosphatidylglycerosulfates

Compound[a]	Chemical Shift (δ ppm)			
	^{31}P–^{1}H		^{31}P–^{1}H	
	Coupled	Uncoupled	Coupled	Uncoupled
Phosphatidylglycerol (bacterial)	3.73, 3.84	3.79		
Phosphatidylglycero-1-sulfate (bacterial)	3.74, 3.86	3.80	3.98	3.98
Phosphatidylglycero-1-sulfate (synthetic)[b]	3.75, 3.86	3.80	3.98	3.98
Phosphatidylglycero-2-sulfate (synthetic)[b]	3.75, 3.86	3.81	4.01	4.02
Phosphatidylglycero-1,2-disulfate[b]	3.76, 3.87	3.81	4.01, 4.04	4.01, 4.04

[a] All compounds are derivatives of 2,3-di-*O*-phytanyl-*sn*-glycerol.
[b] Synthesized as described in accompanying paper (20).

sn-glycero-1-phosphoryl-3′-*sn*-glycerol) with respect to chromatographic mobility, positive reaction with periodate–Schiff reagent, analytical data, specific rotation, and infrared spectrum (18), and not with the β-PG isomer synthesized here. Since the solvolysis mechanism involves retention of configuration (24), these results show that the phosphosulfolipid is a sulfate ester of 1-*sn*-phosphatidyl-3′-*sn*-glycerol. However, the position of the ester sulfate group still remained to be determined.

Final confirmation of a PG sulfate ester structure was obtained by direct monosulfation of the bacterial diether PG with one equivalent of SO_3–pyridine complex at room temperature (see accompanying paper [20]). The monosulfated product obtained (K salt) was identical with bacterial PGS with respect to chromatographic mobility, analytical data, optical rotation, and infrared spectra of the potassium salt and methyl ester (20); the NMR spectra of the bacterial and synthetic PGS methyl esters were also identical (Table 4).

TABLE 5. Sulfate absorption bands in infrared spectra of methyl esters of phosphatidylglycerolsulfates

Methyl Ester[b]	Absorption[a]		
	νS=O	νC—OS	νCO—S
	cm^{-1}		
Phosphatidylglycero-1-sulfate (bacterial)	1195 (S), 1405 (S)	980 (SH)	840 (W)
Phosphatidylglycero-1-sulfate (synthetic)[c]	1195 (S), 1405 (S)	980 (SH)	840 (W)
Phosphatidylglycero-2-sulfate (synthetic)[c]	1198 (S), 1408 (S)	935 (S)	840 (W)
Phosphatidylglycero-1,2-disulfate[c]	1200 (S), 1415 (S)	940 (S) 995 (SH)	840 (W)

[a] Symmetric S=O stretching modes are obscured by C—O—C and P—O—C absorptions. Abbreviations: S, strong; W, weak; SH, shoulder.
[b] All compounds are derivatives of 2,3-di-*O*-phytanyl-*sn*-glycerol.
[c] Synthesized as described in accompanying paper (20).

Although sulfation at position 1 most likely predominated, the possibility remained that sulfation of bacterial PG gave a mixture of 1- and 2-monosulfates. Indeed, the NMR spectrum of the methyl ester of the synthetic monosulfated PG obtained (20) shows, in addition to the major S—OCH_3 signal at 3.98δ, a minor signal at 4.01δ which is attributable to the —OCH_3 group of a secondary sulfate. Evidence for this discrimination between primary and secondary sulfate —OCH_3 signals was afforded by the NMR spectrum (Table 4) of the trimethyl ester of PG-1,2-disulfate, which was synthesized by sulfation of bacterial PG or PGS with excess SO_3–pyridine complex at 60°C (20). The NMR spectrum showed two sharp S—OCH_3 signals (4.01, 4.04δ) that were not ^{31}P coupled (Table 4), showing that signals from glycerol primary and secondary sulfate —OCH_3 groups differ in chemical shift by 3 Hz.

To establish the assignments for primary and secondary sulfate —OCH_3 signals unambiguously, the NMR spectrum of the methyl ester of the authentic 2-sulfate of PG, synthesized as described in the accompanying paper (20), was examined and found to have an S—OCH_3 signal at 4.01δ (Table 4). Therefore, the S—OCH_3 signal at 4.01δ could now be assigned to the secondary sulfate —OCH_3 group and that at 3.98δ to the primary sulfate group (Table 4). Furthermore, the infrared spectrum of the methyl ester of PG-2-S (20) had a prominent *C—O*—S absorption band at 935 cm^{-1} (Table 5), whereas the bacterial PGS or the synthetic PG monosulfate methyl esters showed only weak absorption in this region but had a pronounced shoulder at 980 cm^{-1} (Fig. 1*B* and Table 5). According to Haines (24), the *C—O*—S stretching vibration mode for primary sulfates is generally around 990 cm^{-1}, whereas that for secondary sulfates is characteristically at 935 cm^{-1}. In accordance with this, the spectrum of the PG-1,2-disulfate showed a *C—O*—S band at both 935 and 1000 cm^{-1} (Table 5). The bacterial PGS clearly must contain a primary sulfate ester.

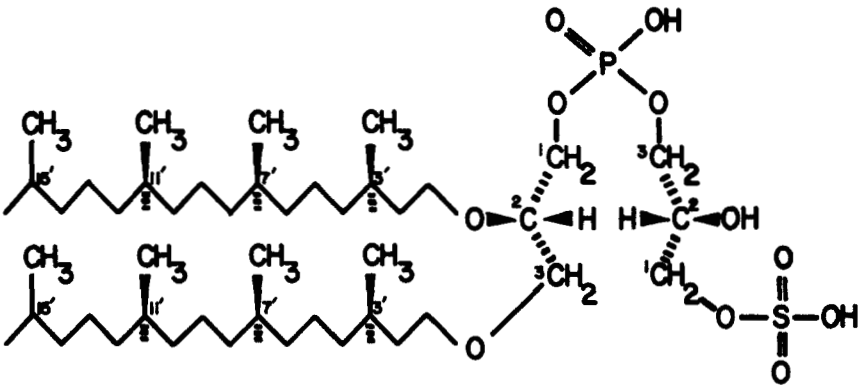

FIG. 4. Stereochemical structure of the phosphosulfolipid in *H. cutirubrum*.

These results thus unambiguously establish the structure of the bacterial phosphosulfolipid as 2,3-di-*O*-(3′*R*,7′*R*,11′*R*,15′-tetramethylhexadecyl)-*sn*-glycero-1-phosphoryl-3′′-*sn*-glycero-1′′-sulfate (Fig. 4).

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