Biochem. J. (1987) 245, 139–143 (Printed in Great Britain)

Structure of two new aminophospholipids from *Methanobacterium thermoautotrophicum*

John K. G. KRAMER,* Frank D. SAUER* and Barbara A. BLACKWELL†

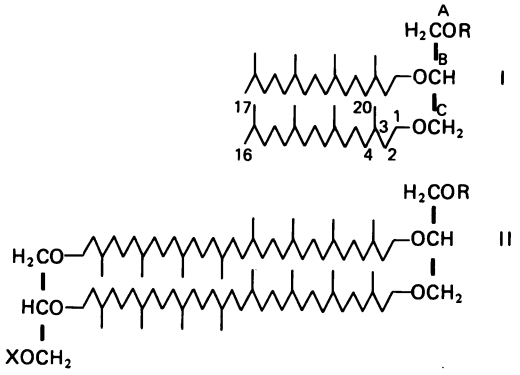
*Animal Research Centre and †Plant Research Centre, Research Branch, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada

The methanogenic bacterium *Methanobacterium thermoautotrophicum* (A.T.C.C. 29183) was shown to contain two new aminophospholipids. These are 2-aminoethyl phosphate ester of diphytanylglycerol diether and a sugar containing bisdiphytanyldiglycerol tetraether. The two aminophospholipids were stable to acid methanolysis except for the sugar on the bisdiphytanyldiglycerol tetraether. Strong acid (6 M-HCl) hydrolysed the alkyl ether and aminophosphate ester bonds. The structure of the phosphate linkage was demonstrated by ^{31}P n.m.r., and the 2-ethanolamine structure was elucidated by ^{1}H- and ^{13}C-n.m.r. spectroscopy and by fast-atom-bombardment m.s.

INTRODUCTION

The lipid core of methanogenic bacteria (Makula & Singer, 1978; Tornabene & Langworthy, 1978; Kushwaha *et al.*, 1981*a*,*b*; Grant *et al.*, 1985; De Rosa *et al.*, 1986*a*,*b*), like that of extremely halophilic (Kates *et al.*, 1965, 1966) and extremely thermophilic bacteria (Langworthy, 1977), consists mainly of diphytanylglycerol diethers (Ia) and bisdiphytanyldiglycerol tetraethers (IIa) (Fig. 1). The ethers are linked to positions 2 and 3 of glycerol instead of positions 1 and 2 normally present in bacterial and mammalian phospholipids and diglycerides (Kates & Kushwaha, 1978). In addition, the lipid of one strain of methanogen (*Methanococcus jannaschii*) consists predominantly of a macrocyclic glycerol diether in which two phytanyl chains are covalently linked end to end to form a C_{40} cyclic loop (Comita & Gagosian, 1983; Comita *et al.*, 1984). Some extreme halophiles contain small amounts of 2-*O*-sesterterpanyl-3-*O*-phytanylglycerol diether (C_{25}, C_{20}) (Grant *et al.*, 1985), and some extreme thermophiles contain bidiphytanyl chains with one to three cyclopentane rings (De Rosa *et al.*, 1986*a*). These isoprenoid glycerol ethers were found to be ester-linked to disaccharides, phosphates and glycerophosphate (Kushwaha *et al.*, 1981*a*,*b*).

Grant *et al.* (1985) reported finding lipids in methanogenic bacteria which stained positively with ninhydrin. No attempt was made to identify the structure of these aminolipids. In the present investigation aminolipids of *Methanobacterium thermoautotrophicum* (A.T.C.C. 29183) were isolated and their structures were elucidated by a combination of chemical, chromatographic and spectroscopic techniques.



	X	R
Ia	-	H
Ib	-	$P(O_2H)O\overset{a}{C}H_2\overset{b}{C}H_2NH_2$
IIa	H	H
IIb	H	$P(O_2H)OCH_2CH_2NH_2$
IIc	Sugars	$P(O_2H)OCH_2CH_2NH_2$

Fig. 1. Structures of diphytanylglycerol diethers (I) and bisdiphytanyldiglycerol tetraethers (II) from *M. thermoautotrophicum*

MATERIALS AND METHODS

Cell culture

M. thermoautotrophicum (A.T.C.C. 29183) cells were grown in sorbitol-free medium and harvested as described by Sauer *et al.* (1984, 1986).

Lipid extraction

The bacterial pellets were washed and resuspended in 4 vol. of water, and were disrupted by ultrasonication in a Rosett Cooling Cell (Heat Systems–Ultrasonics, Plainview, NY, U.S.A.) at 70% full power for a total of 10 min, with 1 min of power on followed by 1 min of power off. The ruptured cell mixture was diluted with 2 vol. of water and then extracted for total lipids by the method of Bligh & Dyer (1959). The upper phase (methanol/water) was extracted two more times with chloroform, and the chloroform layers were combined and represented total lipids extracted.

To test whether the methanol/water phase contained additional lipids difficult to extract, as suggested by Tornabene & Langworthy (1978), this phase was evaporated to dryness and the residue was refluxed with hot chloroform/methanol (1:1, v/v) and filtered. To the filtrate was added 0.9 part of water (chloroform/

Abbreviations used: DEPT, distortionless enhancement of polarization transfer; f.a.b., fast atom bombardment.

methanol/water, 1:1:0.9, by vol.), and the chloroform phase was tested for lipids by t.l.c.

G.l.c.–m.s.

The isoprenoid hydrocarbons were separated by g.l.c. on a Durabond DB-5 coated fused-silica capillary column (15 m × 0.25 mm internal diam.) (J & W Scientific Co., Rancho Cordova, CA, U.S.A.), temperature-programmed from 150 °C at 5 °C/min to 250 °C and maintained at 250 °C for 10 min. The carrier gas was He. The mass spectra were recorded with a Finnigan model 1020 automated system operated at 70 eV, temperature at source 80 °C. Spectra ranging in mass units from 30 to 600 were acquired repeatedly in 1 s increments.

N.m.r. spectroscopy

^{1}H-, ^{13}C- and ^{31}P-n.m.r. spectra were recorded with a Bruker WM 250 spectrometer operated at 250, 62.8 and 101.2 MHz respectively. Spectra were measured with a 40 mg/ml sample (5 mm-outer-diam. sample tube) at ambient probe temperature. ^{13}C-n.m.r. spectra consisted of 16000 data points with a spectral width of 5200 Hz and recycle time of 3.0 s and were plotted with resolution enhancement. The solvents were used as shift standards for ^{1}H- and ^{13}C-n.m.r. spectra and chemical shifts were expressed in parts per million (p.p.m.) downfield from tetramethylsilane. ^{31}P chemical shifts were expressed in p.p.m. relative to 85% H_3PO_4.

Deuterated chloroform or methanol alone resulted in poor resolution of phospholipid proton resonances owing to micelle formation, as noted by Murari & Baumann (1981). These authors suggested the use of deuterated chloroform/methanol/water (10:10:3, by vol.) as solvent for measuring n.m.r. of phospholipids. The use of this solvent system greatly improved the n.m.r. signals. There was an additional peak at δ 1.91 p.p.m. in the ^{1}H-n.m.r. spectrum of both phospholipids and isoprenoid ethers of glycerol. This peak varied in relative intensity between measurements, and represented residual acetic acid. Most of this acetic acid could be removed by exhaustive drying under high vacuum.

F.a.b. m.s.

F.a.b. mass spectra were recorded on a Finnigan MAT 312 mass spectrometer with the use of a glycerol mix (Paré *et al.*, 1985).

T.l.c.

All the plates were prewashed before use by developing them in chloroform/methanol (2:1, v/v), and re-activated at 110 °C for 1 h. Lipids were separated by two-dimensional t.l.c. with solvent A [chloroform/methanol/conc. ammonia (6:3:1, by vol.)] followed by solvent B [chloroform/acetone/methanol/acetic acid/water (10:4:2:1:1, by vol.)]. The neutral lipids were separated by one-dimensional t.l.c. with solvent C [hexane/diethyl ether/acetic acid (50:50:1, by vol.)]. Products of acid methanolysis were resolved by solvents C, A and B.

Lipids were located either by charring after spraying with conc. H_2SO_4/ethanol (1:2, v/v) or under u.v. light (long-wave) after spraying with 0.1% 2′,7′-dichlorofluorescein in methanol. Specific stains were used to identify phospholipids (Kundu *et al.*, 1977), glycolipids (Krebs *et al.*, 1969, spray no. 175) and aminolipids (Krebs *et al.*, 1969, spray no. 178).

T.l.c. densitometry was carried out with a Chromaflux model K-495000 densitometer (Kontes, Vineland, NJ, U.S.A.).

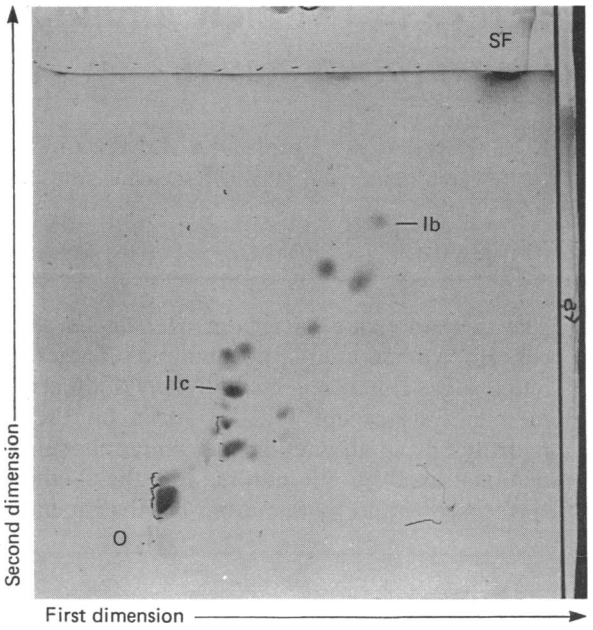


Fig. 2. Two-dimensional t.l.c. of the total lipids from *M. thermoautotrophicum*

The first development was with solvent A and the second development with solvent B. Lipids were located by charring after spraying with H_2SO_4/ethanol (1:2, v/v). Spot Ib is a 2-aminoethyl phosphate ester of diphytanylglycerol diether, and spot IIc is a glyco derivative of a 2-aminoethyl phosphate ester of bisdiphytanyldiglycerol tetraether. Key: O, spot of application; SF, solvent front.

Materials

All solvents and chemicals were of analytical grade and were used without further purification. Deuterated solvents were obtained from Merck, Sharp and Dohme Isotopes, Points Claire, Que., Canada. Precoated silica gel G plates (Redi-plates), 20 cm × 20 cm and 250 μm in thickness, were purchased from Fisher Scientific Co., Ottawa, Ont., Canada. Diphytanylglycerol diether and bisdiphytanyldiglycerol tetraether were isolated from the total lipids of *M. thermoautotrophicum* after acid hydrolysis with 5% (w/w) HCl (gas) in anhydrous methanol for 1 h.

RESULTS AND DISCUSSION

The total lipids extracted from *M. thermoautotrophicum* (A.T.C.C. 19183) cells accounted for 1.7% of cell dry matter. This fraction contained no non-lipid contaminants. Tornabene & Langworthy (1978) reported a lipid content of 2.8% for these bacteria. They used an additional hot extraction with chloroform/methanol (1:1, v/v) for a complete extraction of lipids. In our hands this procedure yielded an additional 0.3% of material, which, however, was non-lipid. It appears that

M. thermoautotrophicum has a lower lipid content than has been reported for other methanogenic, halophilic or thermophilic bacteria (Tornabene & Langworthy, 1978).

The lipids were separated by two-dimensional t.l.c. into about 20 spots (Fig. 2) by using solvent A and solvent B consecutively. This combination of solvent systems gave better resolutions than a neutral solvent system [chloroform/methanol/water (65:25:4, by vol.)] followed by one containing acetic acid [chloroform/methanol/acetic acid/water (80:12:15:5, by vol.)] described by Grant *et al.* (1985). In addition, the use of ammonia instead of diethylamine (Kushwaha *et al.*, 1981*b*) gave a superior separation and permitted development in the second dimension (diethylamine is virtually impossible to remove completely from a t.l.c. plate).

Grant *et al.* (1985) reported the presence of several spots on a two-dimensional t.l.c. of lipids from methanogenic bacteria that stained for both amine and phosphorus (aminophospholipids) and for amine, sugar and phosphorus (aminoglycophospholipids). Three of the spots in Fig. 2 stained positively with ninhydrin, spot Ib, spot IIc and the solvent front (SF). Both spot Ib and spot IIc stained positively for phosphorus, and in addition spot IIc stained positively for sugars. The compounds corresponding to spots Ib and IIc were isolated and purified by preparative t.l.c. first with solvent A. After elution of the bands from the silica gel, the fractions were further purified by t.l.c. with solvent B. As measured by t.l.c. densitometry, the concentrations of compounds Ib and IIc were 3% and 14% respectively of the total polar lipids.

Reaction of compounds Ib and IIc with 5% (w/w) HCl in methanol for 1 h at 80 °C removed only the sugar moieties from compound IIc. Product IIb, produced from compound IIc by acid methanolysis, migrated slightly ahead of compound Ib on t.l.c. with solvent A. Only a small amount of compounds Ib and IIb reacted even after 24 h of acid methanolysis. A more drastic hydrolysis of compounds Ib and IIb with 6 M-HCl/methanol (1:1, v/v) for 24 h at 100 °C gave some diether and tetraether from compounds Ib and IIc respectively, in addition to partially hydrolysed ether linkages. The diether and tetraether products had the same R_F values on t.l.c. (solvent C) as authentic diphytanylglycerol diether and bisdiphytanyldiglycerol tetraether. These two compounds were isolated by t.l.c. and analysed by f.a.b. m.s. The product from compound Ib had an $M+1$ ion (M, molecular ion) of m/z 653.7 (theoretical m/z 653.68), and the product from compound IIb one of m/z 1301.6 (theoretical m/z 1302.32). (exact mass determinations above m/z 1000 were not possible.)

Reaction of compounds Ib and IIc with BBr_3 followed by $LiAlH_4$ as described by Comita *et al.* (1984) gave hydrocarbons that were identified by g.l.c.–m.s. as phytane and diphytane respectively. No diphytane was observed in the mixture from compound Ib, which would have provided evidence for a macrocyclic diphytanyl glycerol ether identified by Comita *et al.* (1984) in *Methanococcus jannashii*. The chemical evidence clearly established the presence of a diphytanylglycerol diether (Ia) and a bidiphytanyldiglycerol tetraether (IIa) backbone in these two lipids (Fig. 1).

The nature of the phosphorus linkage was established by ^{31}P n.m.r. A signal was observed at 2.3 p.p.m. relative to 85% H_3PO_4, which indicated a phosphate ester linkage. This was somewhat surprising, since some phosphate esters in the lipid mixture of these bacteria were so difficult to hydrolyse with 5% (w/w) HCl in methanol, whereas most of the other phospholipids from these and other methanogenic bacteria (Tornabene & Langworthy, 1978; Kushwaha *et al.*, 1981*b*) were hydrolysed completely within 1 h under these conditions. The presence of acid-stable phospholipids in *M. thermoautophicum* was also observed by Makula & Singer (1978), who did not identify the nature of these compounds. A phosphono (–C–P–O–C–) or a phosphino (–C–P–C–) type linkage was considered because of the well-known stability of the C–P bond to acid hydrolysis (Slotboom & Bonsen, 1970; Hori & Nozawa, 1982). However, the signal at 2.3 p.p.m. in the ^{31}P-n.m.r. spectrum of compound Ib would not support a phosphono or phosphino linkage since the ^{31}P signals should have been at -18 to -24 p.p.m. (Hori & Nozawa, 1982) and at -40 to -50 p.p.m. (Mark *et al.*, 1967) respectively. The reason why some phosphate esters are hydrolysed more easily than others is not completely understood. It appears that the structure of some side chains (e.g. a glycerol side chain) may promote the cleavage of the phosphate ester bond whereas other side chains (e.g. 2-aminoethyl) do not have the same effect.

The size of the side chain of these two compounds, containing an amine group, was established by f.a.b. m.s. Each compound gave a strong $M+1$ ion, at m/z 776.7 (compound Ib) and at m/z 1425.1 (compound IIb). The difference between these $M+1$ ions and the corresponding glycerol diether (m/z $776.7-653.7=123.0$) and the diglycerol tetraether (m/z $1425.1-1301.6=123.5$) was basically the same. On the basis of these results, a 2-aminoethyl phosphate side chain having a mass of m/z 123.01 was considered for both compound Ib and compound IIb.

The ^1H-n.m.r. and ^{13}C-n.m.r. spectra confirmed the structure proposed in Fig. 1. The ^1H-n.m.r. spectra of compound Ib and diphytanylglycerol diether are compared in Table 1. In addition to the resonances associated with the isoprenoid chain and the glycerol diether, resonances corresponding to eight protons were observed in the region δ 3.0–4.1 p.p.m. A sharp singlet at δ 3.34 p.p.m. indicated the presence of a terminal amine group. Three resonances at δ 3.10, 3.88 and 4.03 p.p.m. each integrated as two protons, and were assigned to methylene groups adjacent to an N atom (δ 3.10 p.p.m.) or an O–P bond (δ 3.88 and 4.03 p.p.m.). The assignment of the two methylene groups on either side of the phosphate ester were determined by irradiating the resonance at δ 3.10 p.p.m. and observing a sharpening of the resonance at δ 4.03 p.p.m., indicating the presence of coupling.

The ^{13}C-n.m.r. spectrum of compound Ib is shown in Fig. 3. Resonances in the region of δ 20–40 p.p.m. were assigned to the carbon atoms of the isoprenoid chain. These are the same as in the ^{13}C-n.m.r. spectrum of compound Ia and agree closely with those reported for pristane (Lindeman & Adams, 1971). Carbon resonances for the polar head group were similar to those reported for ethers and esters of glycerol and 1,2-ethanediol phospholipids (Murari *et al.*, 1982), and are compared in Table 2. A DEPT (distortionless enhancement of polarization transfer) spectrum (Doddrell *et al.*, 1982)

Table 1. Comparison of 250 MHz ^{1}H-n.m.r. spectra of compounds Ia and Ib isolated from *M. thermoautotrophicum*

Chemical shifts are expressed as p.p.m. from tetramethylsilane; that of [$^2H_4$]methanol was taken as 3.30 p.p.m.

Chemical shift (p.p.m.)	Multiplicity	Assignment	Glycerol diether (Ia)		Compound Ib	
			Calc.	Found	Calc.	Found
0.80–0.90	Complex m	Methyl	30	28.1	30	29.5
0.98–1.43	Complex m	Methylene	40	42.2 and 78.0	40	42.5 and 77.8
1.43–1.65	Complex m	Methine	8	7.7	8	5.8
3.10	Broad s	–C*H*$_2$–NH$_2$ (b)	–	–	2	2.0
3.34	Sharp s	–CH$_2$–N*H*$_2$	–	–	2	2.2
3.42–3.67	Complex t	–C*H*$_2$–O (C,1,1′)	6		6	7.0
3.50	Complex m	–C*H*–O (B)	1	9.0	1	
3.4	Complex t	–C*H*$_2$–OH (A)	2		–	–
3.88 (J = 5.2 Hz)	t	–C*H*$_2$–O–P (A)	–	–	2	1.9
4.03	Broad s	–C*H*$_2$–O–P (a)	–	–	2	2.0

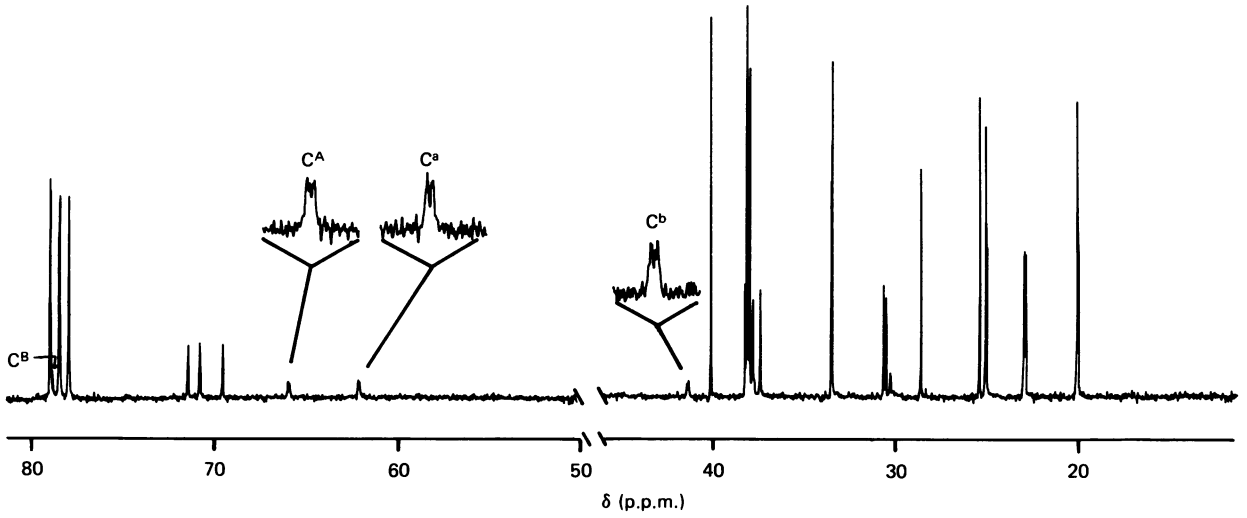


Fig. 3. 62.8 MHz ^{1}H-decoupled ^{13}C-n.m.r. spectrum of compound Ib in deuterated chloroform/methanol (1:1, v/v)

The chemical-shift (δ) assignments of the carbon atoms of the isoprenoid chains in p.p.m. from tetramethylsilane are: 20.08 (C-18, C-19 and C-20); 22.87 and 22.96 (C-16 and C-17); 25.03 and 25.35 (C-5, C-9 and C-13); 28.55 (C-15); 30.45 and 30.58 (C-3); 33.39 and 33.43 (C-7 and C-11); 37.34, 37.74, 37.90, 38.01, 38.05 and 38.16 (C-4, C-6, C-8, C-10 and C-12); 40.02 (C-14). Shift assignments of the remaining carbon atoms associated with the polar head group are given in Table 2. Inserts are enhancements of the resonances due to the two *C*–O–P groups and H$_2$N–*C*–C–O–P to show the presence of C–P couplings.

Table 2. Comparison of ^{13}C-n.m.r. spectra of the phospholipid head groups

Compound Ib was isolated from the *M. thermoautotrophicum* lipids by t.l.c. with the solvent mixtures described in the Materials and methods section. Phospholipid no. 9 is 1,2-di-*O*-hexadecyl-3-*O*-[(2-(trimethylamino)ethyl)phospho]-rac-glycerol, and phospholipid no. 17 is 1-*O*-hexadecyl-2-*O*-[(2-aminoethyl)phospho]ethanediol. The ^{13}C-n.m.r. spectra of these two compounds were reported by Murari *et al.* (1982), who used the deuterated solvent mixture of chloroform/methanol/water (10:10:3, by vol.). $^3J_{CP}$ for C^B of compound Ib was not determined because the resonance could not be resolved from those of [$^2H_3$]chloroform. Abbreviation: n.a., not applicable for comparison purposes. – indicates no carbon.

Head-group carbon	Phospholipid		
	Compound Ib	No. 9	No. 17
C^1 and C$^{1'}$	70.73 and 69.51	72.16 and 71.06	71.90
C^A	65.47 ($^2J_{CP}$ = 4.4 Hz)	65.54 ($^2J_{CP}$ = 5.4 Hz)	65.13 ($^2J_{CP}$ = 5.4 Hz)
C^B	78.68	78.95 ($^3J_{CP}$ = 8.3 Hz)	n.a.
C^C	71.37	72.16	–
C^a	62.20 ($^2J_{CP}$ = 3.2 Hz)	n.a.	62.19 ($^2J_{CP}$ = 5.1 Hz)
C^b	41.25 ($^3J_{CP}$ = 5.0 Hz)	n.a.	40.87 ($^3J_{CP}$ = 6.5 Hz)

was used to determine multiplicity of the carbon resonances and confirm their assignment.

This is the first report of the identification of a phosphoethanolamine-like compound in methanogenic bacteria. One may speculate that these aminophospholipids serve the same function in membranes of methanogenic bacteria as phosphatidylethanolamine and phosphatidylcholine serve in mammalian and other bacterial membranes. The stability of this amino phosphate ester, which is similar to that of the ether linkage itself, probably serves to protect the lipids in these extreme thermophiles from non-enzymic destruction at high temperature.

We are grateful for the technical assistance of Mr. R. C. Fouchard and Mr. P. Lafontaine for performing the f.a.b. m.s. This paper is contribution no. 1421 from the Animal Research Centre and no. 1019 from the Plant Research Centre.

REFERENCES

Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917

Comita, P B. & Gagosian, R. B. (1983) Science **222**, 1329–1331

Comita, P. B., Gagosian, R. B., Pang, H. & Costello, C. E. (1984) J. Biol. Chem. **259**, 15234–15241

De Rosa, M., Gambacorta, A., Lanzotti, V., Trincone, A. Harris, J. E. & Grant, W. D. (1986*a*) Biochim. Biophys. Acta **875**, 487–492

De Rosa, M., Gambacorta, A. & Gliozzi, A. (1986*b*) Microbiol. Rev. **50**, 70–80

Doddrell, D. M., Pegg, D. T. & Bendall, M. R. (1982) J. Magn. Reson. **48**, 323–327

Grant, W. D., Pinch, G., Harris, J. E., de Rosa, M. & Gambacorta, A. (1985) J. Gen. Microbiol. **131**, 3277–3286

Hori, T. & Nozawa, Y. (1982) in Phospholipids (Hawthorne, J. N. & Ansell, G. B., eds.), pp. 95–128, Elsevier Biomedical Press, Amsterdam

Kates, M. & Kushwaha, S. C. (1978) in Energetics and Structure of Halophilic Microorganisms (Caplan, S. R. & Ginzburg, M., eds.), pp. 461–479, Elsevier/North-Holland Biomedical Press, Amsterdam

Kates, M., Yengoyan, L. S. & Sastry, P. S. (1965) Biochim. Biophys. Acta **98**, 252–268

Kates, M., Palameta, B., Joo, C. N., Kushner, D. J. & Gibbons, N. E. (1966) Biochemistry **5**, 4092–4099

Krebs, K. G., Heuser, D. & Wimmer, H. (1969) in Thin-Layer Chromatography (Stahl, E., ed.), 2nd edn., pp. 854–909, Springer-Verlag, Heidelberg

Kundu, S. K., Chakravarty, S., Bhaduri, N. & Saha, H. K. (1977) J. Lipid Res. **18**, 128–130

Kushwaha, S. C., Kates, M., Sprott, G. D. & Smith, I. C. P. (1981*a*) Science **211**, 1163–1164

Kushwaha, S. C., Kates, M., Sprott, G. D. & Smith, I. C. P. (1981*b*) Biochim. Biophys. Acta **664**, 156–173

Langworthy, T. A. (1977) Biochim. Biophys. Acta **487**, 37–50

Lindeman, L. P. & Adams, J. Q. (1971) Anal. Chem. **43**, 1245–1252

Makula, R. A. & Singer, M. E. (1978) Biochem. Biophys. Res. Commun. **82**, 716–722

Mark, V., Dungan, C. H., Crutchfield, M. M. & Van Wazer, J. R. (1967) in Topics in Phosphorus Chemistry (Crutchfield, M. M., Dungan, C. H., Letcher, J. H., Mark, V. & Van Wazer, J. R., eds.), vol. 5, pp. 293–294, Interscience, New York

Murari, R. & Baumann, W. J. (1981) J. Am. Chem. Soc. **103**, 1238–1240

Murari, R., Abd El-Rahman, M. M. A., Wedmid, Y., Parthasarathy, S. & Baumann, W. J. (1982) J. Org. Chem. **47**, 2158–2163

Paré, J. R. J., Greenhalgh, R., Lafontaine, P. & ApSimon, J. W. (1985) Anal. Chem. **57**, 1470–1472

Sauer, F. D., Mahadevan, S. & Erfle, J. D. (1984) Biochem. J. **221**, 61–69

Sauer, F. D., Blackwell, B. A. & Mahadevan, S. (1986) Biochem. J. **235**, 453–458

Slotboom, A. J. & Bonsen, P. P. M. (1970) Chem. Phys. Lipids **5**, 301–398

Tornabene, T. G. & Langworthy, T. A. (1978) Science **203**, 51–53

Received 29 September 1986/18 December 1986; accepted 16 March 1987