

(S)-2-Amino-1,3-propanediol-3-phosphate-carrying diradylglyceroglycolipids Novel major membrane lipids of *Clostridium innocuum*

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(Received March 2, 1994) – EJB 94 0305/5

Two novel aminophosphoglycolipids (I, II) were isolated from *Clostridium innocuum* which constitute 51% (I) and 15% (II) of total polar membrane lipids. The structures, established by quantitative and methylation analyses, fast-atom-bombardment mass spectrometry, and one- and two-dimensional NMR spectroscopy, are (I) *S*-2-amino-1,3-propanediol-3-phospho-6- α -D-galactopyranosyl(1-2) α -D-glucopyranosyl(1-3)diradylglycerol and (II) an acylated derivative of (I) that carries an additional fatty acid ester on O6 of the glucosyl moiety. The stereochemical configuration of the 2-amino-1,3-propanediol 3-phosphate residue was elucidated by conversion to *N*-acetylserine 3-phosphate, with subsequent release and identification of L-serine by HPLC. In addition to diacylglycerol species, both aminophosphoglycolipids contain 15–32% 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol species in which C₁₄, C₁₆, and C₁₈ vinyl ether are combined predominantly with unsaturated C₁₆ and C₁₈ fatty acid ester. Hydrogenation of the vinyl ether was required to desorb the alkyl, acyl-substituted species in fast-atom-bombardment mass spectrometry. Hydrogenation made it further possible to release the alkyl glycerols by acid hydrolysis and to locate the ether bond at O1 of the glycerol moiety.

In contrast to the glycerophosphoglycolipids of other Gram-positive bacteria, the aminophosphoglycolipids are metabolically not related to the lipoteichoic acid of *C. innocuum* and serve, therefore, exclusively as major membrane components. Their large abundance among membrane lipids suggests bilayer-forming physicochemical properties.

Recently a unique membrane lipid pattern was discovered in *Clostridium innocuum* [1]. It consists of glycosyldiradylglycerols, phospholipids, and aminophosphoglycolipids. Several novel lipids have been found, including phosphatidylglycerol acetals of bisphosphatidylglycerol plasmalogen and of phosphatidylglycerol plasmalogen. Structurally remarkable and quantitatively important are two major and two minor aminophosphoglycolipids which represent 51%, 15%, 4% and 1%, respectively, of total polar lipids [1]. We report here on the structure of the two major aminophosphoglycolipids which contain 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol species in addition to diacylglycerol species, and carry as so far unique (*S*)-2-amino-1,3-propanediol 3-phosphate substituent at a dihexosyl diradylglycerol moiety which is derived from the respective membrane glycolipid.

Functionally, these zwitterionic aminophosphoglycolipids seem to be counterparts of aminophospholipids rather than related to the glycerophosphoglycolipids of other Gram-positive bacteria which are minor membrane components and serve as intermediates in lipoteichoic acid biosynthesis [2,

3]. In view of *Clostridium* being a deep (ancient) major phylogenetic unit [4], unusual, possibly ancestral, lipid components are not unexpected.

MATERIALS AND METHODS

Materials

cis-9-Hexadecenal, *cis*-13-octadecenal, fatty acid standards, 1-amino-2,3-propanediol, 1-*O*-dodecyl-*rac*-glycerol, 1-*O*-hexadecyl-*sn*-glycerol, and 1-*O*-octadecyl-*rac*-glycerol were purchased from Sigma Aldrich Vertriebs GmbH. The double bonds of the fatty aldehydes were hydrogenated with PtO₂ as a catalyst and converted into dimethylacetals by treatment with 0.5 M methanolic HCl at 80°C overnight. Bacterial reference glycolipids were available from previous preparations [5]. DEAE-cellulose was purchased from Merck, DEAE-Sephadex A-25 from Pharmacia Biotech, Iatrobeads 6RS-8060 (silica gel) from Iatron Laboratories Inc. (Tokyo, Japan).

Growth of bacteria and extraction and purification of lipids

Clostridium innocuum ATCC 14501 was grown and harvested as described [1]. Bacteria were suspended in 0.1 M sodium acetate pH 4.7 (2.5 ml/g wet mass), disintegrated with glass beads in a Braun disintegrator [6] and extracted

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Abbreviations. COSY, correlation spectroscopy; FAB, fast atom bombardment.

Enzyme. Orthophosphoric-monoester phosphohydrolase (EC 3.1.3.1).

by a modified Bligh-Dyer procedure [7, 8]. The crude lipid extract was fractionated by column chromatography on DEAE-cellulose, acetate form [7]. Hexosylidradylglycerol and dihexosylidradylglycerol were eluted with $\text{CHCl}_3/\text{MeOH}$ 95:5 and 4:1 (by vol.), respectively, most of phosphoglycolipid I with $\text{CHCl}_3/\text{MeOH}$ (2:1, by vol.) and almost pure phosphoglycolipid II with methanol. Final purification was achieved by column chromatography on silica gel (Iatrobeds) from which phosphoglycolipid II and I eluted with $\text{CHCl}_3/\text{MeOH}$ 2.5:1 and 1.5:1 (by vol.), respectively.

Analytical procedures

Carbohydrate, D-galactose, D-glucose, glycerol and phosphorus were determined by standard procedures (for references, see [9, 10]). Fatty acids were identified and quantified as described in [9, 10].

TLC was performed on silica gel plates (Merck 60) with the following solvents: (A) $\text{CHCl}_3/\text{MeOH}$ (3.5:1, by vol.); (B) $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (65:25:4, by vol.); (C) $\text{CHCl}_3/\text{MeOH}/\text{AcOH}/\text{H}_2\text{O}$ (80:12:18:5, by vol.); (D) $\text{CHCl}_3/\text{MeOH}/13.5 \text{ M ammonia}$ (10:10:1, by vol.); (E) propane-1-ol/13.5 M ammonia/ H_2O (6:3:1, by vol.); (F) phenol/ $\text{H}_2\text{O}/\text{AcOH}/\text{EtOH}$ (75:33:4.5:4.5, mass/vol./vol./vol.). Lipids [11], carbohydrate [12], lipid phosphorus [13], non-lipid phosphorus [14], and vicinal hydroxyls [15] were visualized on the plates as described in the respective references.

GLC was performed essentially as described in [9]. For most analyses an HP 5 fused silica-gel capillary column (25 m, inner diameter 0.25 mm, film thickness 0.25 μm) was used. Mixtures of fatty acid methyl esters and alkane dimethyl acetals were separated at 135–230°C with a temperature rise of 6°C/min, alkane dimethyl acetals at 195–250°C with a temperature rise of 2°C/min, aminopropanediol acetates at 150–220°C with a temperature rise of 2°C/min, tris(trimethylsilyl)-2-acetamido-1,3-propanediol phosphate and tris(trimethylsilyl)-2-acetamidoserine 3-phosphate at 135–220°C with a temperature rise of 6°C/min, methylated *O*-alkylglycerols and *O*-alkylethylene glycols at 200–270°C with a temperature rise of 10°C/min. GLC/MS analyses were performed on a Hewlett Packard 5890 A gas chromatograph connected with a mass spectrometer MS-D 5970. The temperature of the transfer line and ions source was 250°C, the ionization potential 70 eV.

Hydrogenation [16]

Phosphoglycolipids were dissolved in $\text{CHCl}_3/\text{MeOH}/0.05 \text{ M KH}_2\text{PO}_4$ (0.5:1.0:0.4, by vol.) in screw-capped tubes and stirred with PtO_2 and H_2 (atmospheric pressure) at room temperature for 1 h. Then, Pt was removed by centrifugation and, after phase separation, the hydrogenated lipids were recovered from the CHCl_3 layer.

N-Acetylation

N-Acetylation of phosphoglycolipids (5–10 μmol) was performed in $\text{CHCl}_3/\text{MeOH}$ (1:1, by vol., 1 ml) with $(\text{C}^2\text{H}_5\text{CO})_2\text{O}$ (100 μl) and pyridine (20 μl), for 15 min at room temperature. 0.15 M NaCl/MeOH (1:1, by vol., 1 ml) was added and the mixture allowed to stand at room temperature for 1 h. After phase separation, the CHCl_3 layer was washed several times with 0.15 M NaCl/MeOH (1:1, by vol.). By preparative TLC in solvent B, *O*-acetylated faster-

moving byproducts were separated from *N*-acetylated phosphoglycolipids.

Peracetylation

Phosphoglycolipid I (5 μmol) was dissolved with sonication in CHCl_3 (1 ml). Pyridine (0.2 ml) and $(\text{CH}_3\text{CO})_2\text{O}$ (0.4 ml) were added at 0°C and the mixture was allowed to stand at 7°C for 48 h with an intermediate addition of $(\text{CH}_3\text{CO})_2\text{O}$ (0.1 ml) after 24 h. Then water (2 ml) was added at 0°C, the resulting CHCl_3 layer washed with water and taken to dryness with several additions of CCl_4 . When peracetylation was carried out at 37°C, a peracetylated phospho-ester derivative was formed which was separated from the peracetylated faster-moving phosphoglycolipid by preparative TLC in solvent A.

Deacylation

Deacylation was performed as described [17] with the exception that cation-exchange resin, NH_4^+ , was used.

Dephosphorylation

Phosphoglycolipids were dephosphorylated in 48% (by mass) HF at 2°C for 36 h. The solvent was removed *in vacuo* over KOH at 2°C.

Oxidation with CrO_3 and methylation analysis

Both procedures were carried out essentially as in previous work [9].

Analysis of the 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol species

For quantitative determination of the diacylglycerol and alk-1-enyl,acyl-glycerol species, the vinyl ether bonds were split by mild acid treatment (90%, by vol., acetic acid, 37°C, 18 h) [18]. The hydrolysate was taken to dryness with several additions of CCl_4 , dissolved in $\text{CHCl}_3/\text{MeOH}$ (4:1, by vol.) and applied to a small column (0.5×3 cm) of silica gel (Iatrobeds). Aldehydes were eluted with the same solvent (2 ml), diacylglycerol and monoacylglycerol with methanol (3 ml). Diacylglycerol and monoacylglycerol were separated by preparative TLC (solvent C), scraped from the plate, hydrolyzed with 2 M HCl, 100°C, 2.5 h, and analyzed for phosphorus and carbohydrate. In a second experiment, diacylglycerol and monoacyl glycerol were eluted from silica gel with $\text{CHCl}_3/\text{MeOH}$ (1:1, by vol.) and analyzed for fatty acid composition.

For the location of the vinyl ether bond at the glycerol moiety, hydrogenated phosphoglycolipids (4 μmol) were hydrolyzed in 1 M HCl, 100°C, 3 h. NaOH was added in excess and the alkylglycerols were extracted with CHCl_3 . One sample was methylated directly. Another sample was treated with methanol/ $\text{CHCl}_3/0.05 \text{ M NaIO}_4$ (1:0.5:0.4, by vol.) at 37°C for 18 h. Periodate was reduced with ethylene glycol and the products reduced with NaBH_4 (3 h). After addition of acetone (50 μl), the products were extracted with CHCl_3 , methylated, and analyzed along with the original material by GLC/MS.

Oxidation with NaIO_4 and β -elimination

Phosphoglycolipid I and II (10 μmol phosphorus) were *N*-acetylated, deacylated (see above), and then oxidized with

0.05 M NaIO₄ (1.6 ml), containing 0.01 M sodium acetate, pH 4.2, at 5°C in the dark. Periodate consumption was followed spectrophotometrically [19]. At timed intervals, samples were withdrawn for the determination of formaldehyde [20] and formic acid [21]. After incubation for 24 h, excess periodate was reduced with ethylene glycol and NaIO₃-precipitated on ice with ethanol (1.6 ml). The supernatant was concentrated to remove ethanol. Then the pH was adjusted to 10.5 with ammonium carbonate and the mixture allowed to stand at 37°C for 24 h. β -Elimination was controlled by determination of inorganic phosphorus before and after treatment with alkaline phosphatase (3.5 U/ml). The reaction mixture was diluted with water (25 ml) and applied to a column (1 cm \times 8 cm) of DEAE-Sephadex A-25, which had been equilibrated with 0.3 M triethylammonium carbonate pH 8.5 and washed before use with water. Elution was performed with a linear gradient of 0–0.3 M triethylammonium carbonate pH 8.5 (150 ml each), and monitored by phosphorus determination. One phosphorus-containing peak eluted at a buffer concentration of 0.09 M. The phosphorus-containing fractions were combined and taken to dryness by several evaporations with water.

Conversion of 2-acetamido-1,3-propanediol phosphate to serine

The 2-acetamido-1,3-propanediol phosphate (2 μ mol), released from phosphoglycolipid I and isolated as described above, was treated with a mixture of 1 M KOH (0.2 ml) and 40 mM KMnO₄ (0.2 ml) at room temperature for 3 h. The reaction was terminated by addition of H₂O₂, and MnO₂ was removed by centrifugation. The supernatant was passed through a small column (0.5 \times 3 cm) of cation-exchange resin, H⁺. The effluent was neutralized with ammonia, rotary evaporated and dephosphorylated with 40% (by mass) HF as described above. After drying the sample *in vacuo* over KOH, the *N*-acetyl bond was split by hydrolysis with 2 M HCl, 100°C, 3 h. The mixture was then dried *in vacuo* and analyzed for amino compounds by TLC and HPLC as described under Results.

Stereochemical analysis of serine

Amino acids (0.5–0.8 nmol) were incubated for 2.5 min at room temperature in 10 μ l 1 M lithium borate pH 9.3 and 5 μ l reagent (10 mg *o*-phthalaldehyde, 10 mg *N*-acetyl-L-cysteine, 1 ml methanol). After addition of 15 μ l 1 M KH₂PO₄, the mixture (20 μ l) was analyzed on a LiChrospher RP-18e column (5 μ m, 4 \times 250 mm, Merck) by elution with a linear gradient (4 min) from buffer A (4%, by vol., tetrahydrofuran, 3%, by vol., acetonitrile in 25 mM sodium phosphate pH 6.8) to 30% (by vol.) buffer B (30%, by vol., acetonitrile in 25 mM sodium phosphate pH 6.8) at a flow rate of 1.5 ml/min. Amino acid derivatives were detected by fluorescence (excitation at 365 nm, emission at > 420 nm).

Fast-atom-bombardment mass spectrometry

Fast-atom-bombardment mass spectrometry (FAB-MS) was performed on a ZAB-HF mass spectrometer (VG Analytical, Manchester, UK) essentially as described in [22]. Native, *N*-acetylated, peracetylated, and hydrogenated phosphoglycolipids were dissolved in CHCl₃/MeOH (2:1, by vol.) and desorbed from the matrix thioglycerol or triethanolamine with xenon as bombarding gas. The mass spectra were ac-

quired as single scans in the upscan mode on a AMD DP10 data system, fitted with SAMII (KWS) hardware and SUSY software (AMD Intectra, Beckeln, Germany). Mass values were obtained after calibration with CsI and represent nominal mass numbers.

NMR spectroscopy

NMR spectra were determined with a Bruker AMX 500 spectrometer and standard acquisition software. Spectra were recorded at 25°C. Phosphoglycolipid I (6 μ mol) was dissolved in C²HCl₃/C²H₃O²H (1:1, by vol., 0.5 ml), phosphoglycolipid II (5 μ mol) in the same solvent, containing 6.5% (by vol.) ²H₂O. Deacylated phosphoglycolipid I (6 μ mol) was dissolved in C²H₃O²H (0.5 ml).

One-dimensional ¹H-NMR spectra (500.13 MHz) were recorded in normal acquisition mode or with ³¹P-decoupling using globally optimized alternating-phase rectangular pulses (GARP) composite pulse decoupling sequence [23]. One-dimensional ¹³C spectra (125.77 MHz) and ³¹P spectra (202.46 MHz) were recorded with proton decoupling. ¹H and ¹³C shifts were measured from tetramethylsilane, ³¹P shifts from 85% H₃PO₄ as external standard.

¹H-¹H correlation spectroscopy (COSY) experiments were recorded with ³¹P-decoupling using a 2K block size in *f*₂ and 512 increments, each with 64 scans in *f*₁, followed by sine-squared multiplication without phase shifting, zero filling of the 2K \times 2K matrix and finally matrix symmetrization. The ¹H/¹³C-heteronuclear-multiple-quantum coherence spectra (HMQC) were recorded using a pulse sequence with bilinear-rotation-decoupling pulse (BIRD) to suppress signals from protons connected to ¹³C [24]. A block size of 2K in *f*₂ and 1024 increments, each with 32 scans in *f*₁ were used in time-proportional-phase-increment mode, followed by zero filling of the 2K \times 2K matrix, processing with a $\pi/2$ phase-shifted sine filter and phase correction in *f*₂ and *f*₁.

RESULTS

Isolation and compositional analysis

Aminophosphoglycolipids I and II represent 51% and 15% of polar membrane lipids and are more abundant than bisphosphatidylglycerol (cardiolipin), the major phospholipid, which constitutes 16% [1]. The two phosphoglycolipids were isolated and purified by successive column chromatography on DEAE-cellulose and silica gel (see Materials and Methods). Both compounds were identified by their staining properties on TLC as aminophosphoglycolipids. The *R_F* values of phosphoglycolipid I and II in solvent C increased, after *N*-acetylation, from 0.11 and 0.26 to 0.17 and 0.34, respectively. On deacylation, both phosphoglycolipids yielded identical products: a major and a minor one (10%) with chromatographic mobilities on TLC relative to α -D-Glcp(1-2)- α -D-Glcp(1-3)glycerol of 1.28 and 2.22 in solvent E, and of 0.42 and 1.34 in solvent F. Both deacylation products stained positively for carbohydrate, amino groups and phosphorus. On phase partition between water and butanol, the minor less polar compound separated into the butanol layer and was identified by FAB-MS as alk-1-enyl ether-containing lysoderivative (see below).

Acid hydrolysis (2 M HCl, 100°C, 2.5 h) and quantification of the released fatty acids suggested that phosphoglyco-

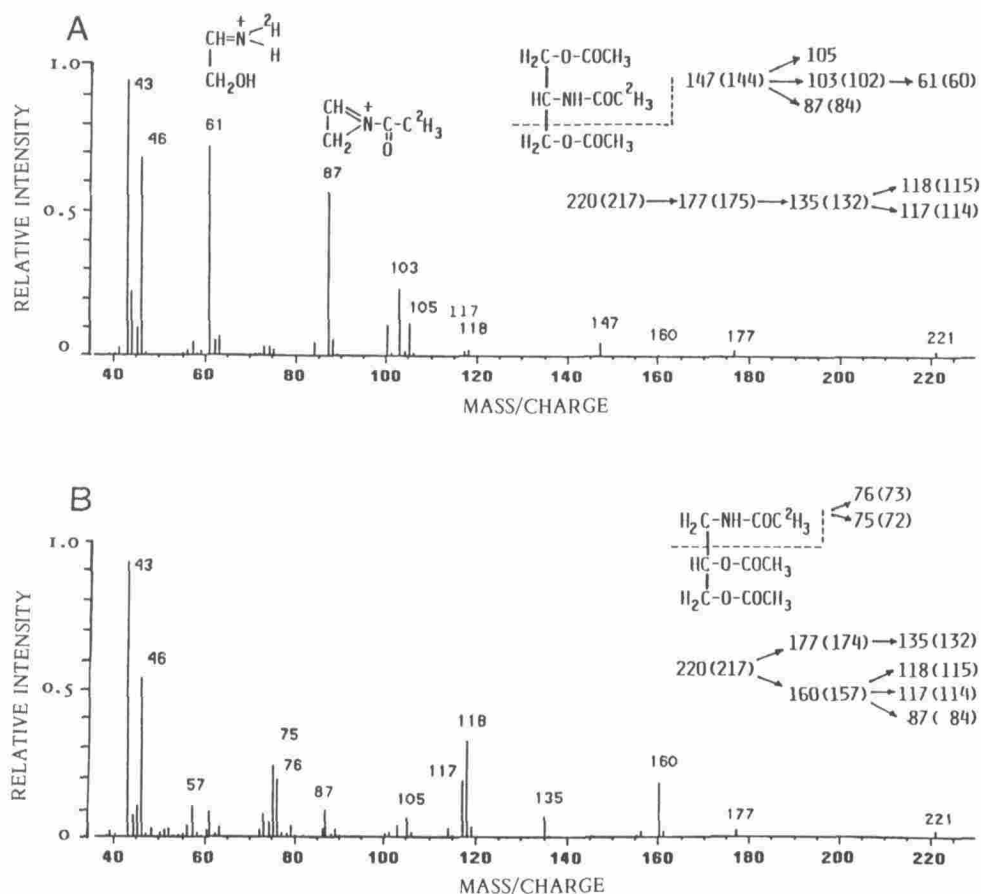


Fig. 1. Electron impact mass spectrum (A) of the peracetylated amino alcohol released from phosphoglycolipid I and II, and (B) of peracetylated authentic 1-amino-2,3-propanediol. Both compounds were first *N*-acetylated with $(\text{C}^2\text{H}_5\text{CO})_2\text{O}$, then *O*-acetylated with $(\text{CH}_3\text{CO})_2\text{O}$. Values in brackets are from compounds peracetylated with $(\text{CH}_3\text{CO})_2\text{O}$.

Table 1. Composition of phosphoglycolipid I and II. D-Galactose phosphate was measured in the HCl hydrolysate as D-galactose after treatment with phosphomonoesterase.

Phosphoglycolipid	Molar ratio to phosphorus			
	D-glucose	D-galactose phosphate	glycerol	fatty acids
I	0.91	0.88	0.89	1.7
II	0.97	0.95	0.99	2.5

lipid I and II differ in the presence of two and three hydrocarbon chains, respectively (Table 1). The water-soluble hydrolysis products were identical: D-glucose, D-galactose phosphate and glycerol in approximately equimolar ratios to total phosphorus (Table 1). The amino-group-containing constituent was purified by passage of the hydrolysis products through a small column (0.5×3 cm) of cation-exchange resin, H^+ , and elution with 1 M HCl. It had a mobility on TLC relative to 1-amino-2,3-propanediol of 2.6 (solvent D) and, after peracetylation, appeared earlier on GLC with a relative retention time of 0.91. As shown in Fig. 1, GLC/MS analysis identified the amino alcohol from both phosphoglycolipids as 2-amino-1,3-propanediol by a fragmentation

pattern, clearly distinct from that of the 1-amino isomer (Fig. 1).

The release by acid hydrolysis of 2-amino-1,3-propanediol and a phosphate ester of galactose suggests that the former is attached by a phosphodiester bond to the galactosyl residue of the glycolipid moiety. Accordingly, dephosphorylation with 48% (by mass) HF released aminopropanediol, inorganic phosphate and the core glycolipid. The phosphodiester bond resisted strong alkaline treatment (0.5 M NaOH, 100°C, 18 h), as was shown with the deacylation product by unchanged mobility on TLC.

Fatty acid ester and alk-1-enyl ether

As shown in Table 2, both phosphoglycolipids had a similar pattern of unbranched saturated and monoenoic fatty acids. In phosphoglycolipid II, tetradecanoate and hexadecanoate were enriched at the cost of unsaturated and saturated C_{18} fatty acids, which suggests that the additional carbohydrate-linked fatty acid of phosphoglycolipid II was predominantly composed of saturated shorter-chain species.

After treatment of phosphoglycolipid I and II with methanolic HCl, GLC revealed three components in addition to fatty acid methyl ester. Two of them had the retention time of hexadecanal dimethylacetal and octadecanal dimethylacetal, respectively. GLC/MS confirmed the identity by a base peak at m/z 75 and (M-31) ions at m/z 255 and 283, respectively.

Table 2. Fatty acid composition of phosphoglycolipid I and II (A) and of their diacylglycerol (B) and alk-1-enyl-acyl-glycerol species (C). Fatty acids were released with methanolic KOH as methyl esters [17] and analyzed by GLC. An asterisk indicates double bond not located.

Fatty acid	Amount in					
	phosphoglycolipid I			phosphoglycolipid II		
	A	B	C	A	B	C
	mol/100 mol					
Dodecanoate	2.6	2.9	2.9	2.8	1.9	2.4
Tetradecanoate*	1.6	1.1	3.3	1.6	1.0	2.0
Tetradecanoate	8.1	9.5	6.3	15.7	14.4	14.8
Hexadecanoate*	4.8	3.9	11.7	4.6	3.4	8.5
<i>cis</i> -9-Hexadecenoate	13.6	12.5	18.3	12.0	12.2	13.8
Hexadecanoate	23.3	27.0	8.9	35.1	37.7	31.4
<i>cis</i> -9-Octadecenoate	25.4	21.4	38.8	13.4	13.1	17.4
<i>cis</i> -11-Octadecenoate	7.0	6.4	7.8	5.1	5.6	5.0
Octadecanoate	13.6	15.3	2.9	9.2	10.7	4.7

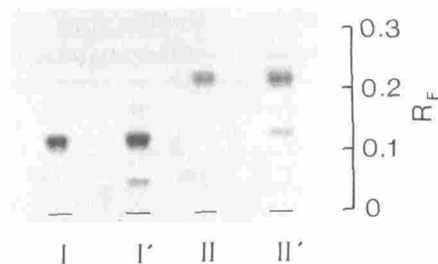


Fig. 2. Phosphoglycolipids, native (I, II) and after treatment with 90% (by vol.) acetic acid (I', II'). TLC in solvent C; visualization with 1-naphthol/H₂SO₄ [12]. R_F, mobility, relative to the front.

The third minor component was characterized as tetradecanal dimethylacetal by peaks at m/z 75 and 227 (M-31). The molar ratio of C₁₄, C₁₆, and C₁₈ aldehydes was 0.1:1.25:1 in phosphoglycolipid I, and 0.3:2.2:1 in phosphoglycolipid II.

The same aldehydes were released from both phosphoglycolipids by mild acid treatment (90%, by vol., acetic acid, 37°C, 18 h) [18]. The resistant diacylglycerol species and the resulting lysocompound were separated by TLC (Fig. 2), quantified and analyzed for their ester-linked fatty acid composition. The alk-1-enyl acyl-glycerol species, measured as lyso compound, was 15–28% in phosphoglycolipid I and 32% in phosphoglycolipid II. As shown in Table 2, the diacylglycerol species of phosphoglycolipid I contained 45% unsaturated fatty acids, the lyso compound 80%, which indicates that the alk-1-enyl ether residues were predominantly combined with unsaturated fatty acids. The accumulation of unsaturated fatty acids was less pronounced in the lyso compound of phosphoglycolipid II, but here the situation may be obscured by the additional carbohydrate-linked predominantly saturated fatty acids.

The location of the vinyl ether bond at the glycerol moiety

By hydrogenation, the vinyl ether bonds of the native phosphoglycolipids were converted into more stable ether

bonds. The alkyl glycerol moieties were released by acid hydrolysis (1 M HCl, 100°C, 3 h) and extracted with CHCl₃. After methylation, GLC revealed three components with retention times relative to authentic 1-*O*-octadecyl-2,3-di-*O*-methylglycerol of 0.34, 0.68, and 1.00, in relative abundances of 0.16, 1.05, and 1.0, respectively. The latter two compounds were identified by GLC/MS as 1-*O*-hexadecyl-2,3-di-*O*-methylglycerol and 1-*O*-octadecyl-2,3-di-*O*-methylglycerol by the aid of the fragments at m/z 312, 340 (M-32), 298, 326 (M-46), 253, 281 (M-91), and 89 [M-CH₃(CH₂)_nOCH₂] (Fig. 3). The fragment at m/z 89 with the intense daughter ions at m/z 58/59 is indicative of an ether bond at O1 of glycerol [25]. Accordingly, the alkyl-glycerols were oxidizable with periodate and the products were, after reduction with NaBH₄ and methylation, identified by GLC/MS as 1-*O*-hexadecyl-2-*O*-methyl- and 1-*O*-octadecyl-2-*O*-methylethylene glycol. Characteristic fragments were observed at m/z 255, 283 (M-45), 224, 252 (M-76) and m/z 77 (M-C_nH_{2n-1}) (Fig. 3).

Linkage analysis of the deacylated glycolipid moiety

Deacylation [17] followed by dephosphorylation with HF [9] produced the same glycoside from both phosphoglycolipids. On TLC (solvent E), it showed the same mobility as α -D-Galp(1-2)- α -D-Glc(1-3)glycerol and reacted rapidly with the periodate Schiff reagent, indicative of a terminal glycol group [15]. Methylation analysis yielded approximately equimolar amounts of 2,3,4,6-tetra-*O*-methylgalactitol and 3,4,6-tri-*O*-methylglucitol. After acetylation, the glycosides were resistant to treatment with CrO₃, which indicates α -glycosidic bonds [26, 27].

The proposed structure is therefore α -D-Galp(1-2)- α -D-Glcp(1-3)Gro, considering that diacylglycerol moieties are usually derived from *sn*-3-phosphatidic acid [28] or the *sn*-1,2-di-*O*-acylglycerol moiety of phosphatidylglycerol [29].

Location and stereochemical configuration of the 1(3)-phospho-2-amino-propane-1,3-diol residue

A sample of phosphoglycolipid I was deacylated, *N*-acetylated, and then subjected to periodate oxidation. Under controlled conditions, which avoid β -elimination [30], 4 mol periodate/mol phosphorus was consumed with the concomitant formation of approximately 1 mol formic acid and 1 mol formaldehyde. These results are consistent with a (1-2)-interlinked dihexopyranosyl residue, attached to O3 of the glycerol moiety. Taken together with the formation of galactose phosphate on acid hydrolysis (Table 1), these results locate the 2-amino-1,3-propanediol phosphate residue at O6 of the galactopyranosyl residue. The location at O6 was confirmed by β -elimination (pH 10.5), which converted 77% of the phosphorus into phosphomonoester. Chromatography on DEAE-Sephadex yielded a single phosphorus-containing peak which proved uniform on TLC (solvent E) and had a mobility of 1.28 relative to glycerol-1(3)-phosphate. It was identified by GLC/MS as tris(trimethylsilyl)-2-acetamido-1,2-propanediol 1(3)-phosphate. The mass spectrum and fragmentation pattern is shown in Fig. 4.

For stereochemical analysis, the 2-acetamido-1,3-propanediol phosphate was oxidized with alkaline KMnO₄ and the mixture worked up as described under Materials and Methods. The products were trimethylsilylated and analyzed by GLC/MS. Among them tris(trimethylsilyl)-*N*-acetyl-ser-

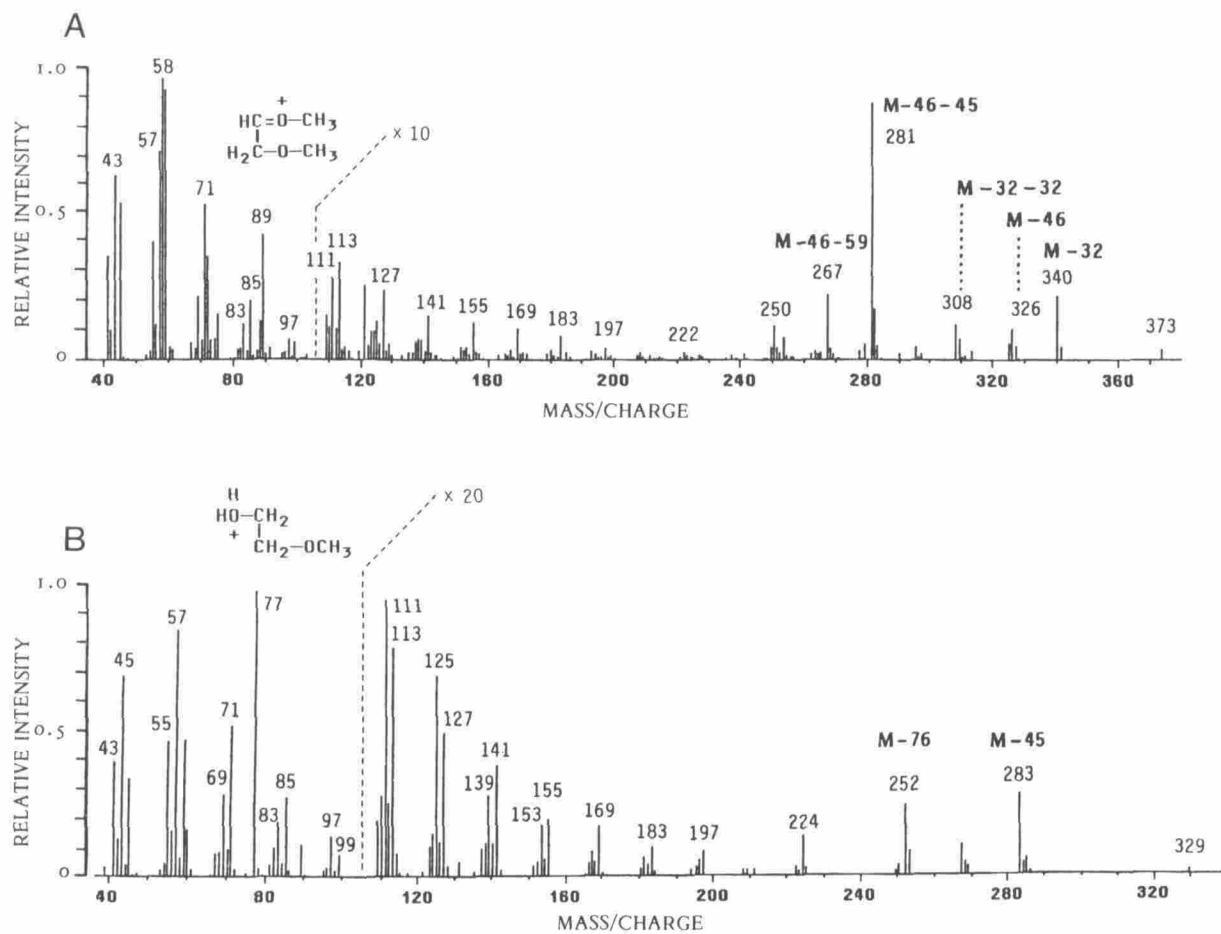


Fig. 3. Electron impact mass spectrum of (A) 1-*O*-octadecyl-2,3-di-*O*-methylglycerol and (B) 1-*O*-octadecyl-2-*O*-methylethylene glycol.

ine 3-phosphate was identified, as shown in Fig. 4. Two less polar byproducts with relative retention times of 0.23 and 0.52 were identified as tris(trimethylsilyl) phosphoric acid [M^+ , m/z 314; (M-15), m/z 399] and tris(trimethylsilyl)-2-hydroxy-2-phospho acetic acid [M^+ , m/z 372; (M-15), m/z 357]. Dephosphorylation of *N*-acetyl-serine 3-phosphate with 48% (by mass) HF, followed by hydrolysis with 2 M HCl (100°C, 2 h), yielded a single ninhydrin-positive compound with the mobility of serine on TLC (solvent D). The *L*-configuration was established by HPLC using precolumn derivatization with *o*-phthalaldehyde and *N*-acetyl-*L*-cysteine (Fig. 5). The substituent of the glycolipid moiety of phosphoglycolipid I has therefore the absolute configuration (*S*)-2-amino-1,3-propanediol 3-phosphate or 2-amino-2-deoxy-*sn*-glycerol 3-phosphate. It was also identified as the substituent of phosphoglycolipid II, using the same sequence of reactions (data not shown).

Summarizing the results of chemical analysis, the proposed structure of phosphoglycolipid I is (*S*)-2-amino-1,3-propanediol 3-phospho-6- α -D-Galp(1-2)- α -D-Glcp(1-3)-1,2-di-*O*-radylglycerol with 15–30% 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol species in addition to di-*O*-acylglycerol species. Phosphoglycolipid II is an acyl derivative of phosphoglycolipid I with similar diradylglycerol species. The location of the third fatty acid on the disaccharide moiety was established by FAB-MS and NMR analysis (see below).

FAB mass spectrometry

Native phosphoglycolipid I

The negative-ion FAB-MS of native phosphoglycolipid I (Fig. 6) is consistent with the proposed structure. The molecular [$M-H^+$] $^-$ ions between m/z 988 and m/z 1098 differ from each other by 28 or 26 Da and reflect various combinations of the constituent fatty acids on the glycerol moiety (Table 3). The addition of the matrix to the double bond of the fatty acids is documented by the ions [$M-H^++108$] $^-$ at m/z 1122, 1150, 1178, and 1206, respectively. The constituent saturated and monounsaturated fatty acids were detectable from their respective carboxylate-type fragment ions of very low abundance at m/z 199 (C_{12}), 227, 225 (C_{14}), 255, 253 (C_{16}), and 283, 281 (C_{18}). The 2-amino-1,3-propanediol 3-phosphate substituent was documented by the abundant primary fragment ion at m/z 170, and located on the non-reducing terminal sugar unit of the disaccharide moiety by the sequence ions at m/z 332 (170+162) and 492 (170+162+162-2). They were accompanied by ions being 42 Da higher at m/z 212 (170+42), 374 (332+42), and 534 (492+42). The increment of 42 Da cannot be attributed to an additional structural element because such mass shifts were not observed in the molecular ion area of the native, *N*-acetylated and peracetylated compound either in negative or positive FAB-MS (Table 3, Fig. 6). Ring cleavage of the

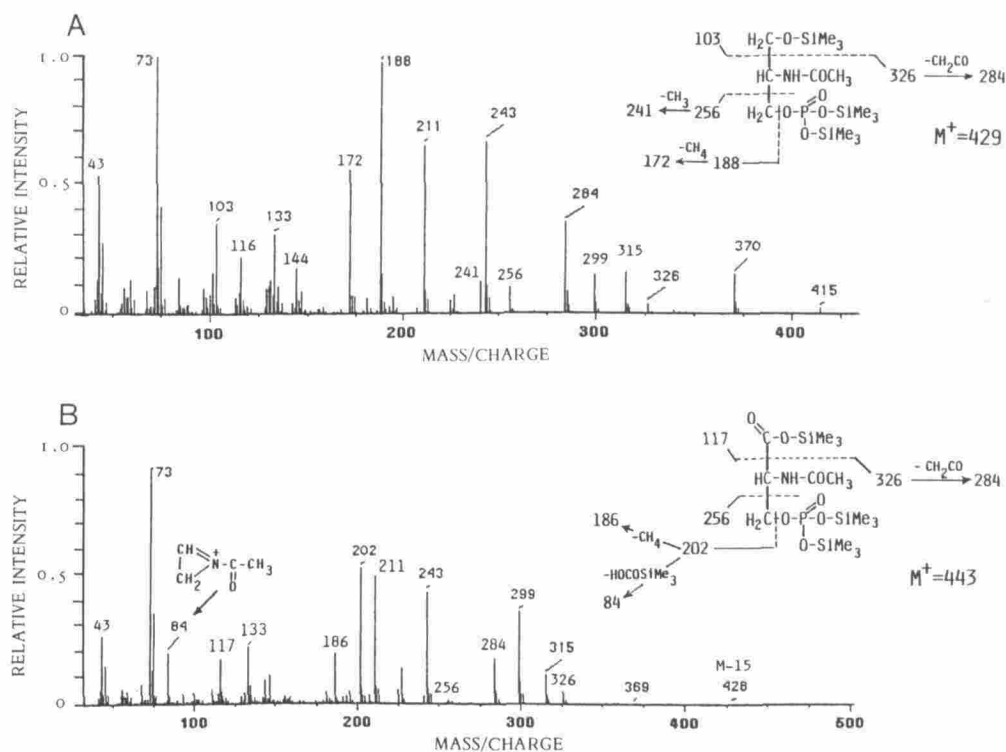


Fig. 4. Electron impact mass spectrum of (A) tris(trimethylsilyl)-2-acetamido-1,3-propanediol phosphate, and (B) tris(trimethylsilyl)-*N*-acetyl-serine phosphate. The ions at m/z 211, 243, 299, and 315 are indicative of phosphate groups [31, 32].

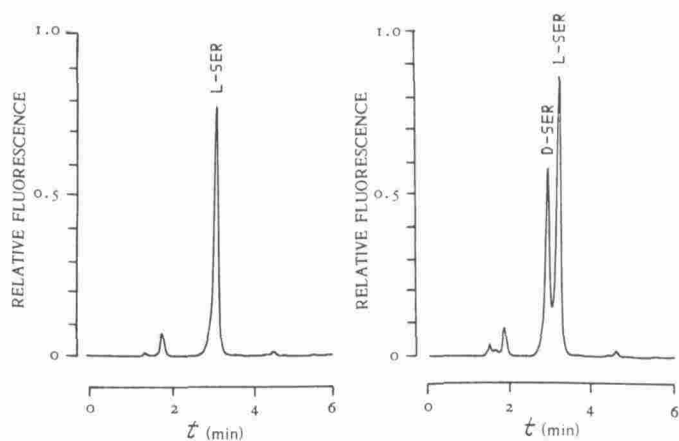
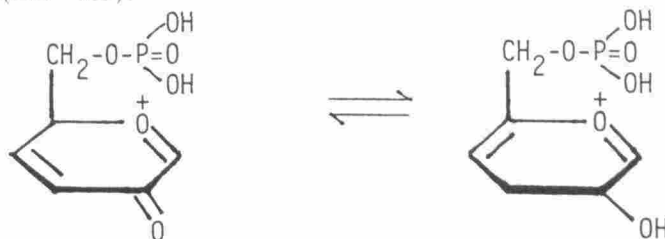


Fig. 5. Identification by HPLC of the amino acid derived from 2-amino-propanediol phosphate. Chromatogram of the amino acid (left) and in admixture with *D*-serine (right). Retention times of *D*- and *L*-serine were 2.9 and 3.2 min, respectively. For procedure see Materials and Methods.

sugar moieties could explain the ions at m/z 212 and 374 but is not compatible with the ion at m/z 534 nor does it conform to the absence of the shifted ions from the mass spectrum of the deacylated derivative (Fig. 7). Further studies are therefore required to elucidate the mechanism by which these ions are formed.

The identification of the aminopropanediol phosphate-substituted disaccharide moiety could be tracked also in the positive-ion MS (not shown) by the help of the abundant primary fragment ion at m/z 172 and the abundant oxonium fragment ions at m/z 316 ($170+146$) and 478 ($170+146+162$). Of diagnostic importance for the phosphate substitu-

tion is apparently the daughter ion observed at m/z 207 ($316-109$):



This fragment ion was present in the positive-ion FAB-MS of the native, *N*-acetylated and peracetylated phosphoglycerolipid (not shown).

N-Acetylated phosphoglycerolipid I

In the negative-ion FAB-MS of phosphoglycerolipid I after *N*-acetylation with $(\text{C}^3\text{H}_7)_3\text{CO}$, the molecular ions (Table 3) and the fragment ions of the aminopropanediol phosphate-substituted disaccharide moiety at m/z 215, $377\rightarrow 359$, $377\rightarrow 419$, and $537\rightarrow 579$ were shifted by 45 Da (spectrum not shown). Fragment ions, not found in the negative-ion FAB-MS of native phosphoglycerolipid I, were observed at m/z 941, 969, 997, which arose by cleavage between the *N*-acetylated side chain and the phosphate group ($M-119$).

Hydrogenated phosphoglycerolipid I

A series of molecular ions which can be attributed to the 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol species of phosphoglycerolipid I were desorbed in negative-ion and positive-ion FAB-MS only after hydrogenation of the double bonds (spectrum not shown). The molecular $[\text{M}-\text{H}]^-$ ions at m/z 1030, 1058,

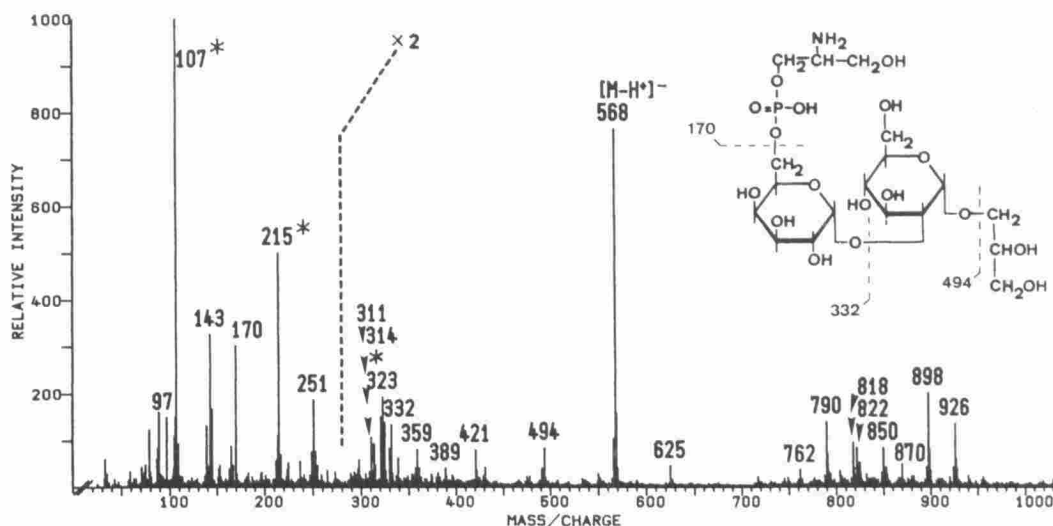


Fig. 7. Structure and negative-ion mass spectrum of deacylated phosphoglycolipid I. Matrix-derived thioglycerol and thioglycerol oligomers are marked by an asterisk.

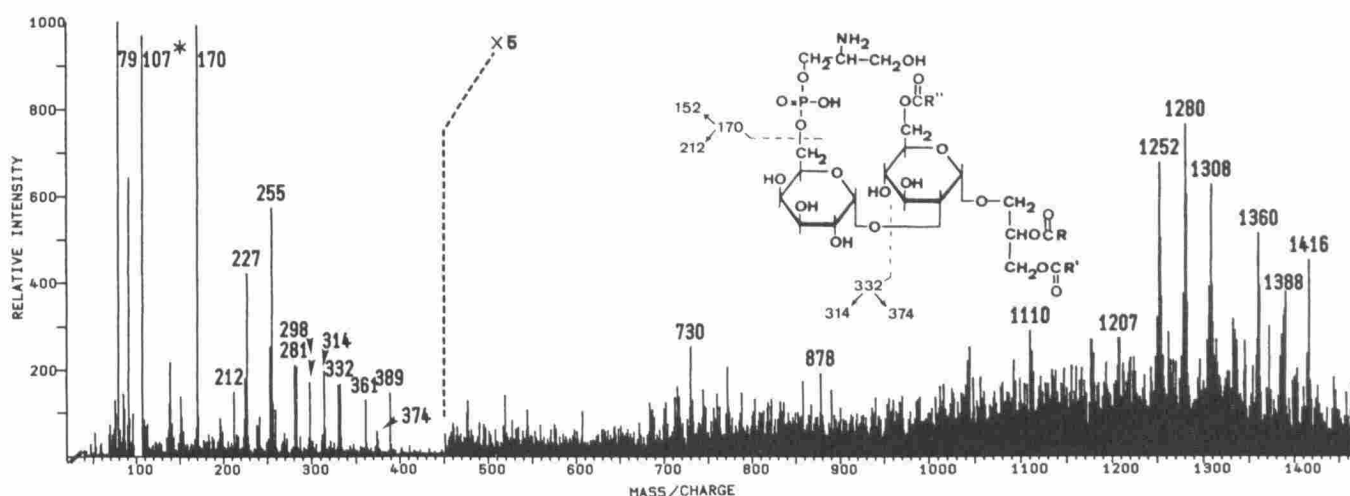


Fig. 8. Structure and negative-ion mass spectrum of native phosphoglycolipid II. For molecular ions and constituent fatty acids, see Table 3. Matrix-derived thioglycerol and thioglycerol oligomers are marked by an asterisk. For discussion of the ions at m/z 212 and 374, see text.

dual bonds in the direction to the diacylglycerol moiety. Cleavage of the aminopropanediol phosphate-bond yielded the fragment ion at m/z 158 and the complementary ions of the glycolipid phosphomonoester at m/z 1245, 1273, and 1301 ($M-158+2H^++22$). Another series of fragment ions at m/z 851, 877, 905 and 933 characterizes the glucosyldiacylglycerol moieties ($M-526+2H^++22$), and a third series at m/z 495, 523, 549, 577, and 605 corresponds to the protonated diacylglycerol species formed by elimination of the aminopropanediol phosphate-containing disaccharide moiety ($M-830$).

Native phosphoglycolipid II

Phosphoglycolipid II was investigated as the native compound by negative-ion FAB-MS (Fig. 8). The similarity to phosphoglycolipid I is evident from the aminopropanediol phosphate fragment at m/z 170 and the aminopropanediol phosphate-substituted hexosyl fragment at m/z 332→314 and 332→374. The fatty acid composition was also similar, as

indicated by the carboxylate ions at m/z 225, 227, 253, 255, 281, 283. The molecular $[M-H]^-$ ions at m/z 1224, 1252, 1280, and 1308 were by 238(236) or 210 Da higher than those of phosphoglycolipid I, suggesting palmitate (palmitoleate) or myristate as and additional carbohydrate-linked fatty acids (Table 3). The presence of the aminopropanediol phosphate-substituted hexosyl fragment at m/z 332 (see above), together with the absence of the respective disaccharide fragment, points to a location of the third fatty acid at the glycerol-linked glucosyl residue, which is confirmed by the acylated aminopropanediol phosphate-substituted disaccharide fragment at m/z 730 ($494-2+238$).

NMR spectroscopy

The results of NMR analyses for phosphoglycolipid I and II and deacylated phosphoglycolipid I are summarized in Table 4. By using 500-MHz 1H -H-COSY (Fig. 9), it was possible to assign all 1H resonances of the individual constituents. ^{13}C chemical shifts were obtained and assigned using a

Table 4. ^{13}C and ^1H chemical shifts of native and deacylated phosphoglycolipid I and native phosphoglycolipid II. For solvents, see Materials and Methods. Shifts were measured from tetramethylsilane. Coupling constants were either $^3J_{\text{C,P}}$ or $^2J_{\text{C,P}}$ as indicated by superscript 3 or 2; (nr), not resolved.

Phospho- glycolipid	Residue	Shift ($J_{\text{C,P}}$) of															
		C1	C2	C3	C4	C5	C6	H1	H1'	H2	H3	H3'	H4	H5	H6	H6'	
		ppm (Hz)															
I, native	α -D-galacto- pyranosyl	98.27	69.43	70.18	69.57	70.70 (nr)	65.31 (nr)	4.95		3.74	3.80		3.94	4.25	3.97	3.97	
I, deacylated		98.78	70.22	71.06	70.45	71.22 (8.5) ³	65.62 (3.8) ²	5.00		3.77	3.83		3.94	4.30	4.01	3.97	
II, native		98.77	69.27	70.30	69.37	70.58 (nr)	64.89 (nr)	4.93		3.73	3.79		3.94	4.24	3.97	3.93	
I, native	2-amino-1,3- propanediol	59.47	53.98 (5.8) ³	63.41 (5.4) ²				3.68	3.75	3.42	3.98	4.03					
I, deacylated		60.03	54.79 (7.8) ³	64.04 (4.9) ²				3.68	3.77	3.44	3.99	4.07					
II, native		59.39	54.05 (5.8) ³	63.21 (5.4) ²				3.66	3.74	3.41	3.96	4.03					
I, native	α -D-gluco- pyranosyl	97.28	78.11	72.61	70.18	72.81	61.57	4.98		3.55	3.75		3.43	3.54	3.79	3.79	
I, deacylated		98.18	78.73	73.50	71.54	73.60	62.55	5.03		3.56	3.79		3.36	3.61	3.68	3.81	
II, native		97.17	77.79	72.59	70.29	70.50	70.86	4.94		3.56	3.72		3.29	3.75	4.18	4.36	
I, native	glycerol	63.34	70.61	66.49				4.16	4.42	5.20	3.62	3.80					
I, deacylated		64.15	72.29	70.75				3.53	3.60	3.83	3.44	3.84					
II, native		63.33	70.95	66.40				4.20	4.42	5.20	3.62	3.78					

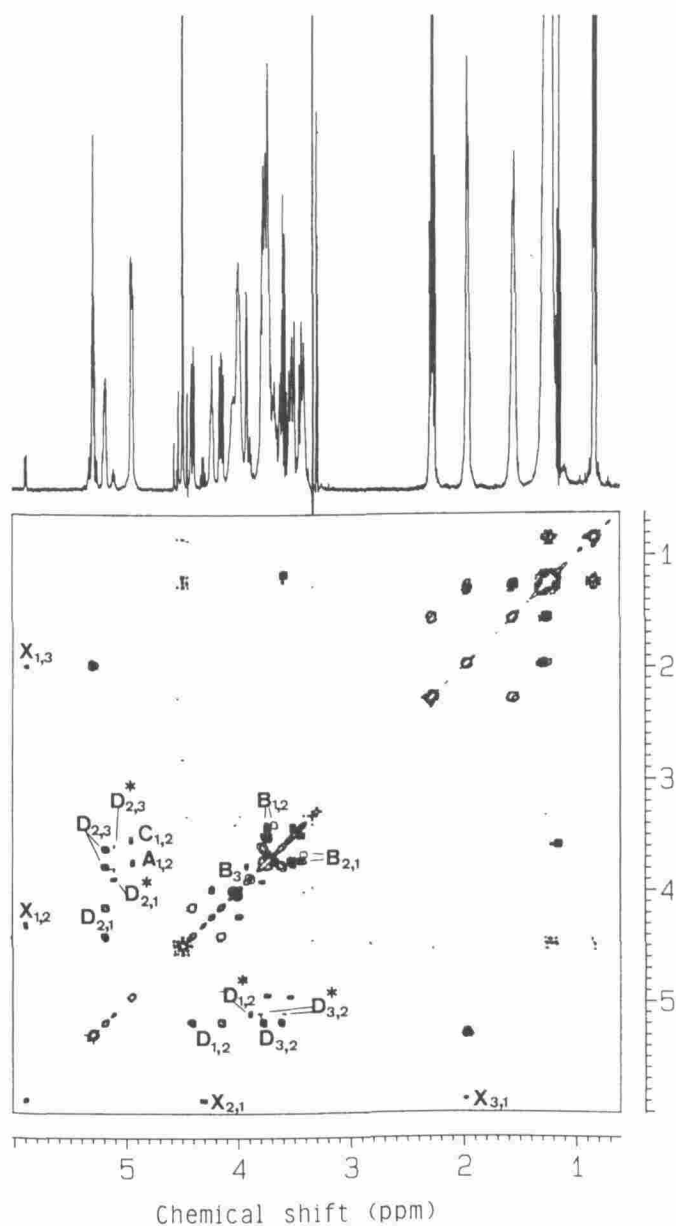


Fig. 9. One-dimensional 500-MHz ^1H -NMR spectrum (top), and $^1\text{H}/^1\text{H}$ -COSY spectrum of native phosphoglycolipid I. Designations: correlation signals of the anomeric protons of the galactosyl ($A_{1,2}$) and glucosyl residue ($C_{1,2}$); correlation signals of the 2-amino-1,3-propanediol residue (B) and the glycerol moiety of the di-*O*-acylglycerol (D) and 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol residues (D^*). Numbering of B, D, and D^* as in Table 4. X, alk-1-enyl ether correlation signals (see text).

$^1\text{H}/^{13}\text{C}$ -HMQC experiment (Fig. 10). The data in Table 4 are consistent with the structure deduced from chemical analyses. Both hexosyl residues show α -anomeric protons between 4.93–5.03 ppm ($^3J_{\text{HH}}$, 3.5 Hz). The glycosidic substitution at O2 of the glucosyl residue is indicated in the ^{13}C experiment by a shift of the C2 signal by 6.5 ppm compared with non-substituted methyl α -glucopyranoside [33]. The protons H1, H1' and H2 of the glycerol moiety are shifted after deacylation by -0.65 , -0.82 , and -1.37 ppm, respectively, which locates the fatty acyl residues at O1 and O2. In the deacylated glycerol residue, the resonances of H3 and H3' are shifted by -0.09 and 0.24 ppm compared with H1 and H1' indica-

tive of the glycosyl moiety linked to O3. The location of the amino group at C-2 of the aminopropanediol phosphate residue is also confirmed, because, compared with glycerol 1(3)-phosphate [34], the signals of C2 and H2 are shifted by -17.3 and -0.3 ppm, respectively. Moreover, *N*-acetylation effected a shift of the C2, C1, and C3 signals by -2.16 , 1.21 , and 0.86 ppm, accompanied by a shift of the H2 signal by 0.58 ppm (not shown). The phosphodiester bond between O3 of the aminopropanediol moiety and O6 of the galactopyranosyl residue is indicated by the respective $^2J_{\text{C,P}}$ and $^3J_{\text{C,P}}$ couplings listed in Table 4. It can also be demonstrated in the ^1H -NMR spectrum by ^{31}P -decoupling which simplifies the signal pattern of H3, H3' of the aminopropanediol moiety and H6, H6' of the galactopyranosyl residue (Fig. 11). The ^{31}P signal appeared in the 206.46-MHz ^{31}P -NMR spectra of native and deacylated phosphoglycolipids at 1.00 ppm.

The NMR data in Table 4 allow the third fatty acid in phosphoglycolipid II to be located at O6 of the glucopyranosyl residue. Compared with the signals of the glucopyranosyl residue of phosphoglycolipid I, those of C6 and C5 are shifted by 9.3 and -2.3 ppm, those of H6, H6', and H5 by 0.44, 0.56, and 0.18 ppm, respectively.

The ^{13}C and ^1H NMR signals of the ester-linked fatty acids, seen in Figs 9 and 10 and summarized in Table 5, are in the expected range [17]. The presence of a third fatty acid in phosphoglycolipid II is evidenced by a third carbonyl signal. Signals arising from the alk-1-enyl ether were observed in the $^1\text{H}/^1\text{H}$ COSY (Fig. 9). Signal X_1 at 5.89 ppm can be assigned to the α -olefinic proton which is coupled with the β -olefinic proton X_2 at 4.31 ppm. The α -methylene protons X_3 at 1.95 ppm are deshielded by the olefinic bond. The location of the ether bond at the glycerol moiety can be identified as follows (Fig. 9): The proton signals D_2^* at 5.12 ppm and D_3^* at 3.82 and 3.60 ppm are close to D_2 and D_3 , which designate the proton signals H2 and H3, H3' of the di-*O*-acylated glycerol moiety (Table 4). Compared to D_1 , the H1, H1' signals of this glycerol moiety, the signal D_1^* at 3.90 ppm shows a significant up-field shift, which locates the vinyl ether at O1 of the glycerol moiety.

DISCUSSION

The structure of the two major aminophosphoglycolipids of *C. innocuum* has been established to be (*S*)-2-amino-1,2-propanediol-3-phospho-6- α -D-galactopyranosyl-(1-2)- α -D-glucopyranosyl(1-3)- α -D-galactopyranosyl-1,3-glycerol and an acylated derivative which carries an additional fatty acid residue at O6 of the glucosyl moiety. The location of this fatty acid was achieved by ^{13}C and ^1H NMR spectroscopy which also confirmed all other structural details except structures and combinations of the fatty acids. These data were obtained by FAB-MS which, particularly in the positive-ion mode, also established the sequence of the individual components. In order to evaluate the absolute configuration of the 2-amino-propanediol phosphate residue, the *N*-acetylated derivative was released from the phosphoglycolipids by periodate oxidation and subsequent β -elimination. The liberated *N*-acetyl-2-aminopropanediol phosphate was oxidized with permanganate to give *N*-acetylserine phosphate. From the latter, serine was released by dephosphorylation with HF and de-*N*-acetylation with HCl, and then analyzed for stereochemical configuration by HPLC (Fig. 5). The linkage of the 2-aminopropanediol residue by a phosphodiester bond to the galactopyranosyl moiety was indicated by the formation of galactose

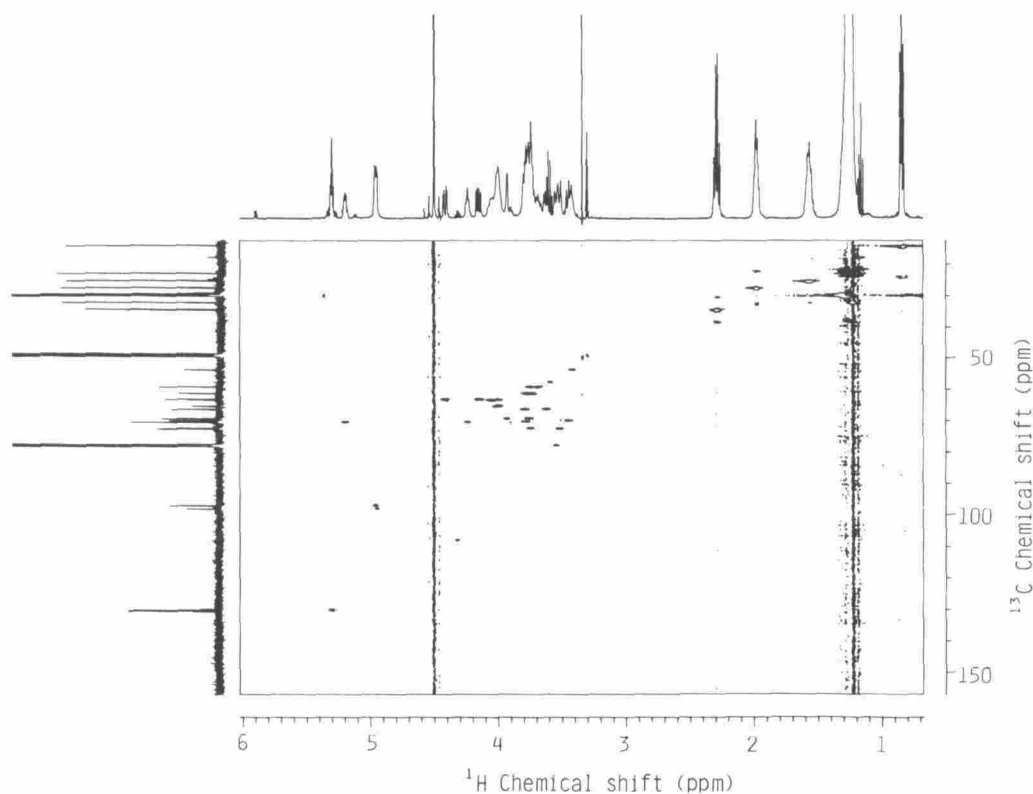


Fig. 10. 500/125-MHz $^1\text{H}/^{13}\text{C}$ -HMQC spectrum of native phosphoglycolipid I.

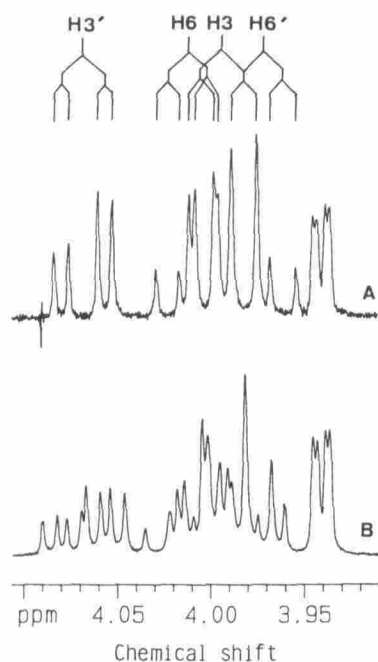


Fig. 11. Partial ^1H -NMR spectrum of deacylated phosphoglycolipid I. (A), ^{31}P decoupled; (B), undecoupled. H6, H6', protons at C6 of the galactopyranosyl residue; H3, H3', protons at C3 of the aminopropanediol residue.

phosphate on HCl hydrolysis, and the release of 2-aminopropanediol phosphate by β -elimination after oxidation of the phosphoglycolipids with periodate located the phosphodiester bond at O6 of the sugar moiety. This point of attachment

Table 5. ^{13}C and ^1H chemical shifts of the ester-linked fatty acids of phosphoglycolipids I and II. $\text{CH}_2\alpha, \beta$, α - and β -position to carbonyl group; $\text{C}_2=$, α -position to olefinic bonds. Range of the aliphatic methylene groups: ^{13}C , 23–32 ppm; ^1H , 1.2–1.35 ppm.

Group	Chemical shift of		
	^{13}C in I	^{13}C in II	^1H in I and II
	ppm		
C=O	—	174.84	
C=O	174.67	174.57	
C=O	174.23	174.03	
HC=CH	130.47	130.47	5.31
HC=CH	130.18	130.18	5.28
$\text{CH}_2\alpha$	34.75	34.75	2.30
$\text{CH}_2\alpha$	34.63	34.63	2.30
$\text{CH}_2\beta$	25.47	25.47	1.57
$\text{CH}_2\beta$	25.43	25.43	1.57
$\text{CH}_2=$	27.69	27.69	1.98
$\text{CH}_2=$	27.54	27.54	1.98
CH_3	14.33	14.33	0.84

was independently identified by ^{13}C and ^1H -NMR spectroscopy (Table 4, Fig. 11).

To the best of our knowledge, (*S*)-2-amino-1,2-propanediol 3-phosphate has so far not been found in nature. One is tempted to speculate that it is metabolically derived from dihydroxyacetone phosphate which may be aminated and then activated presumably in the form of its CDP derivative. Recently 2-amino-1,3-propanediol has been detected as an

O-antigen component in the lipopolysaccharide of *Vibrio cholerae* H11, but in this case it was differently linked by an amide bond to the carboxyl group of D-galacturonosyl residues [35].

Both phosphoglycolipids of *C. innocuum* consist of 15–30% 1-*O*-(alk-1-enyl)-2-*O*-acylglycerol species in addition to diacylglycerol species. The alk-1-enyl residues contain 14, 16, and 18 carbon atoms and are preferentially combined with unsaturated fatty acid residues (16:1, 18:1). The additional sugar-linked fatty acid of phosphoglycolipid II is mostly tetradecanoate and hexadecanoate. The two phosphoglycolipids are related to the glycolipids of this organism which possess a very similar fatty acid pattern and have been identified as α -D-Glcp(1-3)radyl₂Gro and α -D-Galp(1-2)- α -D-Glcp(1-3)radyl₂Gro [1]. These glycolipids represent only a small percentage of total polar lipids in contrast to almost 70% contributed by the aminophosphoglycolipids.

The plasmenyl analogues of conventional phospholipids are present in *C. innocuum* [1] and have been positively identified in numerous clostridia [36]. Although no systematic study of glycolipids in clostridia has been carried out, several examples of glycosyldiacylglycerols have been described [36]. There are, however, only two notes on alk-1-enyl-acylglycerol glycolipids [1, 37] and the location of the vinyl ether bond at the glycerol moiety of these compounds has not been established. In the present study hydrogenation of the vinyl ether bond made it possible to release the alkylglycerols from the aminophosphoglycolipids by acid hydrolysis and to locate the ether bond at O1 or O3 of the glycerol moiety by GLC/MS of the *O*-methylated derivative (Fig. 3). This location could also be derived from the ¹H/¹H COSY (Fig. 9). The position of the alk-1-enyl ether bond is therefore identical in glyceroglycolipids and glycerophospholipids, which suggests a common biosynthetic precursor, possibly 1-*O*-(alk-1-enyl)-2-*O*-acyl-*sn*-glycerol 3-phosphate (plasmenic acid) in analogy to phosphatidic acid, the common precursor of the diacyl-containing glyceroglycolipids and phospholipids.

Bacilli, enterococci, lactobacilli, lactococci, listeriae, staphylococci, and some streptococcal species contain glycerophosphoglycolipids which are also derived from the respective membrane glycolipids but carry a *sn*-glycero-1-phosphate residue at O6 of the glycosyl residue at the non-reducing terminus [2, 3]. These glycerophosphoglycolipids constitute, however, only a small percentage of total membrane lipids and are involved as intermediates in the biosynthesis of the poly(glycerophosphate) lipoteichoic acids of these bacteria [3, 29]. Unpublished data (Fischer, W.) suggest for the lipoteichoic acid of *C. innocuum* a poly(galactosyl glycerophosphate) chain with positively charged glucosaminyl residues or their *N*-acetylated derivatives as substituents at O2 of the glycerophosphate moieties. The lipid anchor is β -D-GlcNp(1-3) α -D-Glcp(1-3)radyl₂Gro, a so far unique glycolipid which is unrelated to the dihexosyl glycerolipid and the aminophosphoglycolipids of *C. innocuum*. In contrast to the glycerophosphoglycolipids of other Gram-positive bacteria, the aminophosphoglyceroglycolipids of *C. innocuum* are therefore unrelated to lipoteichoic acid and serve exclusively as membrane constituents. Their high abundance, 70% of the polar membrane lipids, predicts physicochemical properties that enable them to form stable bilayer structures.

On the basis of DNA-rRNA pairing [38], rRNA oligonucleotide cataloguing [39], and recent sequence analysis of 16 rRNA genes [40], clostridia are a major highly divergent phylogenetic unit, encompassing over a hundred species. The recommended division into different genera will require phe-

notypic criteria [40] and among them may be the lipid composition which has been proved among other Gram-positive bacteria of high taxonomic value [41]. However, with the exception of the apolar chains, the lipids of clostridia have not been profoundly studied [36]. Both the unique lipid pattern and the unique lipoteichoic acid structure found in *C. innocuum*, appear promising. For example, zwitterionic aminophospholipids, such as phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, and their respective plasmalogens which are major phospholipids of *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, and related species [42] are in *C. innocuum* apparently replaced by the zwitterionic aminophosphoglycolipids described in this report. Moreover, the presumed ancient age and evolutionary depth of clostridia [39, 43] suggest that, in some of them, ancestral lipid structures and membrane compositions may have been preserved.

W. F. thanks Norah Johnston and Howard Goldfine for initiating a joint study into clostridial lipids and for the generous gift of cells and crude lipid extract from *C. innocuum*. W. F. further thanks Prof. E. Hannappel for stereochemical analysis of L-serine, Dr A. Haag for valuable discussions, G. Distler for performance of GLC mass spectrometry, and Edeltraud Ebnert, Birgitta Brunner and Christian Emilius for excellent technical assistance. This work was supported by the *Deutsche Forschungsgemeinschaft* (Fi 218/4-8; Eg 39/10-1) and the *Fond der chemischen Industrie* (H. E.).

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