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Natural occurrence of archaetidic acid and caldarchaetidic acid (di- and tetra-ether analogues of phosphatidic acid) in the archaebacterium *Methanobacterium thermautotrophicum*[1]

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A minor phospholipid designated as PL5, assumed to be a precursor of phospholipid biosynthesis, was isolated from *Methanobacterium thermautotrophicum*. The structures of this lipid and another closely related phospholipid (PL4) were elucidated by infrared spectra, fast atom bombardment mass spectra, ^{31}P-nuclear magnetic resonance spectra, and chemical and enzymatic analyses. These lipids were identified as archaetidic acid (PL5) and caldarchaetidic acid (PL4) (diether and tetraether analogues of phosphatatidic acid, respectively).

Key words: archaetidic acid, caldarchaetidic acid, *Methanobacterium thermautotrophicum*, archaebacterium, ether lipid.

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Un phospholipide mineur, appelé PL5, est isolé de *Methanobacterium thermautotrophicum*. Ce phospholipide serait un précurseur de la biosynthèse des phospholipides. Nous avons élucidé les structures de ce lipide et d'un autre phospholipide (PL4) étroitement apparenté par spectrométrie infrarouge, spectrométrie de masse avec bombardement atomique rapide, spectrométrie de résonance magnétique nucléaire du ^{31}P et analyses chimique et enzymatique. Ces lipides sont l'acide archaetidique (PL5) et l'acide caldarchaeditique (PL4) (analogues diéther et tétraéther de l'acide phosphatidique).

Mots clés : acide archaetidique, acide caldarchaeditique, *Methanobacterium thermautotrophicum*, archaebactérie, analogues éther de lipides.

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Introduction

In 1960's Kates and his co-workers pioneered studies on ether lipids of *Halobacterium* (Sehgal *et al.* 1962; Kates *et al.* 1963). These were the first studies on archaebacterial ether lipids. Structures of tetraether lipids from *Thermoplasma* and *Sulfolobus*, the second group of archaebacteria, were described in 1970's (Langworthy *et al.* 1974; Langworthy 1977; De Rosa *et al.* 1977). Polar lipids of methanogens, the third group of archaebacteria, were first studied in 1980's (Kushwaha *et al.* 1981; Morii *et al.* 1986). The polar ether lipids of each group of archaebacteria have specific characteristics; that is, halobacteria have only diether lipids, while *Thermoplasma* and sulfur-dependent archaebacteria contain mainly tetraether lipids (Langworthy 1985), with the exceptions of *Thermococcus celar* which have only diether lipids (De Rosa *et al.* 1987) and *Sulfolobus* in which one of glycerol groups of the tetraether lipids is replaced by nonitol in many lipids. Ether lipids containing amino groups were first identified in methanogens (Morii *et al.* 1986), but not in other archaebacteria. Because methanogens contain both diether and tetraether types of polar lipids as major lipid components, a greater diversity of lipid species have been detected by TLC than in other archaebacteria (Koga *et al.* 1987).

Many biochemical studies have been made on *Methanobacterium thermautotrophicum* in these 10 years, but the major lipids of the organism have just been identified, and the structural regularities and the biosynthetic relationship have been described (the heptad concept, Nishihara *et al.* 1989). In addition to the three heptads of lipids (13 lipids), several minor lipids are present in the total lipid extract (Nishihara and Koga 1987). Because one of them (PL5) showed rapid metabolic turnover in a ^{32}P pulse-labelling and chase experiment (Nishihara *et al.* 1989), it was assumed to be a possible intermediate of ether phospholipid biosynthesis. Therefore, structure determination of PL5 and a related lipid (PL4) were undertaken. This communication describes the structures of the two lipids as diether and tetraether analogues of phosphatidic acid (archaetidic acid and caldarchaetidic acid). Temporary designations of polar lipids of *M. thermautotrophicum* shown in Fig. 2 of our previous paper (Nishihara and Koga 1987) and the nomenclature of archaebacterial lipids proposed by us (Nishihara *et al.* 1987) are used in this text.

Materials and methods

Materials

Phosphatidic acid (diacyl type, prepared from egg yolk phosphatidylcholine), phosphatidylcholine (egg yolk), phosphatidylethanolamine (egg yolk), phosphatidylinositol (soybean), and phosphatidylglycerol (prepared from egg yolk phosphatidylcholine) were purchased from Sigma. DL-α-glycerophosphate and β-glycerophosphate were obtained from Tokyo Kasei Kogyo Co.

Abbreviations: *sn*-G-3-P, L-α-glycerophosphate (*sn*-glycerol 3-phosphate); TLC, thin-layer chromatography; GLC, gas–liquid chromatography; IR, infrared; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; *sn*-G-1-P, *sn*-glycerol 1-phosphate.

[1]This paper is dedicated to Dr. Morris Kates in honour of his valuable contributions to biochemistry in Canada.

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(Tokyo, Japan) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. *sn*-G-3-P, NAD, and *sn*-G-3-P dehydrogenase (rabbit muscle) were purchased from Boehringer. Bis(trimethylsilyl) trifluoroacetamide was from Gasukuro Kogyo Co., Ltd. (Tokyo, Japan). $CDCl_3$ (99.8% atom D) was from Aldrich. Standard archaeol, caldarchaeol, phytane, and biphytane were prepared as described in the previous paper (Nishihara *et al.* 1989).

Growth of the bacterium and extraction of lipids

Growth conditions of *M. thermautotrophicum* ΔH (DSM1053) and extraction method of lipids with the acidified solvent were described previously (Nishihara and Koga 1987).

Thin-layer chromatography

TLC of lipids was carried out on a silica gel 60 plate (Merck) using the following solvents (compositions in volume ratios): solvent A, chloroform - methanol - 14 M aqueous ammonia (60:35:8); solvent B, chloroform - methanol - acetic acid - water (85:30:15:5); solvent C, light petroleum - diethyl ether - acetic acid (50:50:1). Lipid spots were detected as described in the previous paper (Nishihara and Koga 1987). Water-soluble dealkylated products were chromatographed two dimensionally on a cellulose TLC plate (Merck) with solvent D (ethanol - 14 M aqueous ammonia, 3:2), and solvent E (phenol–water, 100:38) in first and second dimensions, respectively. Spots on the cellulose plates were detected with salicylsulfonic acid - $FeCl_3$ reagent for phosphate esters (Vorbeck and Marinetti 1965).

Purification of lipids

Total lipids (400–500 mg, extracted from the cells of approximately 10 L of culture) were fractionated on a column (2.5 × 40 cm) of DEAE-cellulose (Brown, acetate form) as previously reported (Morii *et al.* 1986). After washing the column with methanol, PL4 and PL5 were eluted with 2000 mL each of acetic acid and 0.25 M ammonium acetate in methanol. Since PL4 and PL5 were present as only 2% each of total polar lipid (Nishihara and Koga 1987), the DEAE-cellulose column chromatography was performed eight times to obtain enough quantity of the lipids for analyses. The contaminants in the fractions (mainly inositol-containing phospholipids and phosphoglycolipid) were removed by TLC with solvent B. PL4 and PL5 were isolated each other by second TLC with solvent A. The purified preparation of each lipid showed one spot on two-dimensional TLC with solvents A and B.

Gas–liquid chromatography

GLC was performed by the use of a Shimadzu GC 9A gas–liquid chromatograph equipped with flame ionization detectors. Alkyl chlorides, alkyl iodides, and hydrocarbons prepared from PL4 and PL5 were analyzed on a 2% OV-11 on Chromosorb W AW-DMCS (80–100 mesh) column (1 m) at a temperature increasing from 100 to 340°C and a rate of 20°C/min. Trimethylsilyl derivatives of glycerophosphates were prepared and chromatographed as described previously (Nishihara *et al.* 1982).

Analytical methods and degradative procedures

Determination of phosphorus (Bartlett 1959), acid methanolysis, and acetolysis (Nishihara and Koga 1987) were carried out as described previously. Mild alkaline methanolysis was performed as described by Kates (1986). Glycerol ether bond was cleaved with BCl_3 according to the method of Nishihara and Koga (1988). A portion of the resultant aqueous methanol-soluble product (glycerol or glycerophosphate ester) was chromatographed two-dimensionally on a cellulose thin-layer plate with solvents D and E. Another portion of the fraction was subjected to hydrolysis with 2 M HCl at 100°C for 6 h and then glycerol was determined by the method of Lambert and Neish (1950). Hydrocarbon was prepared by HI cleavage of the ether linkage and subsequent reduction by $LiAlH_4$, as described by Nishihara *et al.* (1989). *sn*-G-3-P was determined by the use of *sn*-G-3-P dehydrogenase according to the method of Michal and Lang (1974).

Physical measurements

Infrared spectra of the lipids were recorded as thin films using a Shimadzu IR spectrometer (IR450S). Optical rotations were measured at 25°C at 589 nm with a high sensitivity polarimeter PM-201 (Otsuka Electronics Ltd. Japan). FAB mass spectra were obtained with a mass spectrometer (JMS D-300, Japan Electron Optics Laboratory, Japan) and thioglycerol as a matrix in a positive ion mode. ^{31}P-NMR spectra were measured by using a JNM-GX270 spectrometer (Japan Electron Optics Laboratory, Japan) operating at 109.25 MHz, with complete proton decoupling at 25°C. The spectrometer was equipped with deuterium field frequency stabilization and an automatic field homogeneity adjustment capability. The lipid sample was dissolved in $CDCl_3$–CH_3OH–H_2O (3.4:1.7:0.2, by volume) in a tube of 10 mm diameter. pH of the lipid solution was measured with pH test papers and adjusted by the addition of a trace amount (less than 20 μL) of 2 M HCl or 2 M KOH aqueous solution. The error of pH value of each lipid solution was less than ±0.2 pH units. Chemical shifts are given relative to the usual standard of external 85% H_3PO_4.

Results

PL5

This lipid was positive to molybdate reagent and negative to ninhydrin, α-naphthol, and periodate-Schiff reagents. The IR spectrum of the free acid form of PL5 showed absorptions corresponding to groups of methyl $-CH_3$ and methylene $-CH_2-$ (2830–2960, 1460, and 1375 cm^{-1}), $-CH(CH_3)_2$ (1360 cm^{-1}), ether C-O-C (1110 cm^{-1}), and phosphate P=O, $P-O^-$, and P-O-C (1210, 1100, and 1050 cm^{-1}). No ester carbonyl (1740 cm^{-1}) was detected. R_f values of this lipid on TLC with solvents A and B were 0.41 and 0.85, which were close to but slightly higher than those (0.37 and 0.82) of the diacyl type of phosphatidic acid. Archaebacterial ether phospholipids consistently showed slightly higher mobilities on TLC compared with the corresponding ester phospholipids with the same polar head groups. Acid methanolysis caused no change in PL5, while the diacyl type of phosphatidic acid was completely degraded. This was due to the fact that ether bonds resisted acid methanolysis, while the ester bonds of diacyl phospholipids could be readily methanolyzed. Acetolysis completely removed the phosphate group from PL5. An acetyl group, which had been attached during acetolysis, was removed by mild alkaline or acid methanolysis that yielded one compound which showed the same mobility as archaeol on TLC with solvent C. The hydrocarbon chain of PL5 was identified as phytane by GLC after HI cleavage and reduction. That is, PL5 had archaeol as a core lipid. The ratio of phosphate to glycerol, 1:1.07, indicated that PL5 had one phosphate group in one molecule. TLC of the aqueous methanol-soluble product of BCl_3 cleavage showed that it comigrated with the authentic α-glycerophosphate (R_fs were 0.23 and 0.30 with solvents D and E, respectively). Because BCl_3 does not affect the phosphodiester linkage (Nishihara and Koga 1988), the polar head group of PL5 was concluded to be the only phosphate that was bound to glycerol through a phosphomonoester linkage. ^{31}P-NMR spectra of PL5 in alkaline, neutral, and acidic conditions are shown in *a*, *b*, and *c* of Fig. 1, respectively. The chemical shifts of PL5 were quite similar to those of diacyl type of phosphatidic acid at respective pH (*d*, *e*, and *f* of Fig. 1), while PL5 showed much broader peaks at neutral and weakly acidic conditions (Table 1). The chemical shifts of both phospholipids were dependent on the pH of the solu-

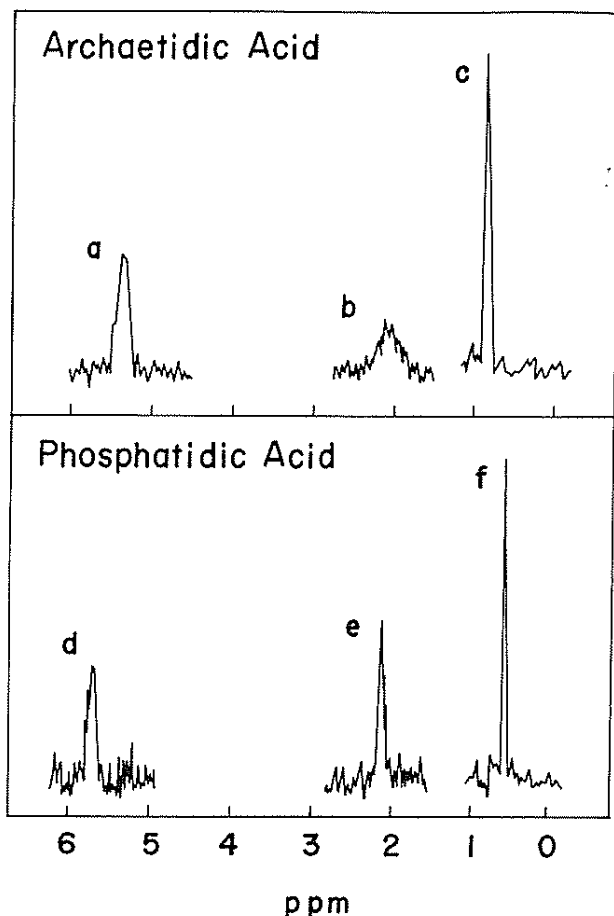

FIG. 1. ^{31}P-NMR spectra of PL5 (archaetidic acid, *a–c*) and authentic phosphatidic acid (diacyl type, *d–f*). Spectra were recorded at pH 10.1 (*a* and *d*), pH 7.5 (*b* and *e*), and pH 1.6 (*c* and *f*). Numbers of acquisitions and concentrations of lipids were the same as described in the Note of Table 1. Because each spectrum revealed only one peak, that portion near each peak was shown in this figure.

TABLE 1. ^{31}P chemical shifts of diacyl and diether types of phospholipids at various pH

	pH				
Lipid	1.6	3.8	7.5	10.1	12.8
PC	NR	0.28(9)	0.28(4)	0.28(5)	NR
PI	NR	0.28(10)	0.86(8)	0.89(3)	NR
PG	NR	1.50(13)	1.48(6)	1.73(3)	NR
PE	NR	1.20(7)	1.22(6)	1.45(5)	NR
PA	0.56(4)	1.25(8)	2.06(10)	5.72(15)	NR
PL5	0.92(10)	1.47(70)	2.07(45)	5.38(25)	5.63(3)
PL4	0.92(8)	1.36(36)	2.05(26)	5.30(13)	5.66(4)

NOTE: Chemical shifts are given in parts per million and peak widths at half height of a signal are given in Hertz in parentheses. Numbers of acquisitions were 50–200 for standard diacyl phospholipids (10–100 mg), 1000 for PL5 (14 mg), and 7000 for PL4 (3.7 mg). PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; NR, not recorded.

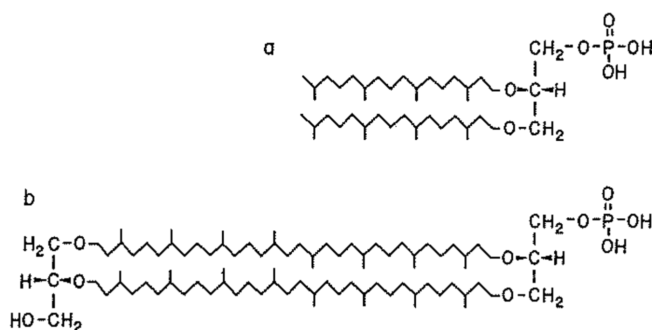

FIG. 2. Proposed structures of PL5 (archaetidic acid, *a*) and PL4 (caldarchaetidic acid, *b*).

tion; the peaks shifted downfield with increasing pH, in contrast with the NMR spectra of other phospholipids with phosphodiester linkage (Table 1). These spectra confirmed the presence of a phosphomonoester group in the molecule of PL5. The molecular weight of archaetidic acid should be 732.6. As expected, FAB mass spectra of PL5 gave m/z 733.8 ($(M + 1)^+$) in the positive ion mode. It is, therefore, concluded that PL5 is a diether analogue of phosphatidic acid (archaetidic acid, Fig. 2*a*).

PL4

The lipid designated as PL4 was detected on a TLC plate at the location close to PL5. The R_f values of PL4 (0.45 and 0.88 with solvents A and B, respectively) were slightly higher than that of PL5. This lipid gave the same responses to acid molybdate, α-naphthol, periodate–Schiff, and ninhydrin reagents on a TLC plate as PL5. Because PL4 seemed to be a lipid closely related with but different from PL5 in structure, identification of PL4 was undertaken. The IR spectrum of PL4 showed absorptions almost identical to those of PL5, except the presence of absorptions corresponding to hydroxy OH group (3400, 1040 cm^{-1}) and the absence of -$CH(CH_3)_2$ group (1360 cm^{-1}). No ester carbonyl (1740 cm^{-1}) was found. While methanolysis caused no effect on the structure of PL4, acetolysis and subsequent acid methanolysis gave caldarchaeol as a chloroform-soluble product. The hydrocarbon chain was identified as biphytane. The molar ratio of phosphorus to glycerol was 1:2.14. BCl_3 cleavage yielded α-glycerophosphate as the water-soluble product. ^{31}P-NMR signals of this phospholipid in acid, neutral, and alkaline solutions were identical to those of PL5 (Table 1). These results suggest that PL4 is caldarchaeol having one phosphate group at one free hydroxyl group of one of the glycerol moieties (molecular weight = 1381.3). The positive ion FAB mass spectrum of PL4 gave a peak of m/z 1381.9 ($(M + 1)^+$), confirming the structure of PL4 as caldarchaetidic acid (Fig. 2*b*).

Stereochemical structure of PL5 and PL4

Because of the small optical rotation and the small quantities of the lipids purified, measurement of optical rotation was not able to be employed for the determination of stereochemical structure of the lipids. The use of the stereospecific enzyme, *sn*-G-3-P dehydrogenase, was an alternative method for the determination of enantiomeric configuration of glycerophosphate moieties of PL5 and PL4. Glycerophosphate prepared from either lipid by BCl_3 treatment was not dehydrogenated with *sn*-G-3-P dehydrogenase in the presence of NAD (Table 2). GLC of trimethylsilylated glycerophosphate prepared from PL4 or PL5 showed that the glycerophosphate was α-glycerophosphate and not the β-form. Therefore, the glycerophosphate derived

TABLE 2. Stereochemical configuration of glycerophosphate derived from PL5 and PL4

Source of glycerophosphate	Glycerophosphate[a] (μmol)	sn-G-3-P[b]	
		μmol	%
Phosphatidic acid[c]	0.533	0.527	98.9
	0.899	0.795	89.4
PL5	0.372	0.010	2.8
	1.116	0.026	2.3
PL4	0.275	0.004	1.4
DL-α-Glycerophosphate	0.992	0.509	51.3
	1.488	0.744	50.0
β-Glycerophosphate	3.55	0	0

[a]Measured by phosphorus determination.
[b]Measured by the use of *sn*-G-3-P dehydrogenase.
[c]Diacyl form.

from PL5 or PL4 was *sn*-G-1-P, which was identical to the stereochemical structure of glycerophosphate residue of the other archaebacterial phospholipids.

Discussion

The structures of two archaebacterial ether phospholipids were elucidated. Although archaetidic acid has been known as an HF degradation product of archaetidylserine (Morii *et al.* 1986), a degradation product of archaetidylglycerol in the ion source of the mass spectrometer (Frederickson *et al.* 1989), or a chemically synthetic compound (Stewart *et al.* 1988), and labelled "prephosphatidic acid" was tentatively identified (Moldoveanu and Kates 1988), this paper is one of the first to describe the natural occurrence of these lipids. Archaetidic acid was recently found also in a halophilic bacterium (Lanzotti *et al.* 1989). However, there is a possibility that these lipids may be artefacts of extraction, isolation, or storage. None of these appear to be the case. The lipids were reproducibly extracted from *M. thermautotrophicum* by different methods (extraction from intact cells or disrupted cells with neutral or acidified solvents; data not shown). Some acid-labile phospholipids and phosphoglycolipids were present in this bacterium (e.g., gentiobiosyl caldarchaetidylinositol (PGL1)). In that case, the degradation product did not retain phosphate group because the acid hydrolysis required free hydroxy component group on the inositol residue adjacent to the phosphate moiety (the 2-OH of the glycerol residue was not liberated by acid hydrolysis because of its highly stable ether bond). This resulted in the cleavage of the phosphoester linkage between glycerol and phosphate groups. Moreover, these lipids showed a profile of metabolic turnover that was different from those of the other cellular phospholipids (Nishihara *et al.* 1989). These facts indicate the natural occurrence of these lipids.

Moldoveanu and Kates (1988) have found labelled prephosphatidic acid (an unsaturated derivative of archaetidic acid) in *Halobacterium cutirubrum* that had been pulse labelled with radioactive precursors. We have shown that PL5 underwent rapid turnover during the chase period after pulse labelling with $^{32}P_i$, and we tentatively identified PL5 as archaetidic acid from its mobility and staining responses on TLC and the ratio of its constituents (Nishihara *et al.* 1989). That is, unsaturated or saturated archaetidic acid are presumably present in archaebacteria including both an extreme halophile and a methanogen. The data presented in this paper confirmed the natural occurrence of this lipid, as well as caldarchaetidic acid. The role of the latter lipid is not clear. In spite of its small quantity, the diacyl type of phosphatidic acid in eukaryotes and eubacteria is a very important lipid, being the key intermediate in biosynthesis of glycerophospholipids. In archaebacteria, archaetidic acid may also play an important role in phospholipid biosynthesis; however, the complete biosynthetic pathway of ether phospholipids remains to be elucidated.

^{31}P-NMR data shown in Fig. 1 and Table 1 confirmed the NMR spectra of standard diacyl phospholipids reported in the previous papers (Henderson *et al.* 1974; London and Feigenson 1979; Meneses and Glonek 1988). In these papers the effect of pH on the chemical shift of phosphatidic acid was not clearly described. The dependency on pH of ^{31}P-NMR of the lipid and its diether and tetraether analogues is shown in the present report. One of the characteristics of the spectra of archaetidic acid and caldarchaetidic acid was the broadening of peaks (Table 1). This forced us to take spectra under condition requiring a large number of acquisitions. The peak broadening of ^{31}P-NMR spectra was most likely due to the formation of micelles or similar mesomorphs. This phenomenon appeared more with archaetidic acid and caldarchaetidic acid, which had ether bonds and highly branched hydrocarbon chains, than with the diacyl analogue (Table 1).

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