An Asymmetric Archaebacterial Diether Lipid from Alkaliphilic Halophiles

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Archaebacterial halophiles from alkaline soda lakes were shown to possess substantial amounts of a core diether lipid differing from the C_{20},C_{20} diether lipid characteristic of *Halococcus* and *Halobacterium* spp. This novel diether lipid was shown to be an asymmetric C_{20},C_{25} diether (2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol). The implications of this unusual lipid for membrane structure are discussed.

INTRODUCTION

Extremely halophilic bacteria of the genera *Halobacterium* and *Halococcus*, the methanogens and the thermoacidophilic genera *Sulfolobus* and *Thermoplasma* have been designated members of the urkingdom Archaebacteria based on sequencing studies of 16S rRNA (Magrum *et al.*, 1978; Woese *et al.*, 1978). Archaebacteria characteristically lack peptidoglycan in their cell walls and ribothymidine in the TΨC loop of tRNA (Ψ = pseudouridine). Equally characteristic of all the archaebacteria whose lipids have so far been described is a cell membrane based on unusual ether-linked isopranyl lipids (De Rosa *et al.*, 1977*b*, 1980*a*; Langworthy, 1977; Langworthy *et al.*, 1974; Makula & Singer, 1978). Among the halophiles, *Halobacterium* and *Halococcus* spp. possess only C_{20},C_{20} glycerol diethers (Kates, 1976), but the recently described alkaliphilic red halophiles (Tindall *et al.*, 1980; Ross *et al.*, 1981) have been shown to possess an additional ether lipid component which is chromatographically distinct from the well-characterized C_{20},C_{20} glycerol diether (Ross *et al.*, 1981). We report here that this novel ether lipid component is a glycerol diether with one C_{20} phytanyl chain and one C_{25} 'sesterterpane' (2,6,10,14,18-pentamethyleicosane) chain.

METHODS

Cultures. Alkaliphilic halophile strains SP1, SP2 and MS3 (Ross *et al.*, 1981) were grown in liquid medium as previously described (Tindall *et al.*, 1980). Cultures were harvested in the early-stationary phase of growth by centrifugation and were freeze-dried. After screening lipid extracts of the strains for the presence of the new compound (Ross *et al.*, 1981), strain MS3 was chosen for further investigation as it contained the greatest amount.

Extraction and hydrolysis of lipids. Dried cells were extracted continuously by the Soxhlet process for 12 h with chloroform/methanol (1:1, v/v). The total lipid extract, about 8% of the lyophilized cells, was treated with methanolic HCl for 6 h under reflux (De Rosa *et al.*, 1977*b*). The hydrolysate was dried *in vacuo*.

Purification and characterization of diethers. The preliminary purification of diethers in the hydrolysate was performed on a silica gel 60 column [Merck, 230–400 mesh: 30 mm (i.d.) × 450 mm] eluted with $CHCl_3$ and $CHCl_3/Et_2O$ (95:5, v/v). The diether fraction was further resolved into 2,3-di-*O*-phytanyl-*sn*-glycerol and the

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unknown diether by high performance liquid chromatography (Waters Associates) in *n*-hexane/Et_2O (95:5, v/v), using Microporasil columns (3·9 mm × 30 cm, flow rate of 2 ml min^{-1} for analytical work; 7·8 mm × 30 cm, flow rate 6 ml min^{-1} for preparative work).

Molecular weight determinations were performed by vapour pressure osmometry in $CHCl_3$ using a Perkin-Elmer Hitachi model 115 instrument (De Rosa *et al.*, 1980*a*). Infrared spectra of diethers were recorded using a Perkin-Elmer 257 spectrophotometer and optical rotations were measured in $CHCl_3$ using a Perkin-Elmer 115 polarimeter (De Rosa *et al.*, 1980*a*). ^{1}H-n.m.r., ^{13}C-n.m.r. and mass spectra were recorded as described by De Rosa *et al.* (1977*b*). Gas chromatography/mass spectrometry of diether acetates was performed as described by De Rosa *et al.* (1977*b*) with a temperature programme of 180–300 °C at 10 °C min^{-1}. Other gas–liquid chromatography (g.l.c.) analyses were performed as described by De Rosa *et al.* (1977*b*) with a temperature programme of 120–280 °C at 10 °C min^{-1} for isopranoid acetates and at 300 °C for diether acetates. Acetylation and BCl_3 cleavage of the unknown diether were performed as described by De Rosa *et al.* (1980*a*). Isopranoid iodides and acetates were produced by the methods of De Rosa *et al.* (1976, 1977*b*).

RESULTS

The lipid content of the alkaliphilic halophile strains was about 8% of the dry cell weight. Hydrolysis of the complex lipids gave rise to a mixture of two glycerol isopranoid diethers, in which the unknown diether represented 55% (w/w) of the mixture (8·5%, w/w, of the total lipid extract). The optical rotation ($[\alpha]_D = +7·2$) and the infrared spectra of the unknown were almost identical to those for the well-characterized 2,3-di-*O*-phytanyl-*sn*-glycerol (Kates, 1976; Ross *et al.*, 1981).

G.l.c. analysis of the isopranoid acetates derived from the unknown showed two equivalent peaks with retention times of 6·9 and 12·2 min, respectively. Gas chromatography/mass spectrometry of these two peaks yielded cleavage patterns characteristic of a C_{20} and a C_{25} isopranoid acetate, respectively: [$C_{22}H_{44}O_2$, *m/e* 340 (M^+), 280 (M^+ − AcOH, 12%), 252 (4), 210 (10), 196 (20), 182 (10), 140 (40), 126 (80), 125 (100); $C_{27}H_{54}O_2$, *m/e* 410 (M^+), 350 (M^+ − AcOH, 15%), 322 (3), 280 (6), 266 (10), 252 (6), 210 (14), 196 (31), 182 (9), 140 (45), 126 (77), 125 (100)]. G.l.c. analysis of the isopranoid monochlorides produced by BCl_3 cleavage indicated that the two monochlorides were in an equivalent molar ratio with glycerol (glycerol:C_{20}:C_{25} = 0·98:0·97:1·1). ^{1}H-n.m.r. spectra of the monoacetylated diether indicated a signal due to CH_2OAc protons (δ 4·14) coupled with a single C*H*O proton signal (δ 3·5, coupled and overlapping with a single CH_2O). These data establish the substitution of the α and β OH groups of the glycerol moiety by the ether linkages, but do not establish the relative location of the two different (C_{20} and C_{25}) chains, nor do they preclude the presence of a mixture of diethers rather than a single molecular species. Mass spectra of the acetylated C_{20},C_{25} glycerol diether showed a peak at *m/e* 705 (loss of the acetyl group). At *m/e* values higher than 250 there were three critical peaks associated with the loss of aliphatic chains linked at the α or β glycerol carbons. Peaks at *m/e* 453 and 467 are related to the loss of $-CH_2-O-C_{20}H_{41}$ and $-O-C_{20}H_{41}$, respectively, and the former, in particular, showed that the C_{20} residue is located on the α glycerol carbon, whilst the peak at *m/e* 397, associated with loss of $-O-C_{25}H_{51}$, places this residue on the β carbon (Fig. 1*a*). The absence of a peak of significant size at *m/e* 383 showed that these locations are unique.

Confirmatory mass spectra of the alkyl monoacetates showed molecular ions corresponding to C_{20} monoacetate (*m/e* 340), C_{25} monoacetate (*m/e* 410), and fragments at *m/e* 280 and 350 which correspond to the same fragments after loss of AcOH. Both spectra revealed a regular series of cleavage products representing sequential loss of saturated isoprene units. Other series of cleavage products, relating to the loss of methyl groups, indicated the same pattern of location of these groups in the C_{25} monoacetate as in the C_{20} monoacetate. The major fragments of the C_{25} monoacetate (*m/e* 126, 182, 196, 252, 266, 322 and 337) indicated the presence of a regular 'head-to-tail' structure; a 'tail-to-tail' condensation as in the C_{25} isomer 2,6,10,15,19-pentamethyleicosane can be ruled out as this compound does not produce an *m/e* 252 peak, whereas it does give a major fragment at *m/e* 309 which is absent in the fragmentation of the naturally derived C_{25} acetate (Holzer *et al.*, 1979). Accordingly,

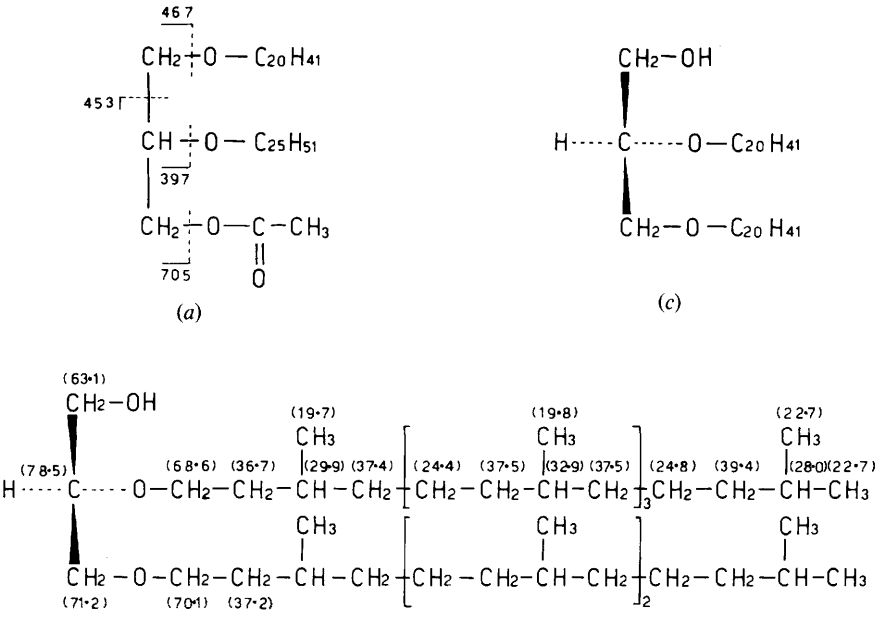

Fig. 1. Mass spectrographic and n.m.r. analysis of glycerol diether. (*a*) Structure of the acetylated C_{20},C_{25} glycerol diether indicating the characteristic mass spectral cleavage pattern due to the position of the C_{25} isopranoid on the C-2 of glycerol (see text). (*b*) Structure of 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol showing characteristic ^{13}C-n.m.r. chemical shifts, indicating a 2,3-dialkoxypropan-1-ol structure with regular 'head-to-tail' isopranoid chains. (*c*) Structure of 2,3-di-*O*-phytanyl-*sn*-glycerol, for comparison.

we believe that this isopranoid has the regular 2,6,10,14,18-pentamethyleicosane (sesterterpane) skeleton.

^{13}C-n.m.r. spectra of the C_{20},C_{25} diether (all signals and multiplicities assigned, Fig. 1*b*) confirmed a 2,3-dialkoxypropan-1-ol structure with regular 'head-to-tail' isopranoid chains; vapour pressure osmometry on the new diether indicated a molecular weight of 729 ($C_{48}H_{98}O_3 = 722$).

These results establish the structure of the new ether lipid as that shown in Fig. 1 (*b*). This differs from the well-known 2,3-di-*O*-phytanyl-*sn*-glycerol (Fig. 1 *c*) in having a C_{25} saturated isoprenoid chain in place of the phytanyl residue at C-2.

All of our isolates of red halophiles from alkaline soda lakes, such as Lake Magadi in Kenya and the Wadi Natrun in Egypt, have afforded substantial proportions of this new diether lipid in addition to the usual 2,3-di-*O*-phytanyl-*sn*-glycerol (H. N. M. Ross & W. D. Grant, unpublished results).

DISCUSSION

Archaebacteria exhibit a considerable range of diversity within the basic scheme of ether lipids, reflected to some extent in morphology, cell wall type and habitat (Balch *et al.*, 1979; Woese *et al.*, 1978). Until now, the lipids have all proved to be based either on 2,3-di-*O*-phytanyl-*sn*-glycerol (glycerol with two ether-linked C_{20} chains) (De Rosa *et al.*, 1977*a*, *b*, 1980*a*; Kates, 1976) or, in the thermoacidophiles and some methanogens, on a type of diglycerol di-biphytanyl tetraether, in which two such C_{20},C_{20} diether moieties are linked 'head-to-head' to give C_{40},C_{40} (di-biphytanyl) units which may also be partly cyclized

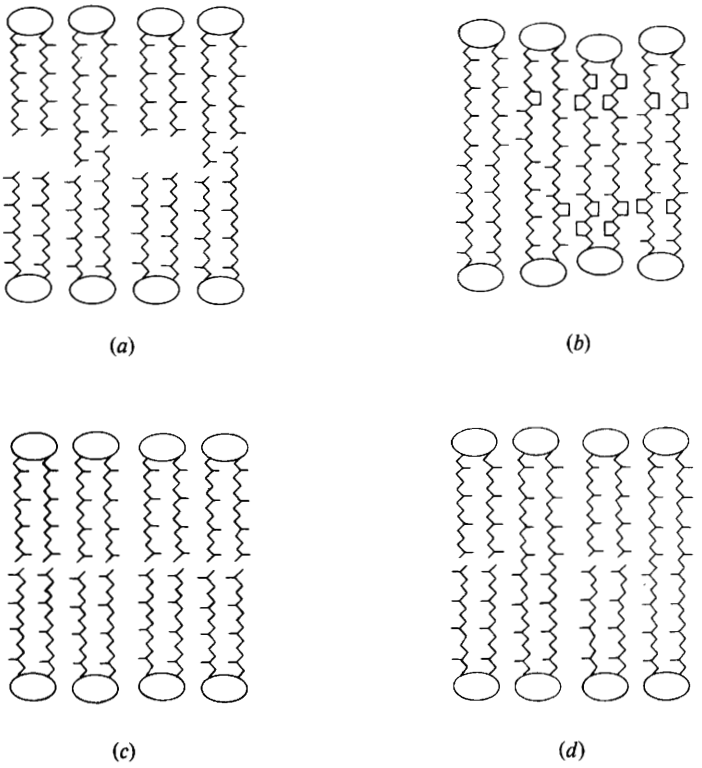

Fig. 2. Proposed archaebacterial membrane types. (*a*) 'Zip' membrane formed by C_{20},C_{25} and C_{20}, C_{20} diether lipids proposed for alkaliphilic halophiles. (*b*) Rigid monolayer membrane structure formed by C_{40},C_{40} tetraether lipids including cyclized forms proposed for the thermoacidophiles *Sulfolobus* and *Thermoplasma* (De Rosa *et al.*, 1980*b*). (*c*) Bilayer membrane formed by the C_{20},C_{20} diether lipids proposed for *Halobacterium* and *Halococcus* spp. and some methanogens (Kates, 1976; Balch *et al.*, 1979). (*d*) Mixed membrane structure formed by a mixture of C_{20},C_{20} diethers and C_{40},C_{40} tetraethers proposed for some methanogens (Balch *et al.*, 1979).

(De Rosa, 1977*b*, 1980*a*; Makula & Singer, 1978; Tornabene & Langworthy, 1978). This is the first report of an archaebacterium possessing asymmetric ether lipids. However, several C_{25} isopranoids including 2,6,10,14,18-pentamethyleicosane have been reported as constituents of various oils, shales and sediments, sometimes in significant amounts (Brassell *et al.*, 1981). C_{25} isopranoids, in particular the isomer 2,6,10,15,19-pentamethyleicosane, have also been described as components of the neutral lipid fraction of certain methanogens (Tornabene *et al.*, 1979), while 2,6,10,14,18-pentamethyleicosane has been detected in small amounts in the neutral lipid fraction of *Sulfolobus* and *Thermoplasma* (Holzer *et al.*, 1979). However, none of these C_{25} isopranoids has previously been detected in ether linkages with glycerol, and it is not clear if these isopranoid hydrocarbons are intermediates or degradative products of larger isopranoids.

In the membrane of the alkaliphilic archaebacteria, strong and unusual interactions are expected to occur between alkyl chains located in opposite faces of the lipid bilayer owing to major penetration by the longer C_{25} chains into the opposite lipid layer, effectively causing a 'zip' effect in the middle of the membrane. Such a type of lipid organization, as illustrated in Fig. 2(*a*), may be intermediate in stability between the rigidly bridged C_{40},C_{40} tetraether monolayer structure found in thermoacidophiles (Langworthy, 1978; De Rosa *et al.*, 1980*b*) (Fig. 2*b*) and the regular bilayer formed by C_{20},C_{20} diethers found in *Halobacterium* and *Halococcus* spp. (Kates, 1976). It may be similar in stability to the mixed C_{20} and C_{40}

membrane structure found in certain methanogens (Fig. 2*d*) (Rohmer *et al.*, 1979), but the 'zip' structure could imply a slightly thicker membrane with gaps at some points between the C_{20} chains. However, the length of the isopranoid chains may not be the sole factor in determining membrane dimensions; for example, the cyclized isopranoid units reported in the thermoacidophiles (De Rosa *et al.*, 1980*b*) presumably would result in a C_{40} chain which is shorter than in their uncyclized congeners (Fig. 2*b*).

The biochemistry of the alkaliphiles remains largely unexplored and these bacteria are particularly interesting in that they cope with the dual stress of high salt concentrations and very high pH. The occurrence of these asymmetric lipids may be a significant feature of membrane stability under these exceptional conditions. However, at least one *Halobacterium* sp. has also been found to have a chromatographically similar lipid component (Ross *et al.*, 1981). It is possible that this particular strain has a high pH optimum and it remains to be established whether the asymmetric polar lipids are restricted to alkaliphilic or alkalitolerant isolates.

It is widely held that C_{20} and C_{40} isopranoids in kerogen, sediments and oils derive, in part at least, from the membranes of archaebacteria. This report of an extant archaebacterium with large amounts of C_{25} isopranoids in its membranes strongly supports the suggestion of a similar origin for the C_{25} compounds found in the same deposits. We still await the discovery of the 'archaebacterial coelacanth' containing ethers based on the C_{15} isopentadecane described in the Messel kerogen by Chappe *et al.* (1979).

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