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ISOPRENOID ETHERS; BACKBONE OF COMPLEX LIPIDS OF THE ARCHAEBACTERIUM *SULFOLOBUS SOLFATARICUS*

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The complex lipids of *Sulfolobus solfataricus* (previously named *Caldariella acidophila*), a thermophilic archaebacterium, are based on 72-membered macrocyclic tetraethers made up from two C_{40} diol units and either two glycerol units or one glycerol and a unique branched-chain nonitol, named calditol. The C_{40} components have the 16,16′-biphytanyl skeleton with up to four cyclopentane rings. Individual isoprenoid ethers, backbone of complex lipids, are separated by high-performance liquid chromatography (HPLC) and the structures of 20 different species are fully described. The structures allow previous observations on these and related lipids to be rationalized biosynthetically.

Introduction

As a result of recent genetic studies [1] and the basis of general biochemical features [2], a third group, the archaebacteria, was introduced into the classification of living organisms, in addition to the prokaryotes and eukaryotes.

Actually the new line of archaebacteria comprises at least three types of microorganisms, namely methanogens, extreme halophiles and thermoacidophiles. One of the most important phyletic markers of archaebacteria is the prevalence of lipids based on isoprenoid glyceryl ethers of structural types 1–13 (Fig. 1).

In the glycerol diethers 1–3 (Fig. 1) the hydrocarbon chains have the perhydrogeranylgeranyl or the sesterterpanyl structure; such types of lipid occur in the extreme halophiles [3–5].

Lipids of methanogens are based on the diether 1 (Fig. 1) and on the tetraether 4 (Fig. 1) [6–8], which contains two C_{40} chains having the 16,16′-biphytanyl skeleton.

Lipids of thermophilic archaebacteria which have been studied up to the present are based on the diether 1 (Fig. 1), on the glycerol-dialkyl-glycerol tetraethers 4–8 (Fig. 1) and on the glycerol-dialkyl-calditol tetraethers 9–13 (Fig. 1) [9–14].

This report describes new types of isoprenoid ethers occurring in the complex lipids of the thermophilic archaebacterium *Sulfolobus solfataricus* [15,16], previously named *Caldariella acidophila* [17].

Materials and Methods

Microorganism and culture conditions. S. solfataricus, strain MT-4, isolated from an acid hot spring in Agnano, Naples, was grown in the standard medium [15] at 87°C in 90-l batch cultures (pH controlled 3.5, low mechanical agitation, aeration at 9 l·min^{-1}) inoculated with 9 l of 12-h broth culture. Cells were harvested in the late exponential growth phase by continuous-flow

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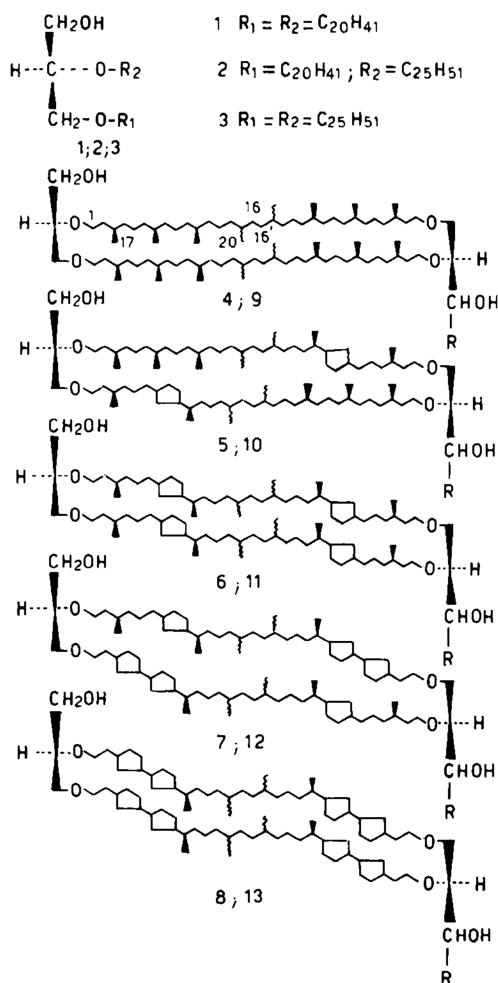


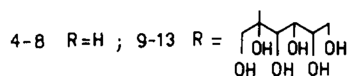


Fig. 1. Structures of isoprenoid ethers, backbone of complex lipids of archaebacteria. Diethers 1–3 occur in extreme halophiles, diether 1 and tetraether 4 in methanogens and diether 1 and tetraethers 4–13 in extreme thermoacidophiles.

centrifugation and lyophilized (yield approx. 0.2 g lyophilized cells $\cdot\, l^{-1}$).

Extraction and hydrolysis of lipids. The microorganism was extracted continuously (Soxhlet) for 12 h with $CHCl_3$/MeOH (1 : 1, v/v). The total lipid extract approx. 8% of dry cells) was treated with methanolic HCl for 6 h under reflux and the hydrolysis mixture dried in vacuo.

Purification of isoprenoid ether components. The chloroform-soluble fraction of the hydrolysis mixture was chromatographed on a silica gel (Si-gel) column (40 cm, internal diameter 10 mm). $CHCl_3/Et_2O$ (98 : 2 v/v) eluted the diether 1 (Fig. 1) (approx. 5.7% of complex lipids), $CHCl_3/Et_2O$ (95 : 5, v/v) eluted the glycerol-trialkyl-glycerol tetraether 14 (Fig. 2) (approx. 3.2%), $CHCl_3/Et_2O$ (9 : 1, v/v) eluted the glycerol-dialkyl-glycerol tetraether fraction (approx. 17.1%) and $CHCl_3$/MeOH (95 : 5, v/v) eluted the glycerol-dialkyl-calditol tetraether fraction (approx. 55.3%). The glycerol-dialkyl-glycerol and the glycerol-dialkyl-calditol tetraether fractions, this last as fully acetylated derivative, were further resolved into single components by HPLC. After HPLC resolution the acetylated glycerol-dialkyl-calditol tetraethers were hydrolyzed with methanolic HCl at reflux for 6 h.

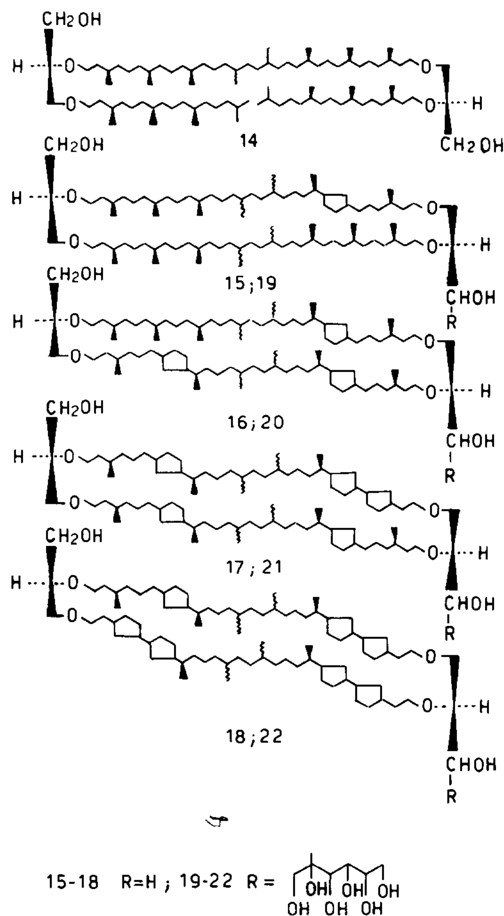
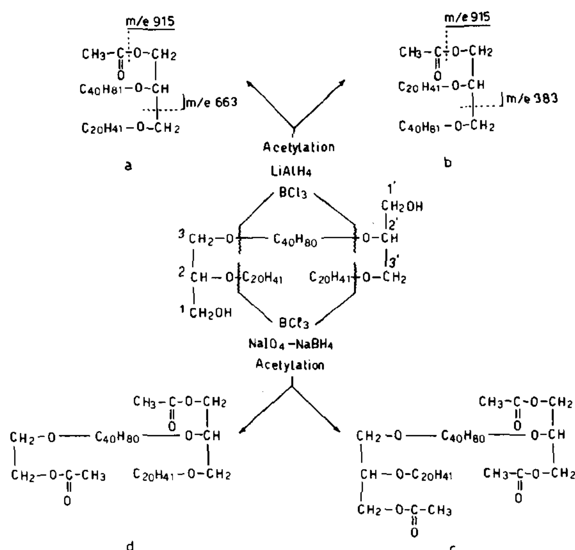


Fig. 2. New structural types of isoprenoid ethers, backbone of complex lipids of *S. solfataricus*.

Hydrolysis of isoprenoid ethers. Cleavage of isoprenoid ethers with 57% HI at reflux for 20 h gave alkyl iodides. These were converted into corresponding alkanes by treatment with excess $LiAlH_4$ in Et_2O [11]. After conventional work-up the hydrocarbons were purified by chromatography on Si-gel in *n*-hexane. Alternatively, the isoprenoid ethers were treated with $BCl_3/CHCl_3$ (1 : 1, v/v) for 14 h at room temperature. Excess reagent was removed at reduced pressure and the residue was chromatographed on Si-gel column; $CHCl_3$ eluted the alkyl chlorides and a linear gradient of MeOH in $CHCl_3$ from 0 to 50% (v/v) eluted polyols (glycerol and calditol). Chromatographic fractions were weighed to evaluate the molar ratio of hydrolysis products. Glycerol was also assayed enzymatically [18].

Degradation of glycerol-trialkyl-glycerol tetraether. Glycerol-trialkyl-glycerol tetraether 14 (Fig. 2) was subjected to a partial hydrolysis in $CHCl_3$ solution (1 mg/ml) adding dropwise an equimolar amount of BCl_3 for 6 h at 5°C. Solvent and unreacted BCl_3 were removed at reduced pressure and the residue was purified on preparative Si-gel TLC developed with $CHCl_3/Et_2O$ (95 : 5, v/v). Fractions with R_F 0.8 and 0.3 were recovered for further chemical work, while intact 14 (Fig. 2) (about 60% of reaction mixture, R_F 0.6) was subjected again to the hydrolysis. Fraction with R_F = 0.8 was treated with excess $LiAlH_4$ in Et_2O for 10 h at room temperature and, after conventional work-up, acetylated to give glycerol diether acetates a and b (Scheme I). The fraction with R_F = 0.3, was subjected to $NaIO_4/NaBH_4$ degradation, as later described for glycerol-dialkyl-calditol tetraethers. The degradation products, after acetylation, were resolved by Si-gel column chromatography eluted with *n*-hexane with increasing proportions of Et_2O; 20 and 30% (v/v) Et_2O eluted, respectively, diacetate d and triacetate c (Scheme I).



Scheme I. Degradation scheme of glycerol-trialkyl-glycerol tetraether 14. On formulas a and b are rationalized some diagnostic fragments of MS spectra. For experimental conditions, see Materials and Methods.

$NaIO_4$ / $NaBH_4$ degradation of glycerol-dialkyl-calditol tetraethers. The glycerol-dialkyl-calditol tetraether fraction dissolved in $CHCl_3$/MeOH (1 : 1, v/v) was treated with excess of 8% aqueous $NaIO_4$ with stirring. The kinetic analysis of $NaIO_4$ oxidation indicates that 25 h are sufficient to perform the full oxidation of susceptible bonds. At the end of the reaction excess reagent was destroyed with glycerol and the oxidation products, recovered by extraction with $CHCl_3$, were treated with excess $NaBH_4$ in $CHCl_3$/MeOH (3 : 7, v/v) with stirring for 3 h. Excess borohydride was destroyed with 1 N HCl and the reduction products purified by Si-gel column, eluted with $CHCl_3/Et_2O$ (95 : 5, v/v).

Isoprenoid ether acetylation. The acetylation of isoprenoid ethers and of their degradation products was performed with Ac_2O/pyridine (9 : 1, v/v) at reflux for 6 h. The reaction mixtures were dried under vacuum and the acetylated compounds were purified by Si-gel column chromatography.

Chromatographic procedures. Thin-layer chromatography (TLC) was performed on 0.25-mm layers of Si-gel F 254, Merck, activated by heating at 100°C for 2 h. Solvents included $CHCl_3/Et_2O$ (95 : 5, v/v) for compounds 1 (Fig. 1) and 14 (Fig. 2), $CHCl_3/Et_2O$ (9 : 1, v/v) for glycerol-dialkyl-glycerol tetraethers; $CHCl_3$/MeOH (9 : 1, v/v) for glycerol-dialkyl-calditol tetraethers; $CHCl_3$/MeOH (7 : 3, v/v) for glycerol; $CHCl_3$/MeOH (3 : 2, v/v) for calditol; *n*-hexane for isoprenoid hydrocarbons and halides. All the compounds were detected either by exposure to I_2 vapour, or by spraying with $Ce(SO_4)_2$. Periodate-Schiff reagent

[19] was used for vicinal glycols. Si-gel column chromatography was performed using Merck Kieselgel 70–230 mesh. HPLC was performed in *n*-hexane/ethyl acetate (6 : 4, v/v) for glycerol-dialkyl-glycerol tetraethers and in *n*-hexane/ethyl acetate (8 : 2, v/v) for glycerol-dialkyl-calditol tetraether acetates using a Microporasil column (3.9 × 30 cm, flow rate of 0.5 $ml \cdot min^{-1}$ for analytical work; 7.8 mm × 30 cm, flow 5 $ml \cdot min^{-1}$ for preparative work). Eluants from HPLC were detected with a differential refractometer.

GLC of isoprenoid hydrocarbons were performed on a glass capillary column (25 m × 0.3 mm) of SE-30, temperature program 100–300°C, $4°C \cdot min^{-1}$.

Instrumental. The MS measurements were performed, according to the method of Constantin et al. [20], with a Thomson-Houston T-H-208 B double focusing instrument, Mattauch-Herzog geometry, equipped with an LKB mass-marker. The sample holder is a gold wire, 0.5–1 mm in diameter, introduced into the ionization chamber (320°C) and so positioned that its extremity is at about 2 mm of the electron beam (70 eV). Infrared spectra (IR) were recorded using a Perkin-Elmer 257 spectrometer and optical rotations were measured in $CHCl_3$ using a Perkin-Elmer 115 polarimeter. GLC-MS was performed at 70 eV with the A.E.I., MS-30 mass spectrometer equipped with a glass column, 1.5 × 3 mm, packed with 1% OV-1 on Gas-Chrom (100–120 mesh), temperature programmed at $6°C \cdot min^{-1}$ over 120–300°C for hydrocarbons and isoprenoid halides and 180–300°C for diphytanyl diether/acetate. ^{1}H and ^{13}C NMR spectra were run on the Bruker WH-500 spectrometer. The samples were spun in 5-mm tubes using $C^{2}HCl_3/C^{2}H_3O^{2}H$ (9 : 1, v/v) as solvent. The deuterium of the solvent provided the lock signal and tetramethylsilane (TMS) the internal standard.

Results

The present work fully defines the skeletal characterization of the principal components of the membrane lipids of the thermophilic archaebacterium *S. solfataricus*. The hydrolysis of complex lipids of the microorganism gave rise to a mixture of isoprenoid ethers, resolved by chromatography on Si-gel column in four fractions, of increasing polarity, characterized as follows.

2,3-Di-O-phytanyl-sn-glycerol. The less polar chromatographic fraction had molecular weight 652 (by MS) and an optical rotation $[\alpha]_D^{20} = +7.2$. It was identical (IR, MS, ^{1}H-NMR, ^{13}C-NMR and TLC) to an authentic sample of 2,3-di-*O*-phytanyl-*sn*-glycerol.

Glycerol-trialkyl-glycerol tetraether 14 (Fig. 2). The second chromatographic fraction was formed by the tetraether 14 (Fig. 2). It had an optical rotation $[\alpha]_D^{20} = +8.1$ and the IR spectrum showed signals corresponding to hydroxyl (3450 cm^{-1}), ether (1115), carbinol (1045) and alkyl (1375, 1460, 2860, 2929 and 2960) groups. The MS spectrum gave M^+ at m/z 1302 and fragments at m/z 1283–1284, 1270–1271 and 1252–1253, indicating the loss of H_2O, CH_3OH and $H_2O + CH_3OH$. The hydrolysis with BCl_3 gave glycerol and a mixture of two alkyl chlorides identified as phytanyl chloride and 16,16′-biphytanyl-dichloride (head-to-head linkage) by comparison (^{1}H-NMR, ^{13}C-NMR and GLC-MS) with authentic samples of 1-chloro-3,7,11,15-tetramethylhexadecane and 1,32-dichloro-3,7,11,15,18,22,26,30-octamethyldotriacontane. The relative molar ratio glycerol : $C_{20}H_{41}Cl$: $C_{40}H_{80}Cl_2$ was 2 : 2 : 1. Hydrolysis of the tetraether 14 (Fig. 2) with HI followed by the reduction of the alkyl iodides with $LiAlH_4$ gave a mixture of C_{40},C_{20} hydrocarbons in a 1 : 2 ratio (by GLC). MS fragmentation pattern of the C_{40} hydrocarbon was identical to that previously reported from the 16,16′-biphytane [11].

Acetylation of the tetraether 14 (Fig. 2) gave a diacetylated derivative; ^{1}H-NMR spectra indicated a signal due to CH_2OAc (4H, δ 4.14) coupled with a CHO proton signal (2H, δ 3.5, coupled and overlapping with a CH_2O signal, 4H). These data established the substitution of the α and β OH groups of the two glycerol by ether linkages. ^{13}C NMR of the tetraether 14 (Fig. 2) confirmed the isoprenoid nature of aliphatic components and α-β localization of ether linkages on glycerol moieties. To locate the isoprenoid components in the molecule, the tetraether 14 (Fig. 2) was degraded as reported in Scheme I. The partial hydrolysis with BCl_3 gave in TLC three major spots, one corresponding to the tetraether 14 (Fig. 2), R_F 0.6, while the remaining two migrated, respectively,

faster (R_F 0.8) and slower (R_F 0.3) than the starting compound.

The fast moving fraction, originating from the cleavage of one of two ether linkages of the C_{40} isoprenoid chain, was treated with $LiAlH_4$, to remove the chlorine in ω position on the C_{40} component, and acetylated. MS of the acetylated fraction did not produce M^+ but showed a peak at m/z 915 (loss of the acetyl group). At m/z values higher than 250 there were four critical peaks associated with the loss of aliphatic chains linked at α or β glycerol carbons. Peaks at m/z 663 and 677 were related to the loss of -CH_2O-$C_{20}H_{41}$ and -O-$C_{20}H_{41}$, respectively, while peaks at m/z 383 and 397 were related to the loss of -CH_2O-$C_{40}H_{81}$ and -O-$C_{40}H_{81}$, respectively. In particular the occurrence of both peaks at m/z 663 and 383 indicated that the analysed sample was a mixture of 2-*O*-16,16′-biphytanyl-3-*O*-phytanyl-*sn*-glycerol acetate (a, Scheme I) and 2-*O*-phytanyl-3-*O*-16,16′-biphytanyl-glycerol acetate (b, Scheme I). The occurrence of both 2,3-isomers was consistent with proposed structure 14 (Fig. 2), characterized by a 3,2′ anchorage of the C_{40} isoprenoid on the glycerol moieties (for numbering, see Scheme I).

Confirmatory evidence for the structure 14 (Fig. 2) resulted from the chemical work performed on the slow-moving fraction (R_F 0.3). This fraction, originating from the removal of one phytanyl residue, was subjected to a prolonged periodate oxidation, followed by $NaBH_4$ reduction and acetylation. By Si-gel column chromatography were obtained the tri- and diacetates c and d of Scheme I. The structures of, such compounds, mainly based on ^{1}H-NMR data (Scheme I), accorded with a 2,3′ localization of the phytanyl residues in tetraether 14 (Fig. 2).

Glycerol-dialkyl-glycerol tetraethers. The third chromatographic fraction was a mixture of glycerol-dialkyl-glycerol tetraethers, that was further resolved into nine compounds 4–8 (Fig. 1) and 15–18 (Fig. 2) by HPLC. The full structural characterization of tetraethers 4–8 (Fig. 1) was previously described [10,13,19]. The tetraethers 15–18 (Fig. 2), in MS, gave M^+ at m/z 1298, 1294, 1290 and 1286, respectively, and peaks corresponding to the loss of H_2O, CH_3OH and $H_2O + CH_3OH$. Structural information on the isoprenoid moieties was obtained by $HI/LiAlH_4$ degradation and by ^{13}C NMR studies of the tetraethers. GLC and GLC-MS of C_{40} hydrocarbons, obtained from the $HI/LiAlH_4$ degradation of tetraether 15–18 (Fig. 2), showed an equimolecular amount of two different C_{40} isoprenoids with n and $n+1$ cyclopentane rings, respectively, on the chain, in accord with the proposed structures.

From ^{13}C NMR spectra of the tetraethers it was possible to obtain information on the orientation of the asymmetric isoprenoids in the macrocycle. In the partial structures A–C of Fig. 3 are reported the chemical shift assignments for the oxygen-bearing carbons of the individual tetraethers. These data showed that the chemical shift of C(1) of the isoprenic chain was higher (by 1.4–1.5 units) when it was linked to the primary carbinol of glycerol and higher (by 1.1–1.2 units) when it was in a chain with a ring at C(3); moreover, the chemical shift of C(3) and C(2) of the glycerol was higher (by 0.07 and 0.13 units, respectively) when they were linked to an isoprenoid with a cyclopentane ring at C(3) of the aliphatic chain. From the data reported in partial structures B and C (Fig. 3) it was possible, therefore, to define that 'bicyclic end' of the tricyclic C_{40} chain in the tetraethers 17 and 18 (Fig. 2) was linked to the primary carbinol of the glycerol; the orientation of monocyclic C_{40} isoprenoid in the tetraethers 15 and 16 followed by simple analogy. Also by analogy with structures 4–8 (Fig. 1), pre-

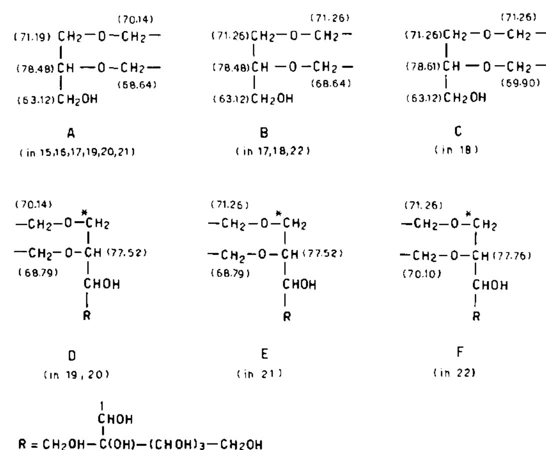


Fig. 3. Partial structure of tetraethers 15–22 (Fig. 2). In brackets are reported ^{13}C-NMR values in δ; for experimental conditions see Materials and Methods. * Overlapped signal.

viously described [11,14], an antiparallel organization of glycerols in tetraethers 15–18 (Fig.2) was proposed.

Glycerol-dialkyl-calditol tetraethers. The fourth chromatographic fraction was a mixture of glycerol-dialkyl-calditol tetraethers, that was further resolved into nine different compounds, 9–13 (Fig. 1) and 19–22 (Fig. 2) by HPLC. To obtain good resolution of these tetraethers in HPLC their polarity was modified by conversion to fully acetylated derivatives. The structural characterization of the tetraethers 9–13 (Fig. 1) has been previously described [12,14]. The MS spectra of tetraethers 19–22 (Fig. 2) showed the absence of M^+ and peaks at m/z 1460, 1456, 1452 and 1448, respectively, corresponding to the loss of H_2O from M^+. Base peaks were observed at m/z 1297, 1293, 1289 and 1285, respectively, corresponding to the loss of $C_6H_{13}O_6$ fragment from the calditol moiety, by α cleavage to the branching. Structural information on the isoprenoid moieties were obtained by GLC and GLC-MS analyses of C_{40} hydrocarbons, obtained by HI hydrolysis of tetraethers 19–22 (Fig. 2), followed by $LiAlH_4$ reduction of C_{40} diiodides.

As previously reported for tetraethers 15–18 (Fig. 2), equimolecular amounts of isoprenoids with n and $n+1$ cycles were observed for each of 19–22 tetraethers (Fig. 2). Structural analogies, with regard to the isoprenoid arrangement in the tetraether macrocycle, occurring between tetraethers 19–22 (Fig. 2) and the corresponding tetraethers 15–18 (Fig. 2), were well supported by the conversion of tetraethers 19–22 (Fig. 2) to the corresponding tetraethers 15–18 (Fig. 2) by exhaustive oxidation with $NaIO_4$ followed by reduction with $NaBH_4$. Tetraethers so obtained gave the same spectroscopic (MS and ^{13}C-NMR) data and chromatographic (HPLC) behaviour as the tetraethers 15–18 (Fig. 2). This structural evidence did not discriminate between structures 19–22 (Fig. 2) and the isomeric possibilities with an opposite orientation of both alkyl chains in the tetraether macrocycle.

Confirmatory evidence for 19–22 isomers (Fig. 2) were derived from ^{13}C-NMR chemical shifts assignments for the oxygen-bearing carbons. In particular, for the tetraether 22 (Fig. 2), containing one tricyclic and one tetracyclic C_{40} unit, the only signals observed were those whose chemical shifts are given in partial structures B and F (Fig. 3), from which it followed that the 'bicyclic end' of the tricyclic C_{40} isoprenoid was linked by an ether bond at the C(1) of calditol. This structural condition also occurred in the tetraether 21 (Fig. 2) containing one bicyclic and one tricyclic C_{40} unit. In fact, for tetraether 21 (Fig. 2) the only signals observed were those whose chemical shifts are given in partial structures A and E of Fig. 3. The structures assigned to tetraethers 19 and 20 (Fig. 2) followed by simple analogy.

Discussion

All the isoprenoid ethers until now identified in the archaebacteria, with the exception of sesterterpanyl ethers 2 and 3 (Fig. 1), occur in *S. solfataricus*. Complex lipids of this microorganism [13] are quite exclusively bipolar amphipathic molecules, organized in the membrane as a covalently bound bilayer in which each molecule, fully stretched, anchors the two polar heads to the inner and outer faces, respectively, of the membrane array [22–25].

Macrocyclic tetraethers 4–8, 15–18 and 9–13, 19–22 (Figs. 1 and 2) represent, respectively, 21 and 68% (w/w) of the isoprenoid ethers obtained from the hydrolysis of complex membrane lipids of the microorganism. At a growth temperature of 87°C tetraethers 6, 7, 17 and 11, 12, 21 (Figs. 1 and 2) represent about 70 and 73% (w/w), respectively, of glycerol-dialkyl-glycerol and glycerol-dialkyl-calditol fractions.

The degree of cyclization in the biphytanyl components is sensitive to environmental parameters, such as, for example, temperature. In accord with previously published data [26], an increase of tetraethers based on the less cyclized isoprenoids was observed when the microorganism was grown at 77°C (De Rosa, M., unpublished data).

In previous work, we obtained information on some aspects of lipid metabolism in *S. solfataricus*, such as the formation of glycerol ether bonds [27] and isoprenoid biosynthesis [11,12,20,21], but many aspects of head-to-head C_{20}–C_{20} coupling and of cyclopentane ring formation on the biphytanyl chains remain unknown.

The structural data reported in this paper could

suggest some plausible hypotheses on these two biogenetic steps. For example, if the tetraethers were formed either from a pool of mixed 16,16′-biphytanols or from variously cyclized diphytanylglycerols, we would expect a much wider range of tetraethers and the occurrence in the lipid extract of free C_{40}-biphytanes or derivatives and of glycerol diethers with cyclopentane rings on phytanyl chains. The absence of both classes of molecules in the microorganism and the high structural specificity of macrocyclic tetraethers suggest that the glycerol-dialkyl-glycerol tetraether biosynthesis proceeds from an initial di-*O*-geranylgeranylglycerol diether, from two units of which, through a head-to-head condensation at 16,16′-methyls of phytanyl chains [28], originate the 72-membered tetraether macrocycle. On this unsaturated intermediate the competition between reduction and cyclizations steps would give the range of glycerol-dialkyl-glycerol tetraethers here reported.

Regarding this last biogenetic step the absence of tetraethers with molecular architecture of structure 14 (Fig. 2), but with cyclopentane rings on the isoprenoid chains, could indicate that the enzyme system devoted to ring formation operates only if the 72-membered macrocycle is closed. Moreover, the regular disposition of cyclopentane rings in the glycerol-dialkyl-glycerol tetraethers, here reported, could indicate that cyclopentanes were closed in an ordered manner by a mechanism that operates in a concerted manner on both alkyl chains, starting from the middle of the isoprenoid system towards ether bonds.

In a similar way, but starting from a 1,2-di-*O*-geranyl-geranyl calditol precursor, tetraethers 9–13 (Fig. 1) and 19–22 (Fig. 2) would be synthesized.

The structures of these tetraethers allow us to hypothesize that the enzyme system, devoted to cyclopentane formation, discriminates between the two polar heads, glycerol and calditol, and closes the cyclopentane rings starting from the phytanyl moiety of C_{40} isoprenoid linked to the C_1 of calditol and then continues in alternate manner on both C_{40} chains as indicated in the structural sequence of tetraethers 9, 19, 10, 20, 11, 21, 12, 22, 13 (Figs. 1 and 2).

Our biogenetic hypothesis, based on structural consideration on the molecular architecture of isoprenoid ether components of complex lipids, was formulated without implications as to stereochemistry, phosphorylation, etc., of the biogenetic intermediates and did not consider the step of the covalent bond formation with inositol, glucose and galactose that characterizes the complex lipids of the microrganism [13].

Studies on biogenetic intermediates based on structural identification of the minor compounds present in the crude lipid extract and on pulse labelling experiments are in progress.

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