A diphytanyl ether analog of phosphatidylserine from a methanogenic bacterium, *Methanobrevibacter arboriphilus*

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Abstract Several ninhydrin-positive lipids were found in methanogenic bacteria and the structure of one of them, designated as PNL2 from *Methanobrevibacter arboriphilus,* was identified as a diphytanyl ether analog of phosphatidylserine. The chromatographic behavior of the lipid on thin-layer plates and on a DEAE-cellulose column was identical to the ester form of phosphatidylserine. The infrared spectra showed the presence of amino, carboxyl, ether, and phosphate groups, and the absence of an ester linkage. The hydrophobic portion of the lipid was identified as diphytanyl glycerol diether on the basis of the mass spectrum of the acetolysis product and gas-liquid chromatography of the iodinated alkyl chain prepared by hydroiodic acid cleavage of PNL2. The fast atom bombardment-ionization and field desorption mass spectrum provided a molecular weight of 819 and several fragment ions consistent with the proposed structure. Hydrofluoric acid hydorlysis resulted in water-soluble products including serine, phosphoserine, and ammonia, which accounted for 95% of hydrolyzed PNL2. The lipid product of the hydrolysis was mainly the diether form of phosphatidic acid. This is the first report on the structural characterization of an amino-containing phospholipid in archaebacteria. Amino lipids have been found in many other methanogenic bacteria. —**Morii, H., M. Nishihara, M. Ohga, and Y. Koga.** A diphytanyl ether analog of phosphatidylserine from a methanogenic bacterium, *Methanobrevibacter arboriphilus. J. Lipid Res.* 1986. **27:** 724–730.

Supplementary key words archaebacteria • di-O-phytanyl glycerophosphoserine • aminophospholipid • crown ether • fast atom bombardment mass spectrometry

Methanogenic bacteria are strictly anaerobic archaebacteria that produce methane from H_2 plus CO_2, formate, or acetate. Alkyl glycerol di- or tetraether lipids are one of the most typical characteristics by which archaebacteria are distinguished from eubacteria (1). Although recent reports (2–6) have shown that a wide variety of methanogenic bacteria contain diphytanyl glycerol diether and dibiphytanyl diglycerol tetraether, polar head groups of the lipids have been studied only in one strain of *Methanospirillum hungatei* (5). Those lipids were phospho-, glyco-, and phosphoglycolipids, all of which did not contain an amino group. Although ninhydrin-positive spots were detected on thin-layer chromatograms of total lipids from two archaebacteria, *Halobacterium halobium* (7) and *Thermoplasma acidophilum* (8), the studies to date on archaebacterial lipids include no full structural characterization of these amino lipids (1–11). Amino lipids are ubiquitous components of biological membranes in other organisms and knowledge of their roles in membranes would be important. We have found ninhydrin-positive phospholipids in several species of methanogenic archaebacteria. The present report describes the structural identification of one of the amino group-containing lipids (PNL2) from *Methanobrevibacter arboriphilus* that has been tentatively identified as a diphytanyl ether analog of phosphatidylserine (**Fig. 1**).

MATERIALS AND METHODS

Growth of the bacterium and extraction of lipids

Methanobrevibacter arboriphilus A2 (DSM2462) was isolated and characterized in our laboratory (12) and maintained on formate-minimal medium (12). It was grown on 500 ml of the complex medium containing 1% sodium formate with H_2 + CO_2 (4:1) as energy and carbon sources as described previously (12). Five liters of the same medium without formate was inoculated with the 500 ml of fully grown culture and incubated for 4 days. The cells were collected at late-log phase and washed with water. Total lipids were extracted as described by Bligh and Dyer (13).

Abbreviations: DEAE, diethylaminoethyl; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; EI, electron ionization; FD, field desorption; FAB, fast atom bombardment; PMR, proton magnetic resonance.

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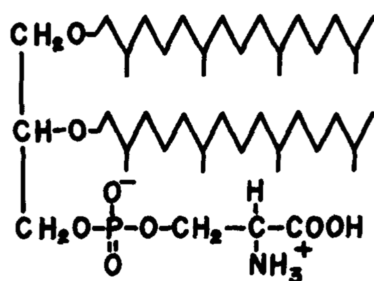

Fig. 1. A proposed structure for PNL2. The molecular weight of the compound is 819.

Chromatography

Total lipids (550 mg) were fractionated on a column (2.5 × 40 cm) of DEAE-cellulose (Brown, acetate form), using the following elution sequence: chloroform (1,600 ml); chloroform-methanol 9:1 (1,800 ml); chloroform-methanol 7:3 (1,000 ml); chloroform-methanol 1:1 (1,000 ml); methanol (1,000 ml); chloroform-acetic acid 3:1 (2,000 ml); and acetic acid (2,000 ml). TLC was carried out on a Silica Gel 60 plate (Merck) using the following solvents (composition in volume ratios): solvent A, chloroform-methanol-7 M aqueous ammonia 60:35:8; solvent B, chloroform-methanol-acetic acid-water 85:30:15:5; solvent C, methyl acetate-l-propanol-chloroform-methanol-0.5% aqueous KCl 25:25:25:10:9; solvent D, chloroform-methanol-water 65:25:4; solvent E, tetrahydrofuran-dimethoxymethane-methanol-2 M aqueous ammonia 10:5:5:1; solvent F, light petroleum (bp 30-70°C)-diethyl ether-acetic acid 50:50:1; solvent G, light petroleum (bp 30-70°C)-diethyl ether-acetic acid 80:20:3. Paper chromatography of water-soluble amino compounds was carried out on Toyo No 51A paper with solvent H, n-butanol-acetic acid-water 3:1:1. Lipid spots were detected by the following spray reagents: ninhydrin spray (0.5% in n-butanol, Tokyo Kasei Industry Co.) for amino group-containing lipids, acid molybdate reagent (14) for phospholipids and subsequent charring for all lipids, and 0.5% α-naphthol reagent (15) for glycolipids.

Determination of lipid composition

The ratio of nonpolar and polar lipid contents was determined by the densitometric tracing of the thin-layer chromatogram, using a microdensitometer 3CS (Joyce-Loebl, England), as follows. Total lipid was developed on a 20-cm-long TLC plate halfway to the top of the plate with solvent B. After drying in a vacuum desiccator, the plate was developed in the same direction to the top with solvent G. In the first development, polar lipids migrated and distributed in the first 10-cm region and nonpolar lipids moved to the first solvent-front line. In the second development, only nonpolar lipids migrated beyond the first solvent-front line. The chromatogram was visualized by charring with 30% H_2SO_4. Phospholipid composition was determined by the measurement of phosphorus of individual spots separated by TLC with solvent B.

Analytical methods and degradative procedures

Phosphorus and sugar were determined by the methods of Bartlett (16) and Dubois et al. (17), respectively. Elemental analysis (C, H, N, O) was performed by use of an elemental analyzer (model 240B, Perkin-Elmer) with acetanilide as a standard. Acetolysis was carried out as described by Renkonen (18). The acetolysis product was deacetylated by methanolysis in 5% HCl-methanol at 100°C for 2 hr. Glycerol ether bonds were cleaved with hydroiodic acid according to the method of Kates, Yengoyan, and Sastry (19). Alkyl iodide was identified by comparison of its retention time with that of a standard on a gas chromatograph, Shimadzu GC6AM. The analysis was carried out at 197°C on a 1.0-m glass column packed with butanediol succinate polyester on Uniport B (Gasukuro Industry Co.). The phosphodiester bond of PNL2 was hydrolyzed in 1 ml of 46% hydrofluoric acid at 0°C for 24 hr (20). The phospholipid was dispersed in hydrofluoric acid by a vortex mixer. After lyophilization of the hydrolyzate, the water- and chloroform-soluble products were partitioned by the extraction method of Bligh and Dyer (13). Phosphate contents of both fractions were determined. Amino compounds in the aqueous phase were identified and determined with the use of an amino acid analyzer (model 835, Hitachi, Japan). The chloroform-soluble phospholipid product was separated from unreacted PNL2 on a TLC plate with solvent B and identified by FAB-mass spectrometry. Treatments with phospholipase C (*Bacillus cereus*, Boehringer) and phospholipase D (cabbage, Boehringer) were carried out as described by Waku et al. (21) and Kates and Sastry (22), respectively.

Physical measurements

Infrared spectra were recorded in KBr pellets on a Shimadzu infrared spectrometer (IR450S). EI-, FD-, and FAB-mass spectra were obtained with a mass spectrometer (JMS DX-300/JMS-3500 data system, Japan Electron Optics Laboratory, Japan). For FAB mass spectrometry of the aminophospholipid, glycerol plus 15-crown-5 was used as a matrix (23). The use of 15-crown-5 made it possible to detect the molecular ion from PNL2. PMR spectra were recorded in $CDCl_3$-CD_3OD 1:1 solution containing tetramethylsilane (TMS) as an internal standard using a JMM-FX200 NMR spectrometer (Japan Electron Optics Laboratory) operating at 200 MHz. Chemical shifts were expressed relative to the internal standard TMS.

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Materials

Authentic samples of diphytanyl glycerol diether, phytanyl iodide, and biphytanyl diiodide were prepared from cells of *M. hungatei* GP1 (DSM1101). The lipids of this organism have been completely analyzed by Kushwaha et al. (5). The organism was obtained from the German Collection of Microorganisms and grown as described by Balch et al. (24). Phosphatidylserine (bovine brain) was the product of Avanti Polar Lipids, Inc.

RESULTS

Lipid composition and chromatographic behavior of PNL2

Total lipid from *M. arboriphilus* A2 consisted of 16% nonpolar lipid and 84% polar lipid determined by a densitometer. The molar ratio of phosphate to sugar in total lipid was 1:1.98. In the polar lipid region on the doubly developed chromatogram, a glycolipid (corresponding to spot #4 in Fig. 2, see below) accounted for almost half of the polar lipid; the other polar lipids were phospholipids and phosphoglycolipids. Because the average number of sugar residues per glycolipid molecule was not known, the molar or weight ratio of phospholipid to glycolipid could not be calculated. Total lipid was developed by TLC with solvents A and B in the first and second directions, respectively (**Fig. 2**). The major lipids were one glycolipid (spot #4) and four phospholipids (spots #1, 5, 7, and PNL2). One of the major phospholipids (designated as PNL2, 35.4% of phospholipid-phosphorus) was ninhydrine-positive. Because the amino group-containing lipid in archaebacteria has not been fully characterized, we decided to elucidate the structure of PNL2 initially. PNL2 gave a positive response to molybdate spray and a negative test with α-naphthol. It migrated almost identically with the diester form of phosphatidylserine in both directions. PNL2 was purified by elution from a DEAE-cellulose column with acetic acid; phosphatidylserine is similarly purified. With solvents C, D, and E, purified PNL2 also showed R_f values almost identical to those of phosphatidylserine (**Table 1**). Thus, the chromatographic behavior of PNL2 suggested that the polar portion of the lipid would closely resemble that of phosphatidylserine. Slightly higher R_f values were consistently observed with the ether form compared to the ester form of the lipid with the identical polar group. The elemental composition of PNL2 was C, 65.2%; H, 11.5%; O, 18.3%; N, 1.77%; and P, 3.24%. The calculated composition for the structure ($C_{46}H_{94}O_8NP$) shown in Fig. 1 was C, 67.4%; H, 11.2%; O, 15.6%; N, 1.71%; and P, 3.79%. The molar ratio of N/P was 1.21.

TABLE 1. R_f values of PNL2 on precoated Silica Gel 60 plates

Lipid	Solvent[a] A	B	C	D	E
PNL2	0.24	0.53	0.25	0.18	0.10
Phosphatidylserine	0.22	0.51	0.24	0.16	0.10
Phosphatidylethanolamine	0.39	0.74	0.47	0.40	0.22

[a] See text for compositions of solvents A–E.

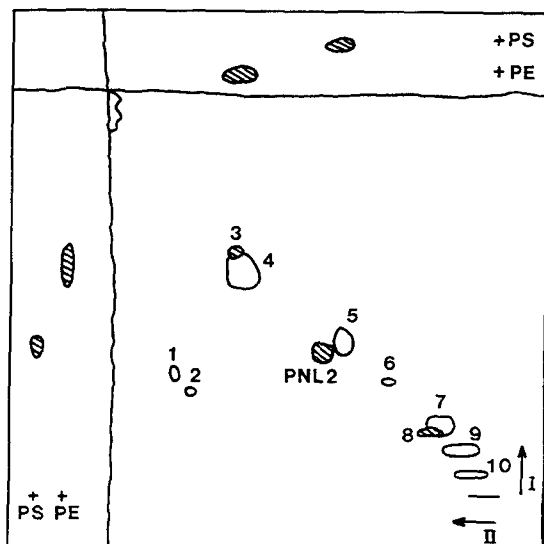

Fig. 2. Two-dimensional TLC of total lipids from *M. arboriphilus* A2. Development was first in the vertical direction with solvent A then in horizontal direction with solvent B. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) were developed as standards in both directions. The hatched spots were ninhydrin-positive. All spots were detected by acid-charring.

Infrared spectra of PNL2

The infrared spectra of the sodium salt and free acid forms of PNL2 were recorded (**Fig. 3**). The spectrum of the sodium salt showed absorptions corresponding to groups of ether C–O–C (1090 cm^{-1}), phosphate P=O and P–O–C (1230 and 1050–1060 cm^{-1}), amino NH_2 (3400–3500 cm^{-1}), carboxyl C–O– (1310 cm^{-1}), isopropyl $(CH_3)_2CH$– (1380 and 1365 cm^{-1}), tertiary –C–H (1350 cm^{-1}), and methyl $-CH_3$ and methylene $-CH_2-$ (1465 and 2850–3000 cm^{-1}). There was no band indicative of ester group (1150–1200 cm^{-1} and 1730–1750 cm^{-1}). The absorption near 1635 cm^{-1} would be assigned to overlapped signals of amino NH and carboxyl anion COO^-. The spectrum of the free acid form of PNL2 showed absorptions identical to that of the sodium salt except that the absorption peak corresponding to carboxylic acid COOH (1720 cm^{-1}) appeared.

Identification of alkyl glycerol diether

The alkyl glycerol diether of polar lipids from archaebacteria is usually isolated by methanolysis followed by

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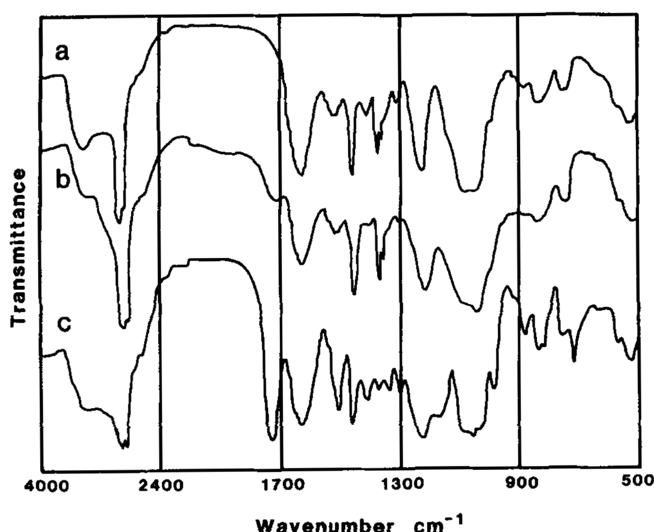

Fig. 3. Infrared spectra of Na salt of PNL2 from *M. arboriphilus* (a), the acid form of PNL2 (b), and bovine brain phosphatidylserine (ester form) (c). The spectra were recorded in KBr pellets.

TLC (2, 4). PNL2, however, was not readily degraded into a polar head group and glycerol diether by methanolysis, probably due to the lack of a free hydroxyl group or easily hydrolyzable ester group adjacent to the phosphodiester bond. Acetolysis was the only procedure by which the glycerol diether was completely released from the phosphate ester. Acetolysis of PNL2 resulted in a single chloroform-soluble product. Its mass spectrum (**Fig. 4**) showed peaks of m/z 694, 679, 634, 415, 397, 383, and 281, which were identical to acetylated diphytanyl glycerol diether (6). The acetolysis product was deacetylated by acid methanolysis. The resultant compound cochromatographed with the authentic 2,3-di-O-phytanyl-*sn*-glycerol diether by TLC with solvent F. The alkyl iodide prepared from PNL2 by hydroiodic acid treatment was compared on GLC with phytanyl iodide and biphytanyl diiodide from *M. hungatei* lipids. It showed a single peak and its retention time coincided with that of phytanyl iodide. Thus, the hydrophobic portion of PNL2 was identified as di-O-phytanyl glycerol.

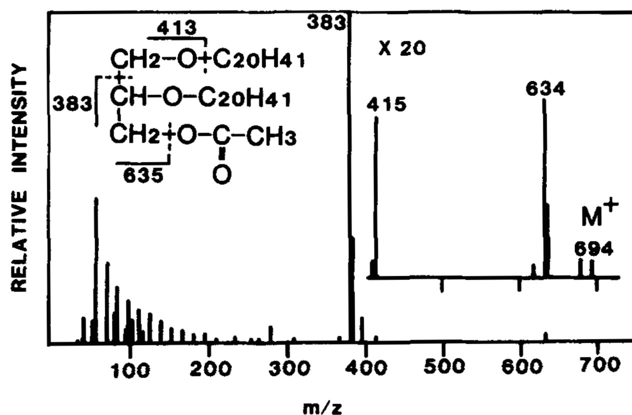

Fig. 4. Mass spectrum of the acetolysis product of PNL2 (acetylated diphytanyl glycerol ether). The molecular weight of the compound is 694.

Identification of the polar head group of PNL2

In order to isolate and identify the head group of PNL2, various cleavage reactions such as acetolysis, methanolysis (in 5% HCl–methanol at 100°C for 2 hr), and hydrolysis (in 6 N HCl at 105°C for 16 hr or in conc. HCl-butanol 1:5 at 125°C for 20 hr) were attempted. Although the glycerol diether portion of PNL2 was recovered by acetolysis, the reaction was not suitable for the isolation of polar head group because of destruction of the amino-containing group at high temperature (145°C). The methanolysis and the hydrolysis under the above conditions resulted in cleavage of about 10% of PNL2. Stronger conditions could not improve the recovery of the intact polar moiety but gave a large amount of ammonia. Phospholipase C or phospholipase D did not attack PNL2 under conditions that resulted in complete hydrolysis of phosphatidylserine. The best recovery of the polar group at moderate cleavage was achieved by the reaction with 46% hydrofluoric acid at 0°C for 24 hr. A typical result of the cleavage reaction is depicted in **Table 2.** Approximately 62% of PNL2 was hydrolyzed. The water-soluble degradation products were analyzed by an amino acid analyzer. Only three peaks were detected. Their retention times were compared with 37 amino acids and amino compounds that were completely resolved on the analyzer. The three products revealed the same retention times as those of serine, phosphoserine, and ammonia, respectively. The main product (76%) cochromatographed with serine by paper chromatography with solvent H. Thus, the water-soluble degradation products of PNL2 were estab-

TABLE 2. Cleavage of PLN2 with hydrofluoric acid

	μmol	%
PNL2 hydrolyzed[a]	0.654	100
Cleavage products		
Diether phosphatidic acid[a]	0.317	
Water-soluble phosphorus-containing compounds[a,b]	0.337	
Serine[c]	0.495	75.7
Phosphoserine[c]	0.023	3.5
NH_3[c]	0.103	15.7

PLN2 (1.06 μmol) dispersed in 1 ml of 46% hydrofluoric acid was incubated at 0°C for 24 hr. The hydrolyzate was lyophilized and partitioned between chloroform and water. Unreacted PLN2 in the chloroform phase was separated from phosphatidic acid (diether form) and glycerol diether by TLC. The water-soluble nitrogenous products were analyzed by an amino acid analyzer. Glycerol diether was not determined.
[a]Determined by phosphate assay.
[b]Water-soluble phosphorous-containing compounds contained phosphoserine and inorganic phosphate.
[c]Determined by an amino acid analyzer.

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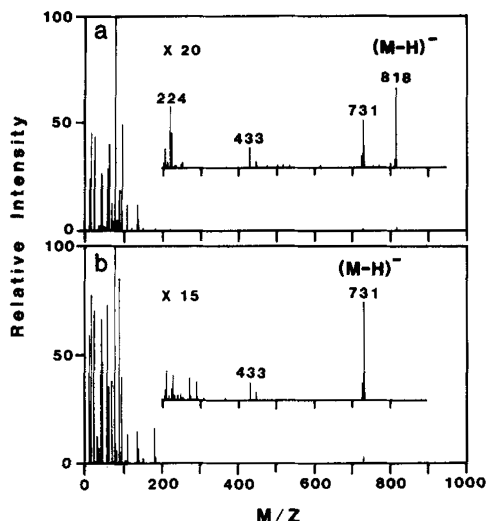

Fig. 5. Negative ion FAB-mass spectra of PNL2 (a), and the product of hydrofluoric acid hydrolysis of PNL2 (ether analog of phosphatidic acid) (b).

lished to be serine as the main component and phosphoserine as a minor component. The three compounds accounted for 95 mol % of hydrolyzed PNL2 (Table 2). No other amino compound was detected by the amino acid analyzer in the aqueous phase of the hydrolyzate. The phosphorus-containing chloroform-soluble product was identified as the diether analog of phosphatidic acid by TLC and FAB-mass spectrum (Fig. 5b, see below). The rather incomplete cleavage of PNL2 is probably due to the insolubility of the lipid in hydrofluoric acid.

Mass and PMR spectra of PNL2

Various ionization methods were attempted to take the mass spectrum of PNL2, such as EI, FD, and FAB (positive and negative ion). The molecular ion peak was detected only by negative ion FAB-mass spectrometry with a crown ether, 15-crown-5, added to glycerol as a matrix. The spectrum (**Fig. 5a**) showed peaks of m/z 818 (M - H), 731 ($M-CH_2CH(NH_3)COOH + H$), 433 ($M - OC_{20}H_{41} - CH_2CH(NH_3)COOH$) and 224 ($M - 2OC_{20}H_{41} - H$), which were absent in the background spectrum of the matrix. **Fig. 6** shows the FD-mass spectrum of the acid form of PNL2, which showed peaks of m/z 733, 653, and 634. The molecular ion was not detected even at the lowest anode temperature (20 mA) at which any ion peaks appeared. As described previously (25), the difficulty in yielding a molecular ion peak seems to be a specific phenomenon of phosphatidylserine. The masses and intensity of the recorded peaks could be interpreted by pyrolysis of PNL2 at both sides of the phosphodiester bond rather than fragmentation, and masses 733, 653, and 634 could be assigned to (diphytanylglycerophosphate + H)$^+$, (diphytanylglycerol + H)$^+$, and (diphytanylglycerol − H_2O)$^+$, respectively. Nevertheless, these peaks are consistent with the presence of a phosphate group being considered in combination with the FAB-mass spectrum described above. The FAB-mass spectrum of a phosphorus-containing chloroform-soluble product of hydrofluoric acid hydrolysis was recorded under the same conditions as that of intact PNL2 (Fig. 5b). A molecular ion peak (m/z 731, M - H) and a peak of m/z 433 ($M - OC_{20}H_{41} - 2H$) suggested that the compound was a diether analog of phosphatidic acid. The PMR spectrum of PNL2 (Na_2 salt, **Fig. 7**) showed signals at δ 0.85, 0.86 and 0.89 (30 H, $-CH_3$); δ 1.1-1.3 (48 H, $>CH_2$ and $>CH-$); δ 3.2-4.4 (12 H, $-CH_2-O-$, $>CH-O-$ and $-NH_2$). These assignments are consistent with PNL2 being derived from the diphytanylglycerol ether and having a serine residue.

DISCUSSION

On the basis of the results described above, the structure of PNL2, one of the major phospholipids in *M. arboriphilus*, is tentatively proposed as di-O-phytanyl glycerophosphorylserine (diether analog of phosphatidylserine, Fig. 1). While the present data cannot exclude the possibility that the backbone structure of PNL2 is α,α'-di-O-phytanyl glycerol, the proposed structure is more likely since the diphytanyl glycerol prepared from PNL2 showed an R_f value completely identical to that of 2,3-di-O-phytanyl glycerol, which would migrate more slowly on a thin-layer plate than 1,3-di-O-phytanyl glycerol. This is analogous to the chromatographic behaviors of 1,2-diacylglycerol and 1, 3-diacylglycerol—the former compound migrates more slowly (26). Stereochemical configurations of the diphytanyl glycerol and serine residues of PNL2 remain to be determined.

Makula and Singer (2) suggested the presence of phosphonolipids in *Methanobacterium.* The difficulty in obtaining complete hydrolysis of PNL2 might imply that PNL2 contained a phosphonate analog. Although the possibility of

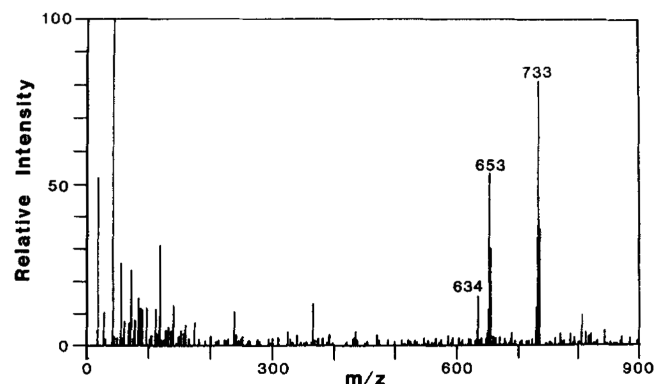

Fig. 6. FD-mass spectrum of PNL2.

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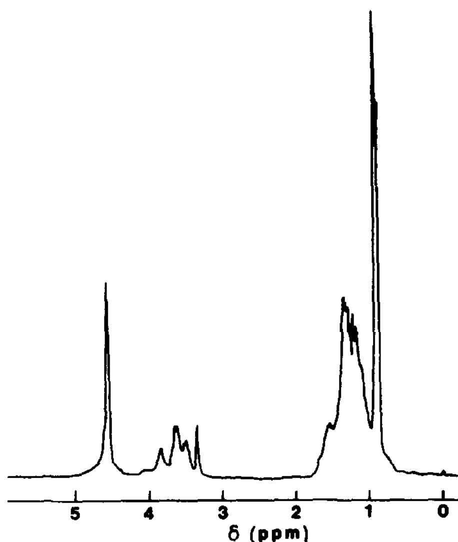

Fig. 7. PMR spectrum of PNL 2 recorded in $CDCl_3$-CD_3OD 1:1.

the presence of a very small amount of the phosphonate analog could not be completely excluded, the following observations suggested that most of PNL2 was in the phosphate form, even if the phosphate and phosphonate analog of PNL2 might be chromatographically copurified. Thus, *1*) carboxyl and amino groups were confirmed by ninhydrin reaction and the infrared spectrum, serine was identified in the water-soluble products of a hydrolyzate, the structure of diphytanyl glycerol was established, and the molecular weight of intact PNL2 was determined; *2*) FD-mass spectrum supported the presence of a phosphate group; and *3*) acetolysis and HF degradation were able to cleave both sides of the linkage of phosphorus.

This is the first report of a rather complete structural characterization of an amino group-containing diether phospholipid found in archaebacteria, although amino lipids occur widely in methanogenic bacteria. A preliminary survey detected ninhydrin-positive lipids in 17 strains of methanogens belonging to 12 species (M. Ohga and Y. Koga, unpublished observations). Many strains seem to possess a similar aminophospholipid which migrates with phosphatidylserine on TLC. For example, a ^{32}P-labeled aminophospholipid isolated from *Methanobacterium thermoautotrophicum* ΔH cochromatographed with PNL2 from *Methanobrevibacter arboriphilus* on a TLC plate (Y. Koga and M. Nishihara, unpublished results). PNL2, therefore, appears to be a rather common lipid among methanogenic bacteria. The distribution of amino lipids in methane-producing bacteria will be reported in detail elsewhere.

The authors are grateful to Dr. R. Isobe, Faculty of Pharmaceutical Sciences, Kyushu University, for obtaining mass spectra, and to Dr. Y. Kodera, Faculty of Sciences, Fukuoka University, for measuring PMR spectra. This work was partly supported by a Grant-In-Aid for Scientific Research (No. 59560094) from the Ministry of Education, Science and Culture of Japan.

Manuscript received 23 September 1985.

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