Structures of minor ether lipids isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6[1]

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Abstract Structures were determined for two phospholipids and three glycolipids purified from chloroform–methanol extracts of *Methanothrix concilii* GP6. Together they accounted for 14% of the total lipid and were based on a $C_{20,20}$-diether core structure consisting of either 2,3-di-*O*-phytanyl-*sn*-glycerol or its 3'-hydroxy analog, namely, 2-*O*-[3,7,11,15-tetramethylhexadecyl]-3-*O*-[3'-hydroxy-3',7',11',15'-tetramethylhexadecyl]-*sn*-glycerol. These two core lipids formed phosphodiester bonds to ethanolamine and glycosidic bonds to β-D-galactopyranose. A third glycolipid consisted of the triglycosyl head group β-D-galactopyranosyl-(1→6)-[β-D-glucopyranosyl-(1→3)]-β-D-galactopyranose in glycosidic linkage to the 3'-hydroxydiether core lipid. —**Ferrante, G., J-R. Brisson, G. B. Patel, I. Ekiel, and G. D. Sprott.** Structures of minor ether lipids isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6. *J. Lipid Res.* 1989. **30:** 1601–1609.

Supplementary key words diether lipid • phospholipid • glycolipid • polar head group • archaeobacteria

Three polar lipids accounting for 78% of the total lipid isolated from the aceticlastic methanogen, *Methanothrix concilii* GP6, have been previously characterized and found to consist of a phospholipid and two glycolipids (1, 2). The phospholipid and one of the glycolipids were found to be based on the standard $C_{20,20}$-diether core lipid with their full chemical designations as 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-1'-*myo*-L-inositol and 2,3-di-*O*-phytanyl-1-*O*-[α-D-mannopyranosyl-(1→3)-β-D-galactopyranosyl]-*sn*-glycerol, respectively. In contrast, the second glycolipid contained a modified hydrophobic $C_{20,20}$-diether core lipid structure in which an hydroxyl group had been introduced on carbon three of the phytanyl chain in ether linkage to the *sn*-3 position of glycerol (2). The full chemical designation given for this structure was: 2-*O*-phytanyl-3-*O*-[3'-hydroxy-3',7',11',15'-tetramethylhexadecyl]-1-*O*-[β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-*sn*-glycerol. Furthermore, at least eight minor lipid components were detected and their structures were partly assigned on the basis of their staining reactions (1). This report provides the full chemical characterization for five of these minor lipids.

MATERIALS AND METHODS

Materials

Silica gel G plates (0.25 mm) and silica gel G for preparative thin-layer chromatography (TLC) were purchased from BDH Chemicals (Canada) and Brinkman Instruments Ltd. (Canada), respectively. Methyl iodide and Ag_2O were obtained from J. T. Baker Chemical Co.

Growth of organism and isolation of lipids

M. concilii, strain GP6 (NRC 2989 = DSM 3671 = ATCC 35969) was first isolated by Patel (3) and grown in 10 l of freshly reduced defined salt medium (3) in a 20-l glass carboy for 3 weeks at 35°C. This subculture was used to inoculate 55 l of fresh medium (10%, v/v) in a 75-l fermentor (Chemap AG fermentor, Switzerland). The fermentor head space was flushed with oxygen-free nitrogen gas (0.3 l/min) during incubation for 35 days at 35°C using an agitation speed of 75 rpm. After 7 days of incubation, the fermentor was re-supplemented every 4 days with 1.0 ml/l of cysteine/Na_2S solution (71.2 mM cysteine and 52 mM Na_2S). The pH of the medium was maintained at 7.3 by the periodic addition of sterile anaerobic glacial acetic acid using a pH stat. At the end of the growth period (A_{660} = 1.0; 0.37 mg cell dry weight/ml) the cells were harvested (1) and the cell pellet was stored at −70°C until lipid extraction (4) was performed.

The lipids were partially purified by column chromatography followed by complete purification by preparative TLC as described in detail elsewhere (1).

Abbreviations: TLC, thin-layer chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TGD, triglycosyldiether; FAB, fast atom bombardment; PED, diether analog of phosphatidylethanolamine; D, diether; D_{OH}, hydroxylated diether; PHG, polar head group.

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Analytical methods and chemical degradation procedures

Phosphorus was estimated by the method of Allen (5) while the sugar content was determined by the phenol-sulfuric acid procedure (6).

Glycolipid (15 mg) hydrolysis was carried out in 20-ml Teflon-lined screw-capped tubes containing 2.0 ml of either 0.18% or 2.5% methanolic-HCl for mild and strong acid hydrolysis, respectively (1). At the end of hydrolysis, 0.2 ml of water and 2.0 ml of petroleum ether were added and the contents were thoroughly mixed. The lipid moieties, recovered in the upper petroleum ether phase, were identified on the basis of optical rotation measurements (2) and TLC mobilities relative to appropriate standards (1). The methyl glycosides present in the lower methanol-water phase were converted to free sugars (7) and analyzed either by paper chromatography (7) or acetylated (7) and analyzed by GLC-MS. A Hewlett-Packard Model 5985 system, with OV-17 capillary column (0.32 mm I.D. × 25 m), was programmed to increase from 180°C to 240°C at 2°C/min. The MS ionization potential was 230v with methane at 0.5 torr as reagent gas.

The sugar sequence in the triglycosyldiether (TGD)[3] was established by methylation (8) followed by GLC-MS analysis of the permethylated alditol acetates, as described above for the acetylated sugars, and by partial acid methanolysis (9). In the latter procedure, acid methanolysis of 15.0 mg of TGD was carried out in a mixture of $CHCl_3$-CH_3OH-2.5% methanolic HCl 9:8:4 (v/v) at room temperature for 120 h. At the end of the incubation time, three major products were observed with TLC mobilities in $CHCl_3$-CH_3OH-HAC-H_2O 85:22.5:10:4 (v/v) identical to triglycosyldiether (starting material), diglycosyldiether ($GalGalD_{OH}$ (1)), and monoglycosyldiether ($GalD_{OH}$) (**Fig. 1**). The individual partial methanolysis products were purified by TLC in $CHCl_3$-CH_3OH-HAC-water 85:22.5:10:4 (v/v). Following a brief exposure to iodine vapor, the lipids were individually removed and eluted from the silica with CH_3OH-$CHCl_3$ 1:1 (v/v). The alditol acetates of the individual partially methylated triglycosyl products were prepared as described elsewhere (8) and analyzed by GLC-MS as described above.

Phospholipids (15 mg) were hydrolyzed in 2.0 ml of 48% HF in 20-ml Teflon centrifuge tubes tightly capped with a cork and incubated overnight at 0°C. The solvent was blown off under a stream of N_2 gas in a gently heated water bath and to the remaining residue was added 2.0 ml of methanol and 0.2 ml of water. The lipid was extracted three times with 2.0-ml portions of petroleum ether and identified by TLC mobility and optical rotation measurements (2). The methanol-water-soluble material was brought to dryness under a stream of N_2 gas, followed by overnight desiccation over KOH pellets. The residue was quantified by weighing, and subsequently analyzed with a Duram-500 amino acid analyzer.

[3]Diether lipid abbreviations were assigned on the basis of hydroxylation of the diether core lipid (D or D_{OH}) (2) and according to the head group components (see Fig. 1).

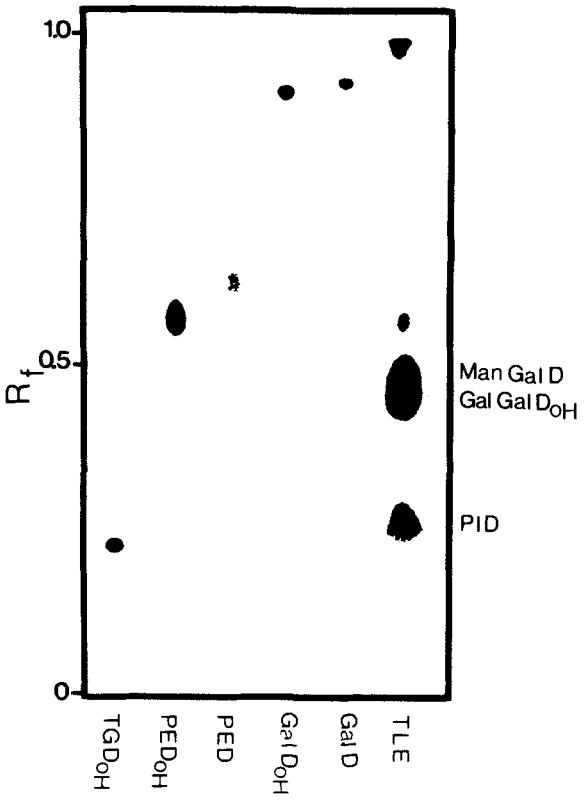

Fig. 1. Thin-layer chromatogram of purified lipids and of total lipid extract (TLE) isolated from *M. concilii*. The lipids were chromatographed on silica gel G plates developed in chloroform-methanol-acetic acid-water 85:22.5:10:4 (v/v). Lipid spots were visualized by acid charring. Core lipid structures are represented as D, 2,3-di-*O*-phytanyl-*sn*-glycerol and D_{OH}, the 3'-hydroxydiether analog of D (2). Polar head groups are shown as TG, triglycosyl; PE, phosphoethanolamine, PI, phosphoinositol, or as the standard abbreviations used for sugar residues.

Physical measurements

Optical rotation measurements, Drift-spectra, and negative and positive ion fast atom bombardment mass spectrometry were performed as described previously (10). NMR spectra were measured at room temperature using a Bruker AM500 spectrometer. Purified lipids, usually 10 mg, were dissolved in 0.5 ml of benzene-d_6-methanol-d_4 7:1(v/v) with tetramethylsilane as the internal reference. ^{13}C-NMR signals were assigned using distortionless enhancement by polarization transfer (DEPT) technique (11) with selective proton decoupling.

RESULTS

Lipid composition

The bulk of the total lipid fraction of *M. concilii* (78% by wt) consists of three structurally characterized diether lipids and several unidentified minor components. On the basis of staining characteristics, two of the minor lipids were described as phospholipids and several others as glycolipids (1). Five of these minor lipids, whose structural features are the subject of this study, are characterized with respect to relative mobility and purity in Fig. 1. One of the previous glycolipid spots, R_f 0.92 (1), has now been resolved into two glycolipids (GalD and $GalD_{OH}$)[3] in the solvent system of chloroform–methanol 99:1 (v/v).

Phospholipids

Two lipid components (PED and PED_{OH}) obtained chromatographically pure (Fig. 1) were found to stain positive with molybdate and ninhydrin spray reagents (1) indicating the presence of both phosphate and free amino groups in the molecule. The infrared spectrum of PED_{OH} showed adsorption bands corresponding to hydroxyl (O–H stretch at 3514 cm^{-1}), amino (NH_2 stretch at 3348, 1742, 1638, and 1552 cm^{-1}), methylene (C–H stretch at 2920 and 2959 cm^{-1}; C–H deformations at 1457 cm^{-1}), isopropyl doublet (CH_3 deformations at 1376 and 1362 cm^{-1}), methyl (C–H stretch at 2870 cm^{-1}), P = O group at 1229 cm^{-1}, P–O^- and ether groups at 1086 cm^{-1} and a strong P–O–C bond at 1033 cm^{-1}. The results obtained from the elemental analysis confirmed the presence of one mole each of phosphate and nitrogen (**Table 1**) in agreement with the infrared and spray reagent results and in accordance with a structure having a molecular formula as indicated (Table 1). The high-field region of the ^{13}C-NMR spectrum (between 20–45 ppm) of lipid PED contained carbon signals that were attributed to the aliphatic phytanyl carbons showing the characteristic modified chemical resonances of C-2, C-4, and C-5, as reported previously for a 3'-hydroxydiether (2). Noteworthy is the presence of C-3 in the low-field part of the spectrum (**Fig. 2, A1; Table 2**) attributed to the quaternary carbon on the phytanyl chain and confirmed by the absence of this signal from the DEPT spectrum (Fig. 2, A2). The chemical shift values of C-1 phytanyl and $C\text{-}1_g$ (where g = glycerol), $C\text{-}2_g$ and $C\text{-}3_g$ (Table 2) were also similar to results reported previously (1, 2). The remaining two methylene signals (multiplicity determined from DEPT, Fig. 2, A2) with chemical shifts of 62.79 ppm and 40.79 ppm (Fig. 2, A1 and A2; Table 2) were attributed to the head group signals, and found to be identical to those of model dipalmitoyl phosphatidylethanolamine. Also, head group signals with similar chemical shift values have been reported for the diether analog of phosphatidylethanolamine from *Methanobacterium thermoautotrophicum* (12). A splitting of $C\text{-}1_g$ ($J_{C,P}$ = 5.0 Hz) and C-2 (CH_2, $J_{C,P}$ = 4.3 Hz) of the head group due to phosphate coupling was observed upon expansion of the spectra (not readily seen in Fig. 2), indicating that the ethanolamine residue was linked to the C-1 position of the 3'-hydroxydiether via a phosphodiester linkage.

TABLE 1. Analytical data for the minor phospholipids of *M. concilii* GP6

	PED		PED_{OH}	
	Found	Calculated	Found	Calculated
Mol wt[a]		775.2		791.2
C%	69.73	69.72	68.11	68.31
H%	11.94	12.09	11.21	11.85
N%	1.76	1.81	1.69	1.77
P%	3.95	4.00	3.99	3.92
Diether (%)	83.95	84.26[b]	84.10[c]	84.57[b]
PHG (%)[d]	16.07	18.07[b]	15.93[c]	17.70[b]
Diether/PHG (mole ratio)	1.12	1.00	1.12	1.00

[a]Calculated for $C_{45}H_{93}O_6PN$ (PED) and $C_{45}H_{93}O_7PN$ (PED_{OH}).
[b]Percent calculated for $C_2H_7O_4PN$ and either $C_{43}H_{88}O_3$ (D) or $C_{43}H_{88}O_4$ (D_{OH}).
[c]Percent determined by weighing.
[d]PHG, polar head group.

Positive FAB–mass spectrometry revealed an ion peak [M + 1] at *m/z* 792 (**Fig. 3A**) in agreement with our postulated molecular weight (Table 1) and in accordance with an hydroxylated diether having phosphoethanolamine as head group. The most important ion fragments were found at *m/z* 774 (loss of water), *m/z* 757 (loss of water and ammonia), and *m/z* 496 (loss of hydroxylated phytanyl chain).

Hydrolysis of the phospholipid with 48% HF at 0°C yielded a petroleum ether-soluble component that was identified as 3'-hydroxydiether on the basis of its TLC mobility and optical rotation values (2). The methanol–water-soluble material was identified as ethanolamine with an amino acid analyzer using appropriate standards. On the basis of these results we conclude that this phospholipid represents the hydroxylated diether analog of phosphatidylethanolamine (PED_{OH}) (**Fig. 4**).

Phospholipid PED gave color reactions with spray reagents and infrared absorption bands similar to those of PED_{OH}. The obvious contrasting difference resided in the aliphatic region of the ^{13}C-NMR spectrum of PED (Fig. 2B; Table 2) which showed that this phospholipid had chemical shift signals typical of a standard diether (2). The presence of the standard diether (D) as the core lipid was in agreement with the analytical data (Table 1) and the results from the negative FAB–mass spectrum which showed an ion peak [M-1] at *m/z* 774 (Fig. 3B). The fragment ion at *m/z* 730 represents the diether analog of phosphatidic acid (10), with the loss of the ethanolamine head group. Hydrolysis of this lipid with 48% HF

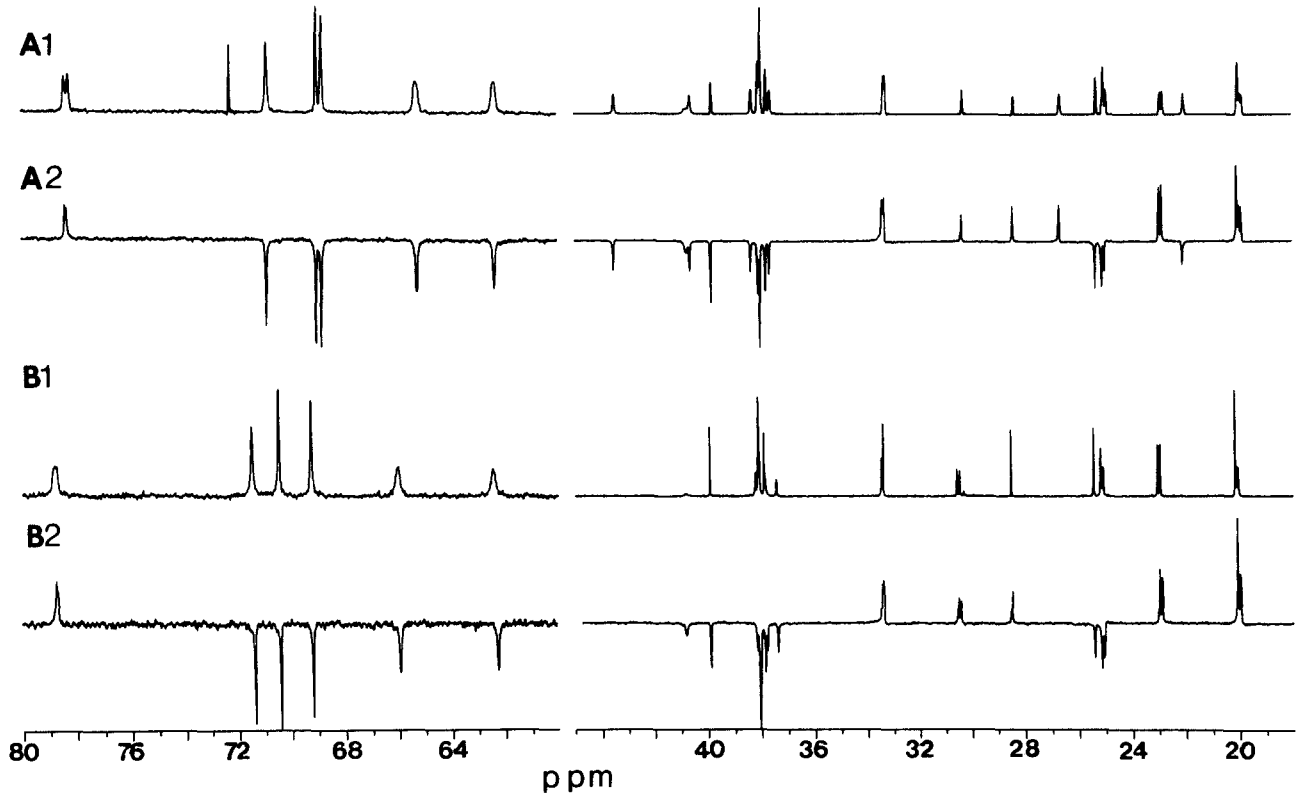

Fig. 2. ^{13}C-NMR spectra of purified minor phospholipids PED_{OH} (A1) and PED (B1), and DEPT spectra for PED_{OH} (A2) and PED (B2). Chemical shift values (ppm) and their assignments are shown in Table 2.

yielded a methanol–water-soluble material identified as ethanolamine with an amino acid analyzer, and a standard diether (D) in the petroleum ether phase as indicated by its TLC mobility (2), and optical rotation values (2). In conclusion, therefore, this phospholipid was found to be the diether analog of phosphatidylethanolamine (PED) (Fig. 4).

Glycolipids

The ^{13}C-NMR spectrum of $GalD_{OH}$ contained a single anomeric carbon signal with a chemical shift value of 104.41 ppm (**Fig. 5, A1**; Table 2), typical of a β-linked galactose (1, 13) and excluding an α-galactose where the anomeric carbon signal is reported as 100.1 ppm (13). This result was confirmed by ^{1}H-NMR which revealed an anomeric signal at 4.33 ppm with a coupling constant of J (1, 2) = 7.85 Hz (1, 13). The J constants for α and β anomers are reported (13) as ca. 3.0 and 8.0 ppm, respectively, giving an unambiguous assignment of linkage configuration. Noteworthy from the ^{13}C-NMR spectrum was the appearance of the quaternary carbon at 72.59 ppm (Fig. 5, A1 and A2; Table 2) and the modified C-2, C-4, and C-5 resonances indicating the presence of a 3'-hydroxydiether core lipid (2). Hydrolysis of the sugar under mildly acidic conditions (0.18% methanolic HCl), gave a methanol–water-soluble material which was identified as galactose by GLC–MS of its alditol acetate derivatives and by paper chromatography, while the GLC–MS of its permethylated alditol acetate gave 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol. The latter observation indicated a pyranoside ring structure with a glycosidic linkage at C-1. The petroleum ether-soluble material was further identified as the 3'-hydroxydiether on the basis of its TLC mobility and optical rotation value (2). Thus, the full chemical designation for this glycolipid ($GalD_{OH}$) is: 2-O-phytanyl-3-O-[3'-hydroxy-3',7',11',15'-tetramethyl-hexadecyl]-1-O-[β-D-galactopyranosyl]-*sn*-glycerol (Fig. 4).

The ^{13}C-NMR spectrum of lipid GalD revealed the presence of a single anomeric signal with a chemical shift value of 104.71 ppm (Fig. 5, B; Table 2) similar to that of $GalD_{OH}$. This result, together with the observation of a single ^{1}H-NMR signal at 4.33 ppm with a coupling constant of J(1,2) = 7.62 Hz, clearly established the presence of a β-linked galactose (1, 13). The presence of galactose as head group was confirmed by paper chromatography, GLC–MS of its alditol acetate derivative (data not shown), and GLC–MS of its permethylated alditol acetate derivative which established the presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol. In contrast to $GalD_{OH}$,

TABLE 2. ^{13}C-NMR chemical shift[a] values for the minor polar lipids of *M. concilii* GP6 measured in benzene-d_6-methanol-d_4 7:1 (v/v)

Carbon No.	PED	PED_{OH}	GalD	$GalD_{OH}$	TGD_{OH}
C-1[b]			104.41	104.41	104.45
C-2			71.99	71.91	69.72
C-3			74.17	74.23	79.97
C-4			69.52	69.73	68.68
C-5			75.55	75.69	75.71
C-6			62.95	62.14	67.96
C-1[c]					104.39
C-2					71.77
C-3					74.20
C-4					69.72
C-5					75.10
C-6					62.40
C-1[d]					103.04
C-2					73.92
C-3					77.35
C-4					70.90
C-5					77.08
C-6					62.12
$C\text{-}1_g$[e]	65.94	65.29	69.07	68.81	69.32
$C\text{-}2_g$	78.45	78.38	78.70	78.20	78.22
$C\text{-}3_g$	71.39	70.89	71.18	71.99	71.55
C-1ph[f] (*sn*-2)	69.20	68.83	69.52	68.93	68.86
C-1ph (*sn*-3)	70.40	69.03	70.39	69.73	69.03
CH_2-NH_2	40.81	40.79			
O-CH_2	62.27	62.79			
C-3ph (*sn*-3)	30.0	72.39	30.0	72.59	72.59

[a]The chemical shift values are given in ppm relative to internal tetramethylsilane.
[b]Sugar linked to the *sn*-1 position of glycerol.
[c]Second sugar.
[d]Third sugar.
[e]g, Refers to carbons of glycerol.
[f]ph, Refers to the carbon atoms of the phytanyl chain.

the aliphatic region of the ^{13}C-NMR spectrum of GalD (Fig. 5,B) contained the standard core diether signals (2). The standard $C_{20,20}$-diether core structure was confirmed by its TLC mobility and optical rotation value following strong acid hydrolysis (2). These results clearly identify GalD as: 2,3-di-*O*-phytanyl-1- *O*-[β-D-galactopyranosyl]-*sn*-glycerol (Fig. 4).

Lipid TGD_{OH} was observed to be much more polar than either $GalD_{OH}$ or GalD (Fig. 1) and its ^{13}C-NMR spectrum revealed three anomeric signals (Fig. 5, C1 and C2; Table 2) indicating the presence of three sugar residues in the hydrophilic region. Also, the chemical shift values of these anomeric signals (Table 2; Fig. 5, C1 and C2) are characteristic of two β-galactose and one β-glucose residues (13). These results were corroborated by ^{1}H-NMR which showed the presence of three anomeric signals at 5.00, 4.71, and 4.56 ppm with coupling constants of J(1,2) = 6.87, 6.88, and 6.98 Hz, respectively (1, 13). In fact, this glycolipid differs from the more abundant diglycosyl diether $GalGalD_{OH}$ (1) only by the inclusion of a Glc residue. Mild acid hydrolysis yielded a methanol-water-soluble material identified as galactose and glucose (2:1, mole %) by GLC-MS of its alditol acetate derivative and by paper chromatography. GLC-MS analysis of the permethylated alditol acetate showed the presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol, 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol, and 2,4,-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylgalactitol. These results agreed with pyranoside ring structures and with one of the following two sugar sequences: Glc*p*-(1→6)-[Gal*p*-(1→3)]-Gal*p*-diether or Gal*p*-(1→6)-[Glc*p*- (1→3)]-Gal*p*-diether. The correct sequence was established by partial acid methanolysis followed by the isolation of the products found to consist of the starting material plus a diglycosyldiether and a monoglycosyldiether with TLC mobilities similar to that of $GalGalD_{OH}$ (1) and $GalD_{OH}$. The permethylated alditol acetates of the diglycosyldiether showed the presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol and 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylgalactitol while the monoglycosyldiether revealed the presence of only 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol. These results confirm the sugar sequence as Gal*p*-(1→6)-[Glc*p*-(1→3)]-Gal*p*-diether.

The aliphatic region of the ^{13}C-NMR spectrum (Fig. 5, C1 and C2; Table 2) revealed the modified chemical signals characteristic of a 3'-hydroxydiether. This observation was confirmed by analysis of the petroleum ether

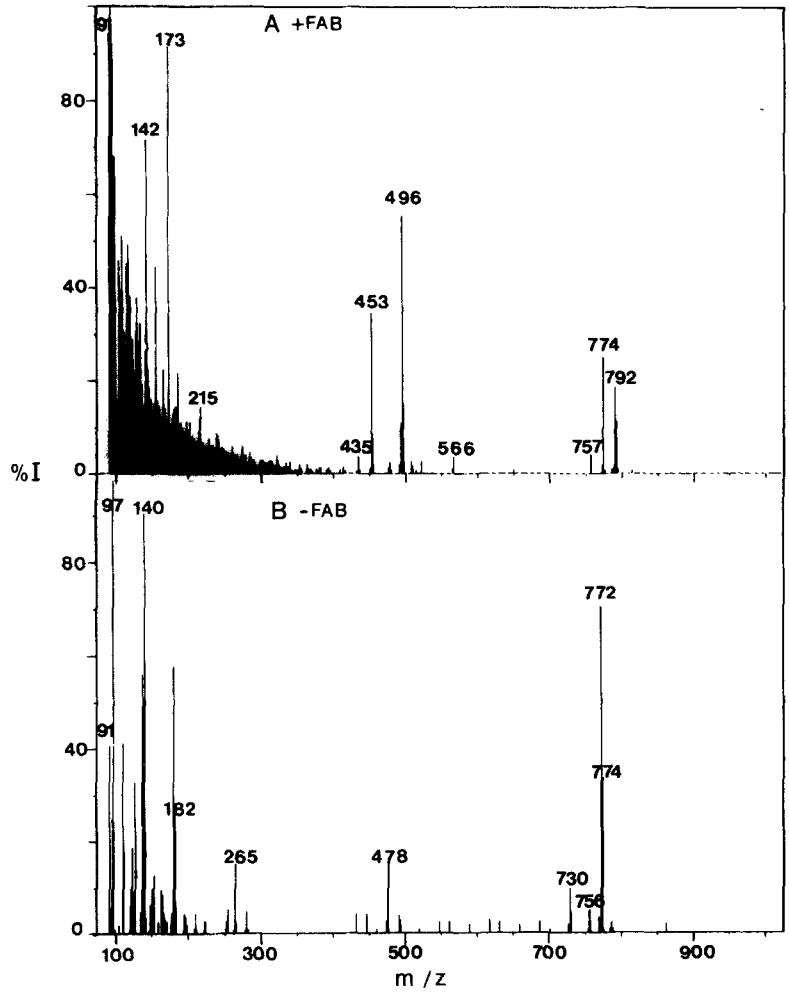

Fig. 3. Positive ion FAB of phospholipid PED_{OH} (A) and negative ion FAB of phospholipid PED (B) illustrating the significant ion fragments.

phase, following mild acid hydrolysis, by TLC and optical rotation measurements (2). Thus, the proposed structure for this triglycosyldiether (TGD_{OH}) is: 2-*O*-phytanyl-3-*O*-[3'-hydroxy-3',7',11',15'-tetramethylhexadecyl]-1-*O*-{β-D-galactopyranosyl-(1→6)-[β-D-glucopyranosyl-(1→3)]-β-D-galactopyranosyl}-*sn*-glycerol (Fig. 4).

DISCUSSION

A characteristic of the methanogens so far examined (1, 8, 10, 14–17) is the preponderance of the polar fraction in total lipid extracts, corresponding in *M. concilii* to ca. 93% by wt (1). Eight diether lipids from *M. concilii* lipid extracts, accounting for 98.6% by wt of the polar lipid fraction, have now been characterized structurally (present study, 1). Of the polar lipids remaining structurally unresolved there are at least four, all present in trace quantities (1).

The $C_{20,20}$-diether lipids of *M. concilii* are based on two core lipid structures, namely, the standard 2,3-di-O-phytanyl-*sn*-glycerol (D) and its 3'-hydroxydiether (D_{OH}) analog (2). The polar head groups consist of phosphoinositol and phosphoethanolamine, common head groups of ester phospholipids of eubacteria and eukaryotes, and various mono-, di-, or trisaccharides linked glycosidically to glycerol at the *sn*-1 carbon.

Prior to the current study showing phosphatidylethanolamine lipids of diethers D and D_{OH} in *Methanothrix concilii* (Fig. 1), this head group had been shown only in two tetraether phosphoglycolipids (17) and a $C_{20,20}$-diether lipid (12) from *Methanobacterium thermoautotrophicum*.

Despite the wide variation among methanogen genera with respect to both the core structure (2, 18, 19) and polar head groups of the ether lipids, it remains unclear

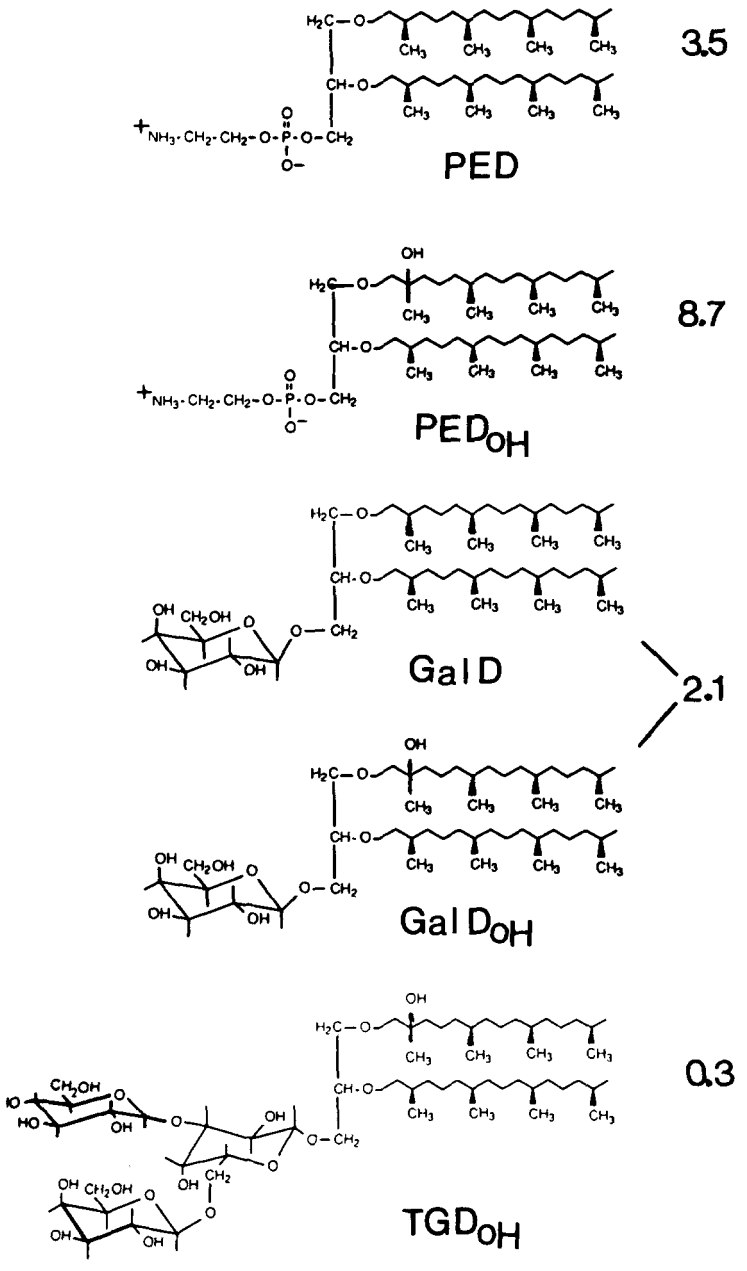

Fig. 4. Proposed structures for the minor polar lipids isolated from *M. concilii* GP6. The weight % of the total polar lipids is indicated (from ref. 1).

at present whether any ether lipids will prove diagnostic at the genus level. It is, however, likely that the entire spectrum of ether lipids formed by each methanogen will provide useful taxonomic data. This has been the case for the lipid structural studies performed to date, including *Methanospirillum hungatei* (8, 14), *Methanococcus voltae* (10), *Methanobacterium thermoautotrophicum* (12, 17), *Methanobrevibacter arboriphilicus* (16), *Methanococcus jannaschii* (19, G. Ferrante, J. Richards, and G. D. Sprott, unpublished results), and *Methanothrix concilii* (1, 2, present study). Diagnostic structural features of the diether lipids of *M. concilii*, in comparison to other methanogens studied, are the 3'-hydroxydiether core (2) and the triglycosyl diether (TGD_{OH}) lipid. A phosphatidyl inositol diether lipid occurs in both *M. concilii* (1) and *Methanobrevibacter arboriphilicus* (16).

Based on pulse labeling studies, the biosynthetic sequence of reactions occurring during synthesis of the diether lipids of *Halobacterium cutirubrum* appears to be alkylation of a three-carbon glycerol derivative to form an

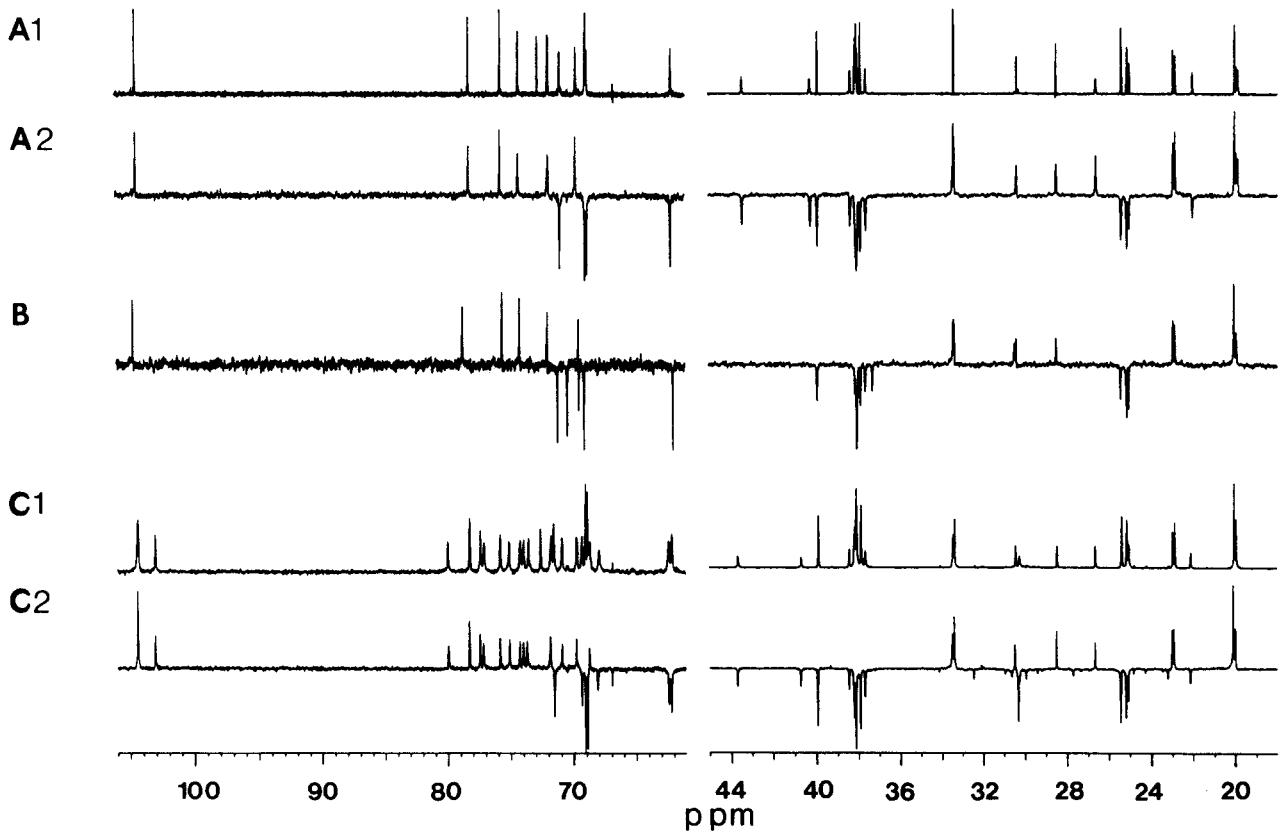

Fig. 5. ^{13}C-NMR spectra of purified minor glycolipids $GalD_{OH}$ (A1) and TGD_{OH} (C1). Spectra from DEPT experiments are shown for $GalD_{OH}$ (A2), GalD (B), and TGD_{OH} (C2). Chemical shift values (ppm) and their assignments are shown in Table 2.

isoprenyl diether compound, followed by either phosphorylation or glycosylation of the *sn*-1 carbon. This molecule undergoes further head group modifications, and finally hydrogenation of the double bonds to form the saturated diether lipids (20). Incorporation of labeled precursors into the ether lipids of *M. hungatei* suggested also that ether bond formation preceded reduction of the double bonds in the geranylgeranyl side chains (21). In the case of *M. concilii*, it is presently unknown whether the introduction of the hydroxyl group to the hydrocarbon chain occurs by a specific C-3 hydration process across the double bond or by a specific hydroxylating system after double bond reduction.

In terms of increasing complexity of the diether lipids extracted from *M. concilii*, the following biosynthetic schemes are possible.

$GalD_{OH} \rightarrow GalGalD_{OH} \rightarrow Gal(Glc)\ GalD_{OH}$ Scheme 1

$GalD \rightarrow ManGalD$ Scheme 2

The order of sugar residue addition in Scheme 1, in which Glc is the last sugar added, depicts the fact that a $GlcGalD_{OH}$ lipid was not detected. Based solely on structural data, a step-wise addition of sugar residues is shown (Schemes 1 and 2) which has yet to be tested by pulse labeling studies. Interconversion of diether and 3'-hydroxydiether lipids, such as GalD, $GalD_{OH}$ and PED, PED_{OH}, is postulated to occur by either specific hydroxylating or dehydroxylating systems.

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