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STRUCTURAL REGULARITIES IN TETRAETHER LIPIDS OF *CALDARIELLA* AND THEIR BIOSYNTHETIC AND PHYLETIC IMPLICATIONS

MARIO DE ROSA,* AGATA GAMBACORTA,* BARBARA NICOLAUS,* SALVATORE SODANO* and J. D. BU'LOCK†

* CNR Laboratorio CMIB, via Toiano 2, Arco Felice, Naples, Italy;
† Weizmann Microbial Chemistry Laboratory, Department of Chemistry, The University of Manchester, Manchester, M13 9PL, U.K.

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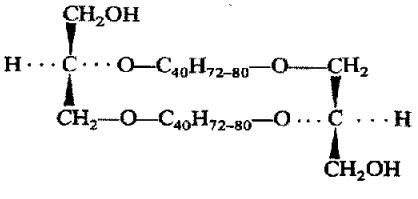
Key Word Index—*Caldariella; Sulfolobus;* archaebacteria; ether lipids; biphytanyl; biosynthesis.

Abstract—Individual di(biphytanyl) diglycerol tetraether lipids from thermoacidophile archaebacteria of the *Caldariella* series, with differently cyclized biphytanyl components, are separated and shown to have structures **8–12**, with the glycerol and biphytanyl components demonstrably both antiparallel and with partial assignments of stereochemistry. Tetraethers with alternative arrangements of the components are absent. The structures allow previous observations on these and related lipids to be rationalized both biosynthetically and phyletically.

INTRODUCTION

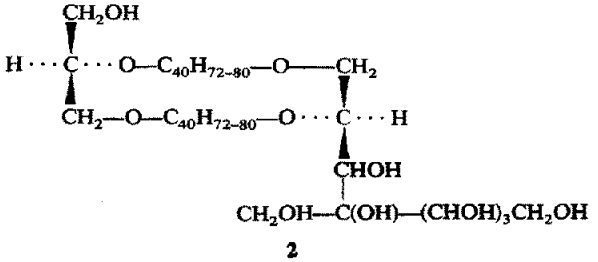
The membrane of extreme thermoacidophile bacteria of the *Caldariella* series is a monolayer based on macrocyclic tetraethers having pairs of bifunctional C_{40} isoprenoid chains [1–4]. This unusual type of lipid has now been recognized in some of the methanogenic bacteria as well, notably in species of *Methanobacterium* and *Methanospirillum* [5, 6]. The parent tetraethers are recovered from the non-saponifiable fraction of acid hydrolysates of the total complex lipids [3, 4] and comprise the glycerol-dialkyl-glycerol type (**1**) and the closely-related calditol-dialkyl-glycerol type (**2**). The details of these structures are justified by evidence presented in the present paper. They are made up of two glycerol molecules (or one glycerol and one calditol) bridged through ether linkages by two C_{40} 16,16′-biphytanyl diols [4, 7], forming the 72-membered macrocycle. The C_{40} components we have shown are represented by structures **3–7** and are made up of two perhydrogeranylgeranyl units joined 'head-to-head' at the 16-16′ position; they differ in the additional feature of up to four cyclopentane rings [3, 4, 7].

Variations in the relative proportions of the different C_{40} isoprenoids are both genotypic and phenotypic. While *Thermoplasma acidophila* [1, 8] and the methanogenic bacteria [5, 6] have high proportions of the acyclic C_{40} isoprenoid (**3**), *Sulfolobus acidocaldarius* and *Caldariella acidophila* strains have high proportions of the cyclized C_{40} components [8]. At the same time the isoprenoid composition of the more thermophilic MT-4 strain of *Caldariella acidophila* is markedly affected by the growth temperature; at 75° the isoprenoids **3**, **4** and **5** predominate, but at 89° the isoprenoids **5**, **6** and **7** are the main components [9]. A molecule such as **1**, made up from the sub-units described, might have any one of a large number of possible structures, even if stereochemical considerations are ignored. From the 5 known isoprenoids, two of which are not symmetrical end-to-end, there are 28 possible pairs, for each of which the two glycerols could be either parallel (↑↑) or antiparallel (↑↓). The present paper resolves these structural ambiguities; chromatographic separation of the mixed tetraethers from *Caldariella acidophila* MT-4 has shown that there are (surprisingly) few components, and spectroscopic data on these permits virtually complete structural assignments.



1

8 (both alkyls as **3**)
9 (both alkyls as **4**)
10 (both alkyls as **5**)
12 (both alkyls as **7**)



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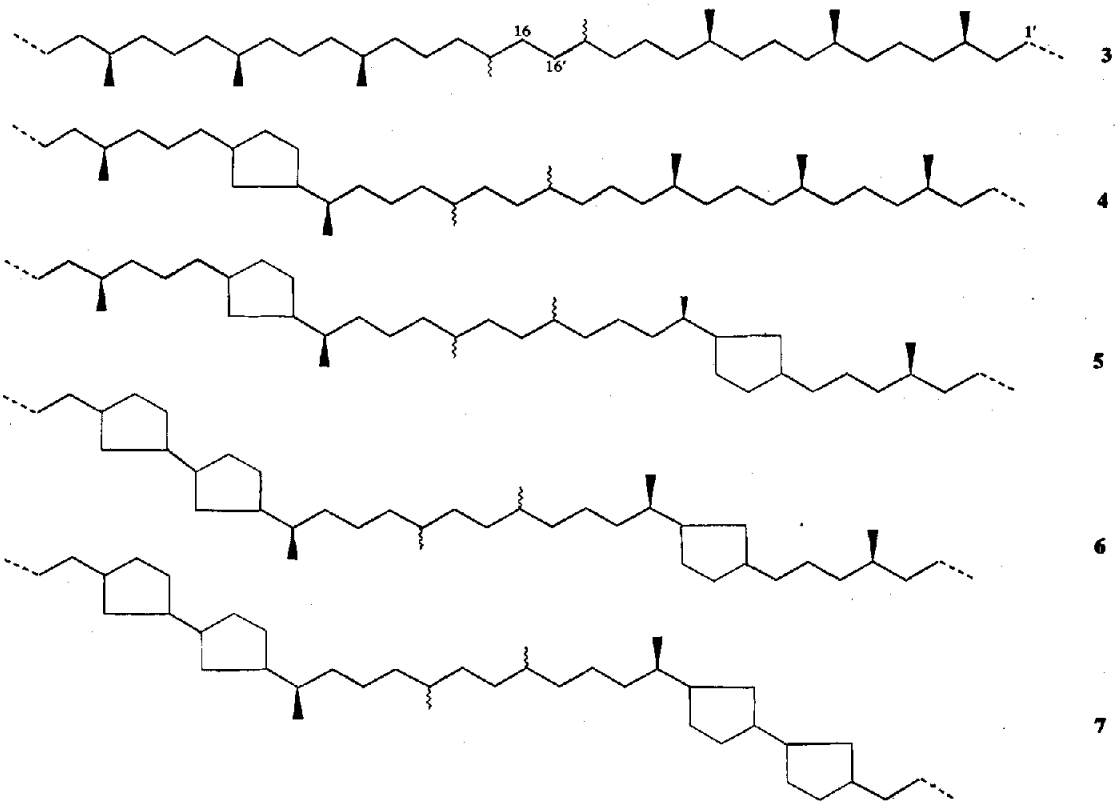


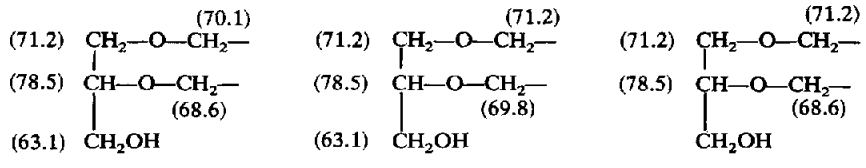
RESULTS

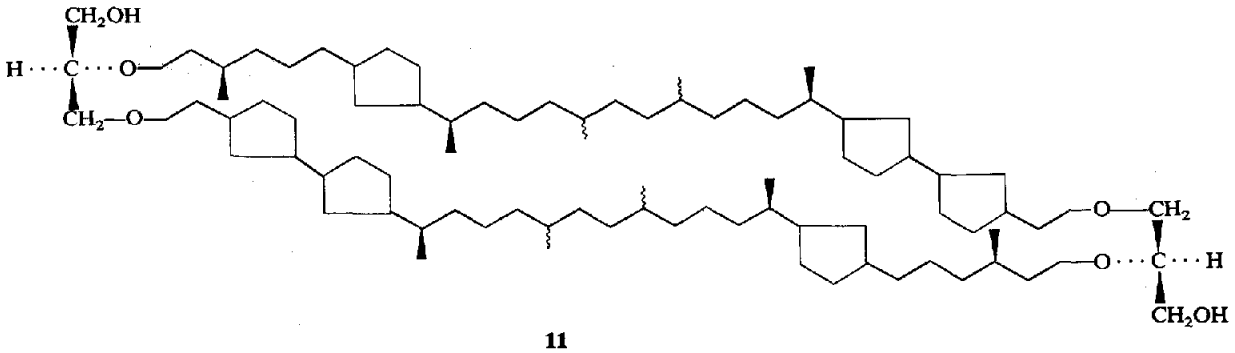
Careful chromatography of **1**, whether derived directly from the complex lipid hydrolysate or indirectly from **2**, revealed significant proportions of only 5 different tetraethers **8–12** as listed in Table 1. The ^{13}C NMR spectra of these tetraethers indicated that each contained only one type of C_{40} component, and GLC of the derived C_{40} hydrocarbons identified these as shown in Table 1.

The ^{13}C NMR chemical shift assignments for the oxygen-bearing carbons of the individual tetraethers are as shown with the partial structures **A–C**. Structure **A** shows the data for tetraethers in which the C_{40} chain is similar at each end, with C(3) and C(3′) both >CHMe—i.e. for **8**, **9** and **10**. Similarly structure **B** shows the data for **12**, in which the C_{40} chains are again similar at each end but with C(3) and C(3′) both part of a cyclopentane ring. These data show that the chemical shift of C(1) of the isoprene chain is (a)

Table 1. Chromatography of diglycerol tetraethers

Component	**8**	**9**	**10**	**11**	**12**
Eluted in $CHCl_3$–Et_2O at	98:2	97:3	95:5	93:7	9:1
R_f on TLC	0.55	0.49	0.43	0.37	0.29
$[\alpha]_D$	+8.3° ($c = 10$)	+8.2° ($c = 20$)	+7.9° ($c = 120$)	+4.2° ($c = 96$)	+1.7° ($c = 11$)
C_{40} component	**3**	**4**	**5**	**6**	**7**





11

higher (by 1.4–1.5 units) when it is linked to the primary carbinol of glycerol, and (b) higher (by 1.1–1.2 units) when it is in a chain with a ring at C(3). The data for the tetraether (**11**), containing two tricyclic C_{40} units (cf. (**6**)) in which one end is —CHMe—CH_2—CH_2—O— and the other is (ring)$>$CH—CH_2—CH_2—O—, are therefore critical; the only signals observed are those whose chemical shifts are given in partial structure **C**, from which it follows that *at both ends* the primary carbinol of glycerol is linked to the 'bicyclic end' of the isoprenoid. Thus structure **11**, in which both the isoprenoid chains and the glycerol units are antiparallel, is determined uniquely. The structures assigned to the remaining tetraethers, i.e. with antiparallel glycerols and [in (**9**)] antiparallel isoprenoids, follow by simple analogy.

Table 1 also lists the optical rotations of the tetraethers, which show a regular trend. It is clear that asymmetry of the disubstituted glycerol units makes the greatest contribution and so comparison with the optical activity of di-*O*-phytanylglycerol, the analogous diether lipid of halobacteria, is valid. For the natural 2,3-di-*O*-(3′*R*,7′*R*,11′*R*,15-tetramethylhexadecyl)-*sn*-glycerol, Kates [12] recorded $[\alpha]_D$+ 8.5° (in $CHCl_3$), and for a mixture of this with the 3′*S*-epimer, +7.6°; we can therefore deduce both the 2,3-*sn*-configuration in the glycerol units and the *R* configuration for the CHMe groups at C(3) and C(3′). The configuration of the remaining CHMe groups is either assigned by simple analogy with the halobacterial phytanyl chains or, at C(15) and C(15′), is unknown; equally the configurations at the —CH$<$(cyclic) positions are unknown. Note that as cyclic structures closer to the glycerol units are introduced in **11** and **12**, the $[\alpha]_D$ is substantially lowered. The rotations of diglycerol tetraethers derived chemically from the calditol glycerol tetraethers (**2**) were identical to those of the 'native' **8**–**12** so that a partial assignment of stereochemistry in the calditol residue can also be made, as shown in **2**.

DISCUSSION

The observation that so few of the 'possible' tetraethers actually occur in the *Caldariella* lipids clearly arises through–and to that extent clarifies–their biosynthesis. We have already shown that this follows the standard isoprenoid pathway as far as a biogenetically *trans*-C_{20} (geranylgeranyl) chain [13] and for the further steps of C—C bond formation, at the 16,16′-link and in the cyclizations, we can comment that although the mechanisms are unknown both types of reaction involve carbon centres which are allylic at the geranylgeranyl stage. We have also shown [9] that the extent of cyclizations in the biphytanyl chains is influenced by the growth temperature. Less direct but still relevant data are first, the existence of a minor lipid category in *Caldariella* based on tri-*O*-(geranylgeranyl) glycerol and showing evidence for stepwise hydrogenations of C=C starting at the carbinol end [14]; secondly, the phyletic consideration that in 'archaebacteria' [15], diphytanyl diether lipids are widespread, di-(biphytanyl) tetraether lipids less so, and the di-(cyclized biphytanyl) tetraethers quite specialized.

The structural data now established suggest that some biosynthetic hypotheses would be more plausible than others. For example, if the tetraethers were formed either from a pool of mixed 16,16′-biphytanols or from variously-cyclized diphytanyl-glycerols, we would expect a much wider range of products. Those actually found all show the same (diad axis) symmetry, with each of the antiparallel biphytanyl chains (cf. **11**) having the same steric relationships to each of the antiparallel *sn*-glycerol units, and the cyclizations seem to have been introduced in conformity with that symmetry (as expressed in presumed enzyme–substrate interactions).

We conclude that the biosynthesis proceeds from an initial di-*O*-(geranylgeranyl) glycerol diether, which by direct reduction would afford diphytanyl glycerol diether (cf. [12]). Competition between complete stepwise reduction and 16,16′-coupling (between methyls on the last double bond to be reduced) would give the diether/tetraether mixture found in some methanogenic bacteria [5, 6], while general 16,16′-coupling with competition between earlier reduction steps and cyclizations would give the range of tetraethers here categorized for *Caldariella*. Not only does such a sequence lead to the structures actually found, it also permits both the phenotypic variations and the phyletic relationships to be formulated in acceptable terms.

EXPERIMENTAL

The organism used was the MT-4 strain of *C. acidophila*, and culture conditions have been fully described [10]. To obtain the widest possible spread of isoprenoid composition, it was grown in two separate batches at 75 and 89°, respectively, each in 90 l. batch cultures (pH controlled 3.5, low mechanical agitation, aeration at 9 l./min), inoculated with 9 l. of a 12 hr broth culture. The specific growth rate was

0.07 hr^{-1} at 75° and 0.036 hr^{-1} at 89°. The cells were harvested in the late exponential phase by continuous-flow centrifugation (Alfa Laval LAB-102 B-25) and the two batches of cells combined and lyophilized. Extraction of the lipids, acid hydrolysis and chromatographic separation of the tetraether mixtures **1** and **2** were as previously reported [3, 6]. The calditol tetraethers (**2**) proved to be too polar for further chromatographic resolution and were converted into the corresponding glyceroldialkyl-glycerol derivatives (**1**) (yield 70%) by oxidation of the calditol moiety with $NaIO_4$ for 25 hr, followed by reduction with $NaBH_4$, as previously described [3, 7]. Cleavage of the diglycerol tetraethers with HI, conversion of the resulting C_{40} di-iodides into the corresponding hydrocarbons, and the GLC analysis of the hydrocarbons **3**–**7** were as previously described [3, 7]. The tetraether mixtures (**1**) were resolved by Si gel column chromatography. A 2.5×40 cm glass column packed in $CHCl_3$ with 80 g Merck Kieselgel (70–230 mesh), previously activated for 12 hr at 150°, was employed and the sample (0.3 g) was applied pre-adsorbed on 5 g of adsorbent. If the sample was applied directly to the column, the initial high lipid concn resulted in poor separation of the individual tetraethers. The elution was performed with 3 l. of a linear gradient of Et_2O in $CHCl_3$ from 0 to 10%. Diglycerol tetraethers were analysed by TLC on Merck Kieselgel 60-F254 (activated for 12 hr at 150°) in $CHCl_3$–Et_2O (9:1), and visualized either by exposure to I_2 vapour or by $Ce(SO_4)_2$ spray at 100°. The ^{13}C NMR spectra were recorded on a WH-270 Bruker spectrometer operating in the FT mode; multiplicity was determined by off-resonance decoupling. Samples (20–60 mg) were spun in 5 mm tubes using $CDCl_3$ (0.5 ml) as solvent; solvent D provided the lock signal and TMS the int. standard. Chemical shifts are accurate to within ±0.02 ppm. These differ slightly from previously-reported values because the scale calibration used earlier required corrections; in the earlier report ([11], p. 1910) there is also a proof error; for the chemical shift of glycerol $\underline{C}H_2OR$, read 69.6 for 66.9.

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