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A complex lipid with a cyclic phosphate from the archaebacterium *Natronococcus occultus*

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Polar lipid extract from the haloalkaliphilic archaebacterium *Natronococcus occultus* contains a new type of phospholipid. Spectroscopic methods establish the structure as a phosphatidylglycerol phosphate derivative with a cyclic phosphate (2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycero-1′,2′-cyclic phosphate). A 2-*O*-sesterterpanyl-3-*O*-phytanyl (C_{25},C_{20}) glycerol diether form of the novel phospholipid is also present.

Introduction

All halophilic archaebacteria have lipids based on 2,3-di-*O*-phytanyl-*sn*-glycerol (C_{20},C_{20}) [1], and the haloalkaliphilic isolates also have substantial amounts of lipids based on 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol (C_{25},C_{20}) [2,3]. The complex lipids of these organisms have diphytanyl glycerol linked to different polar groups, such as glycerol phosphate, glycerol sulfate, glycerol diphosphate, sugars and sugar sulfates [4–6].

The haloalkaliphilic archaebacteria of the genus *Natronobacterium* and *Natronococcus* have a relatively simple polar lipid composition, the major species of polar lipids being C_{20},C_{20} and C_{25},C_{20} derivatives of phosphatidylglycerol (PG) and phosphatidylglycerol phosphate (PGP) [7,8]. However, there are also several minor unidentified phospholipids present [7,9] in examples of these genera. In particular, *Natronococcus occultus* has a significant amount of a phospholipid designated PL2 by Morth and Tindall [9]. This phospholipid has a mobility intermediate between those of PG and PGP when analysed by TLC in $CHCl_3$/MeOH/H_2O (65 : 25 : 4, v/v.) (Fig. 1).

Recently, spectroscopic methods, including ^{13}C-NMR, have been used to unequivocally establish the structures of C_{20},C_{20} and C_{25},C_{20} derivatives of PG and PGP from *Natronobacterium* and *Natronococcus* spp. [8]. Here, we report a similarly detailed characterization of the polar lipid PL2 from *Nc. occultus*.

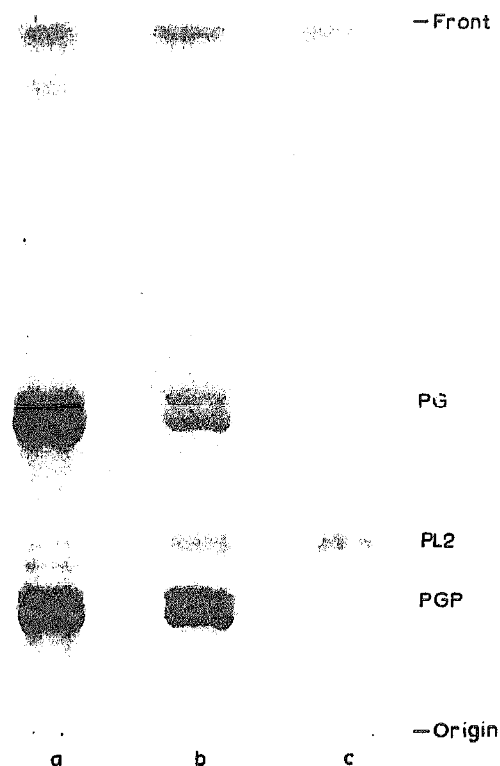

Fig. 1. TLC of mixture of standards PG and PGP (a); extract of polar lipid of *Natronococcus occultus* (b); PL2 (TLC-pure) (Fig. 2) (c). Solvent system $CHCl_3$/MeOH/H_2O (65 : 25 : 4, v/v).

Abbreviations: PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate.

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Materials and Methods

Microorganism and culture conditions

Natronococcus occultus (NCMB 2192) was obtained from the National Collection of Marine Bacteria, Torry Research Station PO Box 31, Aberdeen AB9 8DG, U.K., and was grown as described by Tindall et al. [7]. Cells were harvested in the late exponential phase by centrifugation, washed with a basal salts solution, and lyophilized.

Extraction and isolation of lipids

Lipids were extracted from wet cells (30 g) with $CHCl_3$/MeOH (1 : 1, v/v) and MeOH/H_2O (1 : 1, v/v) as described elsewhere [8], purified on a silica gel column (70 g, Kieselgel, 70–230 mesh, Merck) (40 cm × 10 mm, i.d.). The column was eluted with 3 l of a step gradient of 0–50% (v/v) MeOH in $CHCl_3$ in 5% increments (250 ml for each gradient step). The minor compound PL2 was eluted with $CHCl_3$/MeOH (95 : 5, v/v) and the major polar lipids PGP and PG were eluted, respectively, with $CHCl_3$/MeOH (90 : 10, v/v) and $CHCl_3$/MeOH (85 : 15, v/v), and all were weighed to evaluate their percentages [8].

Chemical procedures

The lipids were permethylated with dimethylformamide/NaH/MeI using a standard procedure [10].

Acid methanolysis of lipids was done in dry methanolic 2 M HCl. The reaction mixture was heated at 110 °C in a stoppered reaction tube for 16 h [11]. After being cooled, the hydrolysis products were dried under vacuum and then extracted with $CHCl_3/H_2O$ (1 : 1, v/v). After thorough mixing, the $CHCl_3$ phase was analysed by HPLC using a Waters Associates apparatus equipped with a differential refractometer. 2,3-Di-*O*-phytanyl-*sn*-glycerol and 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol were detected in the $CHCl_3$ phase by elution with *n*-hexane/diethyl ether (8 : 2, v/v) using a Microporasil column (flow rate 1 ml/min) [3]. Glycerol was identified in the aqueous phase by elution with acetonitrile/H_2O (9 : 1, v/v) on a carbohydrate column (flow rate 2 ml/min).

Thin-layer chromatography

Polar lipids were analysed by two-dimensional thin-layer chromatography on silica gel (0.25 mm, F 254, Merck) using as solvent system $CHCl_3$/MeOH/H_2O (65 : 25 : 4, v/v) in the first dimension, and $CHCl_3$/MeOH/CH_3COOH/H_2O (80 : 12 : 15 : 4, v/v) in the second dimension [12].

Compounds were detected by exposure to I_2 vapour, or by spraying with 0.1% $Ce(SO_4)_2$ (w/v) in 2 M H_2SO_4, followed by heating at 150 °C for 5 min. Dittmer and Lester reagent [13] was also used for phospholipids.

Quantitative analysis

Phosphorus was determined by the method of Ames [14] and glycerol was assayed enzymatically [15]. Glycerol diethers were determined gravimetrically after drying to constant weight.

^{13}C-NMR spectroscopy

Samples for NMR analysis were prepared by dissolving each sample in 0.5 ml $C^2HCl_3/C^2H_3O^2H$ (7 : 3, v/v).

All the ^{13}C-NMR spectra were run at 125 MHz on a Bruker WH-500 spectrometer. ^{13}C Fourier transform NMR spectra, with sweep width and transmitter frequencies optimized for the lowest possible digital resolution (Hz/data point), were obtained from 32 K free induction decay signals. Exponential multiplication was applied previously by Fourier transformation, and line broadening constants were adapted for the digital resolution.

Analysis of multiplicities was achieved by distortionless enhancement by the polarization transfer (DEPT) technique, using a commercially available microprogramme. Two DEPT experiments were performed using polarization transfer pulses of 90 and 135°, respectively, obtaining in the first case only signals for methine groups and in the other case positive signals for methine and methyl groups, and negative signals for methylene groups.

Mass spectrometry

MS analyses were done with a Kratos MS-50 instrument equipped with a Kratos fast atom bombardment (FAB) source. The negative FAB mass spectra were obtained by dissolving the samples in a glycerol matrix on the probe prior to bombardment with Argon atoms having a kinetic energy equivalent to 2–6 keV.

Results and Discussion

The new phospholipid PL2, eluted with $CHCl_3$/MeOH (95 : 5, v/v), represents approx. 10% of the lipid extract and has an $R_F \approx 0.3$ on TLC ($CHCl_3$/MeOH/H_2O, 65 : 25 : 4, v/v) (Fig. 1).

Acid methanolysis yielded glycerol, phosphate and glycerol diether in the molar ratio shown by PGP, i.e., 1 : 2 : 1. Glycerol and phosphate were quantified and identified as described in Materials and Methods; glycerol diethers were quantified and identified by comparison with authentic samples [2]. The proportions of the C_{25},C_{20} and C_{20},C_{20} forms of PL2 were determined by HPLC as 38 : 62, respectively, after hydrolysis to release the core structure similar to those reported for PG and PGP derivatives [8].

Negative fast atom bombardment-mass spectra of PL2 showed the same peaks as PG, i.e., identical to

those of PGP following the loss of the additional phosphate group [8].

^{13}C-NMR assignments are reported in Table I. ^{13}C data were obtained from a proton noise decoupled spectrum and from DEPT experiments that allowed us to determine the multiplicity of each signal. ^{13}C-NMR spectra of PGP and PL2 showed practically identical resonances, except that in PL2, carbon 2′ was shifted by 4.3 ppm and appeared as a doublet with $J_{C\text{-}O\text{-}P} = 1$ Hz in the proton noise decoupled ^{13}C-NMR spectrum.

These results suggested that the structure of PL2 differed from PGP only in an additional substitution on carbon 2′ of the second glycerol molecule. Since the quantitative analyses indicated a phosphate composition identical to PGP, the most likely explanation was that a single terminal phosphate group was linked both to carbon 1′ and to carbon 2′ of the second glycerol molecule.

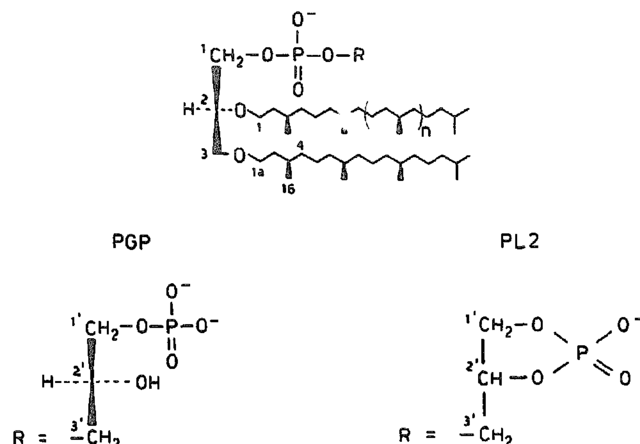

Fig. 2. Chemical structures of C_{20},C_{20} ($n = 1$) and C_{25},C_{20} ($n = 2$) PGP and PL2. PGP, 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycero-1′-phosphate. PL2, 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphoryl-3′-*sn*-glycero-1′,2′-cyclic phosphate. C_{25},C_{20} forms have sesterterpanyl chains replacing phytanyl chains on C-2 of the glycerol moiety.

TABLE I

^{13}C-NMR assignments for phospholipid PL2

^{13}C-NMR spectra were recorded in $C^2HCl_3/C^2H_3O^2H$ (7:3, v/v) at 125 MHz; the chemical shifts (δ) are in ppm with respect to tetramethylsilane. Chemical shifts data on carbons 21–25 of the isopranic unit in the C_{25} chain were the same as carbons 16–20 in the C_{20} chain. Multiplicities were obtained from DEPT experiments: s = singlet; d = doublet; t = triplet; q = quartet.

Position	Carbon atom [a]	PGP [b] δ	PL2 δ
Glycerols	1	65.7 [c]	65.5 [c] t
	2	78.3	78.1 d
	3	71.2	71.5 t
	1′	66.6 [c,d]	66.8 [c,d] t
	2′	70.3	74.6 [c] d
	3′	66.7 [c,d]	66.9 [c,d] t
Isopranoid chains	1	69.4	69.5 t
	1a	70.7	70.8 t
	2	37.3	37.4 t
	2a	37.8	37.9 t
	3	30.4	30.3 d
	3a	30.6	30.6 d
	4,6,8,10,12	38.0	38.1 t
	5	25.0	25.1 t
	7,11	33.3	33.4 d
	9	24.9	25.0 t
	13	25.3	25.5 t
	14	39.9	40.1 t
	15	28.5	28.6 d
	16	20.1	20.2 q
	17,18	20.2	20.3 q
	19	22.9 [e]	23.1 [e] q
	20	23.0 [e]	23.2 [e] q

[a] The carbon atom numbers refer to those given in Fig. 2.
[b] De Rosa et al. [8].
[c] In the proton noise decoupled ^{13}C-NMR spectrum, signals are doublets with $J_{C\text{-}O\text{-}P} = 1$ Hz.
[d] Assignments are interchangeable with one and other.
[e] Assignments are interchangeable with one and other.

Confirmation of such a structure was obtained by comparing the permethylated derivative of PL2 with that of PGP. Permethylation of PGP produced a derivative in which the chemical shift of C-2′ was shifted from δ 70.3 to δ 78.4, thus indicating that this position was methylated. A new signal also appeared at δ 58.4 due to the presence of the corresponding methoxyl group [16]. The corresponding derivatized PL2 preparation showed no change in any of the assignments. Accordingly, we believe that these results indicate that the structure of PL2 is as shown in Fig. 2.

This is the first report of a cyclized phosphate in an archaebacterial complex lipid. Such compounds are produced as intermediates during the mild acid hydrolysis of glycerol ester phospholipid derivatives that possess a free hydroxyl adjacent to phosphoric acid [17], but we are not aware of their participation in the biosyntheses or bioconversions of such phospholipids. It is unlikely that PL2 is an intermediate in phospholipid biosynthesis or turnover in *Nc. occultus*, since it is present in significant amounts at various stages during the growth of the organism. Furthermore, we have not detected this phospholipid in lipid extracts from other halophilic archaebacteria (all of which contain PG and PGP). The function of this structure in relation to those of PG and PGP remains to be established.

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Natronobacterium gen. nov. and Natronococcus gen. nov., Two New Genera of Haloalkaliphilic Archaebacteria

Ref. 110

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