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Iso- and Anteiso-Branched Glycerol Diethers of the Thermophilic Anaerobe *Thermodesulfotobacterium commune*

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Summary

Thermodesulfotobacterium commune is an extremely thermophilic, anaerobic, sulfate-reducing bacterium that grows optimally at 70 °C and neutral pH. Total lipids comprise about 6.7% of the cell dry weight and consist of 17.1% neutral lipids. 12.6% glycolipids and 70.3% phospholipids. Hydrophobic residues of the glycolipids and phospholipids were released by acid methanolysis and analysed by thin-layer and gas chromatography, infrared and mass spectrometry and by chemical analyses. The hydrophobic residues consisted of *sn*-1,2-dialkylglycerol diethers (82%), *sn*-1-alkylglycerol monothers (11%) and fatty acids (7%). Five principal diether species were present containing C_{16}:C_{16}, mixed C_{16}:C_{17}, C_{17}:C_{17}, mixed C_{17}:C_{18}, and C_{18}:C_{18} alkyl side chains. The alkyl chains of the diethers and monoethers were comprised of primarily *anteiso*-C_{17} and lesser amounts of *iso*-C_{16}, *n*-C_{16}, *iso*-C_{17}, *iso*-C_{18}, *n*-C_{18}, *anteiso*-C_{18}, *iso*-C_{19} and *n*-C_{19} hydrocarbons. Among the neutral lipids were identified free fatty acids and a series of C_{16} to C_{35} hydrocarbons.

In contrast to the isopranoid-branched *sn*-2,3-diphytanylglycerol diethers and dibiphytanyldiglycerol tetraethers that characterize the lipids of thermoacidophilic, halophilic and methanogenic archaebacteria, the diethers of *Thermodesulfotobacterium* are levorotary (M_D −16.7°) indicating the *sn*-1,2-glycerol stereoconfiguration, and they contain *O*-alkyl chains of variable carbon number (C_{16} to C_{19}). The presence of glycerol diethers suggests that this organism may have had an evolutionary episode similar to archaebacteria, but the apparent stereochemistry and absence of isopranoid-branched side chains suggests that this organism is perhaps more likely a eubacterium capable of ether lipid biosynthesis. This new class of diethers indicates that a thorough chemical analysis is required when using ether lipids as a chemical marker for the identification of archaebacteria.

Key words: *Thermodesulfotobacterium*-Archaebacteria-Lipids-Diethers-Monoethers-Monomethyl-branched-Thermophile-Anaerobe-Taxonomy-Evolution

Introduction

Thermodesulfotobacterium commune is a thermophilic, obligately anaerobic, sulfate-reducing bacterium recently isolated from thermal muds, springs and algal bacterial mats associated with volcanic activity (*Zeikus* et al., 1982). The organism is a Gram-negative, non-sporulating rod which grows between 40 °C to 85 °C (optimum, 70 °C) and between pH 6.0 to 8.5 (optimum, pH 7.0). It can utilize lactate, pyruvate or hydrogen as electron donors and sulfate, thiosulfate or sulfite as electron acceptors. The organism is catalase negative, contains cytochrome C_3, lacks desulfoviridin and has a DNA base composition of 34.4% G + C.

Using lipid analysis as an approach for the identification of archaebacteria (*Langworthy* et al., 1982) we initiated an analysis of the lipids of *Thermodesulfotobacterium* to determine whether this organism might expand the archaebacterial grouping, currently comprised of the extreme halophiles, the methanogens and certain thermoacidophilic bacteria (*Fox* et al., 1980; *Zillig* et al., 1981). Archaebacteria are thus far the only organisms known to possess dialkylglycerol ether lipids, namely diphytanylglycerol diether and dibiphytanyldiglycerol tetraethers (*Langworthy* et al., 1982). Although we found *Thermodesulfotobacterium* to contain ether lipids, phytanyl-based isopranyl ethers were absent. We report herein the identification of a new class of naturally occurring straight-chain and monomethyl-branched glycerol ethers in *Thermodesulfotobacterium*.

Materials and Methods

Culture Conditions

Thermodesulfotobacterium commune strain YSRA-1 (ATCC 33708, DSM 2178) was cultivated in a modified liquid medium for sulfate-reducing bacteria described by *Postgate* (1963) with lactate as the energy source. The medium contained per liter: 0.5 g KH_2PO_4, 1.0 g NH_4Cl, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 5.0 g Na-lactate, 1.0 g yeast extract, 1.0 g ascorbic acid, 1.0 g thioglycolic acid, 5.0 g $FeSO_4 \cdot 7H_2O$ and 3.0 g Na_2SO_4. Final pH was adjusted to 6.8. Cultures were grown at 65 °C in a 14 liter New Brunswick Microferm fermentor. Anaerobic conditions were maintained by constant mixing and continuous gassing at a rate of 50 cc/min employing a gas mixture consisting of N_2/CO_2 (95:5). After 2 days incubation, cells were harvested in a Sorvall centrifuge equipped with a KSB continuous flow adaptor, washed with deionized water and lyophilized.

Extraction of lipids

Lipids were routinely extracted from 1 g dried cells by the method of *Bligh* and *Dyer* (1959). Cells were stirred at room temperature for 3 h in 114 ml chloroform-methanol-water (1:2:0.8, v/v). Following centrifugation, the cell residue was re-extracted and the combined supernatants partitioned by addition of 60 ml chloroform and 48 ml water. After drying the lower chloroform phase in vacuo at 40 °C, the lipid residue was taken up in chloroform-methanol-water (60:30:4.5, v/v) and passed through Sephadex G-25 to remove non-lipid contaminants (*Wells* and *Dittmer*, 1963).

Fractionation of lipids

Total lipids were separated into lipid classes by chromatography on silicic acid columns (1 × 12 cm) prepared with Unisil (100/200 mesh, Clarkson Chemical Co., Inc., Williamsport, PA). Columns were eluted with chloroform (100 ml) for neutral lipids, acetone (100 ml)

for glycolipids and methanol (100 ml) for phospholipids. The neutral lipids were fractionated further on 1 × 25 cm columns of Unisil (325 mesh) by elution with one column volume each of *n*-hexane for hydrocarbons, benzene for fatty acids followed by chloroform for more polar components.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on 0.25 mm layers of silica gel H activated by heating at 115 °C for at least 1 h. Developing solvents included: (i) *n*-hexane-diethyl ether-acetic acid (70:30:1, v/v) for acid methanolysis products, glycerol diethers, monoethers, fatty acid esters and neutral lipid components; (ii) *n*-hexane for alkyl halides; (iii) chloroform-methanol-formic acid (65:25:10, v/v) for glycerol; (iv) chloroform-methanol (9:1, v/v) for glycolipids; and (v) chloroform-methanol-water (65:25:4, v/v) for phospholipids. For preparative TLC, isolated bands were scraped and eluted through sintered glass filters with chloroform-methanol (2:1, v/v). Prior to infrared or optical analyses, those eluted components were taken up in *n*-hexane, washed with methanol-water (1:1, v/v) containing several crystals of $Na_2S_2O_3$ and finally dried over Na_2SO_4. Lipid components were visualized by exposure to I_2 vapors or by charing with 50% methanolic-H_2SO_4. Periodate-*Schiff* reagent was used to detect vicinal OH groups (*Baddily* et al., 1956) and the reagent of *Vaskovsky* and *Kostetsky* (1968) used for phospholipids.

Degradative procedures

The following procedures are essentially those fully detailed elsewhere (*Kates* et al., 1965; *Langworthy*, 1977, 1982a). Degradation of lipids was carried out in 13 × 100 mm or 18 × 120 mm screw cap tubes with Teflon-lined closures unless otherwise noted.

Acid methanolysis of lipids (5–15 mg) was carried out in anhydrous 1 M HCl (gas) in methanol (5 ml) at 100 °C for 18 h. After addition of water (2.5 ml), methanolysis products were extracted with *n*-hexane.

Alkaline methanolysis of lipids (1–5 mg) employed 0.25 M KOH in methanol (1.0 ml) at 45 °C for 30 min. After addition of an equal volume of water and chloroform, the chloroform-soluble products were taken for analysis.

Alkyl iodide derivatives of the O-alkyl side chains of the glycerol ethers (1–5 mg) were generated by digestion in 55% hydriodic acid (1.5 ml) at 100 °C for 18 h under an atmosphere of N_2. Alkyl iodides were extracted with *n*-hexane and the extract washed with water, 10% Na_2CO_3 and finally 50% $Na_2S_2O_3$.

Alkane derivatives were generated through reduction of the alkyl iodides (1–5 mg) by heating at 100 °C for 18 h in zinc dust (200 mg) and acetic acid (1 ml). After neutralization with 10% Na_2CO_3, alkanes were extracted with *n*-hexane.

Alkyl acetates were produced by refluxing alkyl iodides (1–5 mg) with silver acetate (250 mg) in acetic acid (7 ml). After addition of water, acetates were extracted with *n*-hexane followed by washing with 10% Na_2CO_3. Alcohols were prepared from the acetates by alkaline methanolysis in 0.5 M KOH in methanol at 100 °C for 3 h.

Alkyl chlorides and glycerol were released from glycerol ethers (1–5 mg) by incubation in liquified boron trichloride in chloroform (1:1, v/v) at room temperature for 18 h. After evaporation under N_2, alkyl chlorides and glycerol were distributed between *n*-hexane (1 ml) and water (0.5 ml) respectively.

Gas chromatography

Gas chromatography (GC) was performed on a Hewlett-Packard F & M 402 or a Hewlett-Packard 5840A gas chromatograph both equipped with flame ionization detectors and a digital electronic integrator. Analyses were carried out using the following GC columns: 1.8 m × 4 mm (i.d.) glass column packed with 5% SP-2100 on 100/120 mesh Supelcoport; 1.8 m × 4 mm (i.d.) glass column packed with 10% OV-11 on 100/120 mesh

Supelcoport; 2.0 m × 3.1 mm (i.d.) glass column packed with 3% OV-101 on 100/120 mesh Gas Chrom Q; 10 m × 0.25 mm (i.d.) fused quartz capillary column coated with SP-2100; 1 m × 0.25 mm (i.d.) fused quartz capillary coated with SE-30. Oven temperatures for isothermal separations were 320 °C for diethers and derivatives; 245 °C for monoethers and derivatives; 205 °C for fatty acid esters and 185 °C for alkanes, alkyl halides and alcohol derivatives. In some instances samples were analysed by temperature programming from 60–250 °C at a rate of 6 °C/min. Derivatives for GC analysis included: trimethylsilyl (TMS) derivatives prepared by incubating samples at room temp for 1 h in a mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane/bis-trimethylsilyl trifluoroacetamide (2:2:1:1, v/v); acetates prepared by incubation at 100 °C for 30 min in pyridine-acetic anhydride (3:1, v/v); and trifluoroacetates (TFA) prepared by incubation in trifluoroacetic anhydride at room temperature for 1 h.

Physical measurements

Electron impact (E.I.) and chemical ionization (C.I.) mass spectra were recorded with a Hewlett-Packard 5985 gas chromatograph-mass spectrometer combination system (GC-MS). Infrared spectra were recorded as liquid films between NaCl crystals using a Beckman IR-18A infrared spectrophotometer. Optical rotations of samples in chloroform were measured at 22 °C at 589 nm with a Bendix Ericsson automatic polarimeter. Quantitation of lipids separated by TLC and visualized by charring was estimated using a Zeineh soft laser scanning densitometer.

Materials

All solvents were freshly distilled before use. Available reference compounds including *n*-alkanes, isoheptadecane, anteisooctadecane, fatty acids, 1-octadecylglycerol, 1-hexadecylglycerol and 1,2-dioctadecylglycerol ethers were purchased commercially. Synthetic *rac*-dihexadecylglycerol ether was a gift from Dr. *M. Kates*. Authentic 2,3-diphytanylglycerol ether, phytane, and dibiphytanylglycerol tetraethers were prepared from *Halobacterium salinarium* and *Thermoplasma acidophilum* as described herein and elsewhere (*Langworthy*, 1977; 1982a).

Results

Total lipids extracted from *Thermodesulfotobacterium* accounted for about 6.9% of the cellular dry weight and consisted of 17.1% neutral lipids, 12.6% glycolipids and 70.3% phospholipids (Table 1). Examination of the glycolipid and phospholipid fractions by TLC indicated an apolar glycolipid component(s), R_f 0.95 (solvent iv) and three major phospholipids, R_f 0.53 (33%), R_f 0.67 (36%), R_f 0.74 (24%) along with three minor phospholipids, R_f 0.81, 0.87 and 0.95 (solvent v). When re-examined by TLC following alkaline methanolysis, all polar lipid components were resistant with the exception of one minor phospholipid (R_f 0.95) suggesting the presence of ether lipids. To release hydrophobic residues, the glycolipid and phospholipid fractions were subjected to acid methanolysis and the *n*-hexane-soluble products examined by TLC (solvent 1). As shown in Fig. 1, acid methanolysis of the phospholipids released three hydrophobic components designated 1, 2 and 3. The glycolipid fraction appeared to contain material that underwent severe degradation causing considerable tailing, although small amounts of components 1 and 2 were discernable. Accordingly, components 1, 2 and 3 from the phospholipid fraction were isolated by TLC and subjected to detailed analyses.

Table 1. Lipid composition of *Thermodesulfotobacterium**

Fraction	% Cell Dry Weight	% Total Lipid
Total lipid	6.96	100
Neutral lipid	1.19	17.1
Glycolipid	0.88	12.6
Phospholipid	4.89	70.3

*Lipid percentages were determined gravimetrically after fractionation by silicic acid chromatography.

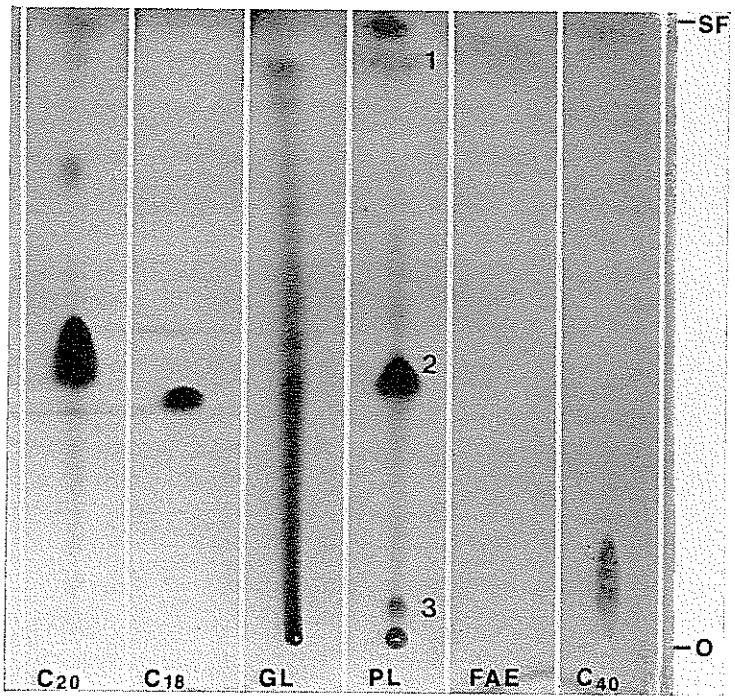

Fig. 1. Comparative thin-layer chromatogram of the *n*-hexane-soluble components released by acid methanolysis of the glycolipids (GL) and phospholipids (PL) from *Thermodesulfotobacterium.* (C_{20}) = 2,3-diphytanylglycerol ether from *H. salinarium;* (C_{18}) = synthetic 1,2-dioctadecylglycerol ether; (FAE) = mixture of C_{16} and C_{18} fatty acid esters; (C_{40}) = dibiphytanyl-diglycerol tetraethers from *T. acidophilum.* The solvent was *n*-hexane-diethyl ether-acetic acid (70:30:1 by volume). SF, solvent front; O, origin.

1. Fatty acid methyl esters

Component 1 (Fig. 1) migrated as fatty acid methyl esters (R_f 0.95) and represented about 7% of the material released from the phospholipids by acid methanolysis. The fatty acid methyl esters were identified by GC on columns of 5% SP-2100 and 10% OV-11 and found to consist primarily of *n*-C_{14} (1.2%), br-C_{15} (3.1%), *n*-C_{15} (1.9%), br-C_{16} (9.6%), *n*-C_{16} (15.1%), br-C_{17} (47.2%), br-C_{18} (6.5%), and *n*-C_{18} (14.5%) fatty acids.

2. Glycerol diethers

Component 2 (Fig. 1) represented about 82% of the hydrophobic residues and had a chromatographic mobility (R_f 0.42) similar to 2,3-diphytanylglycerol diether (R_f 0.41). The component was periodate-*Schiff* negative indicating the absence of vicinal OH groups and degradation with boron trichloride in chloroform released alkyl monochlorides (R_f 0.90, solvent ii) and glycerol (R_f 0.70, solvent iii). Its infrared spectrum (Fig. 2) was essentially identical to that of 2,3-diphytanylglycerol and showed absorption bands for the following groups: OH (3400 cm^{-1}), CH_2 and CH_3 (2930, 2860, 1465 cm^{-1}), C-CH_3 (1380 cm^{-1}), C-$(CH_3)_2$ (1380 and 1365 cm^{-1} doublet), ether C-O-C (1115 cm^{-1}) and primary C-OH (1045 cm^{-1}). There were no bands indicating double bonds or secondary OH groups. At this point the data indicated an α, β-dialkylglycerol ether indistinguishable from 2,3-diphytanylglycerol diether.

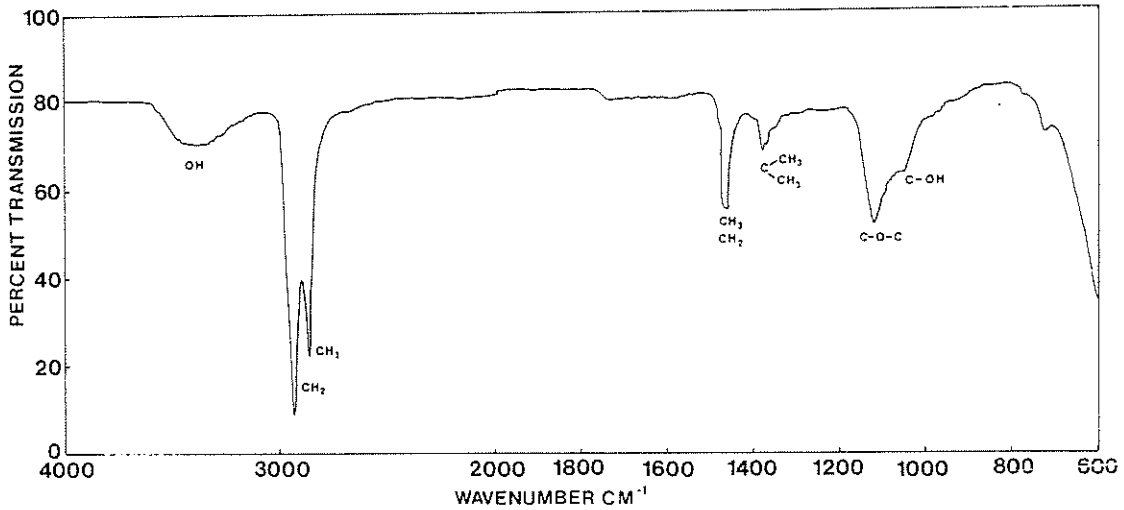

Fig. 2. Infrared spectrum of the glycerol diethers from *Thermodesulfotobacterium.*

GC analysis of component 2, however, on a 5% SP-2100 column failed to show the single peak expected for 2,3-diphytanylglycerol. Instead, chromatograms revealed four major diether peaks (Fig. 3) designated 1, 2, 3 and 4. In addition, peaks 1, 2 and 4 had slightly separated shoulders designated 1a, 2a and 4a. None of the diethers had retention times equivalent to phytanyl, octadecyl or hexadecylglycerol diethers either as the free diethers, TMS, TFA or acetate derivatives (Table 2).

GC-MS analysis of the diethers using an SE-30 fused quartz capillary column (1 m × 0.25 mm) showed the mixture to consist of 5 components. The methane C.I. mass spectra of the eluting substances, equivalent to peaks 1, 2, 3, 4 and 4a (Fig. 3) respectively, showed M + 1 peaks at m/e = 541, 555, 569, 583 and 597. These corresponded to a C_{16}:C_{16} chain glycerol diether (M = 540), a mixed C_{16}:C_{17} chain glycerol diether (M = 554), a C_{17}:C_{17} chain glycerol diether (M = 568), a C_{17}:C_{18} chain glycerol diether (M = 582), and a C_{18}:C_{18} chain glycerol diether (M = 596). The major constituent was the C_{17} diether (58.0%), followed by the mixed C_{16}:C_{17} diether (19.0%), mixed C_{17}:C_{18} diether (12.8%), C_{18} diether (5.4%) and the C_{16} diether (4.7%).

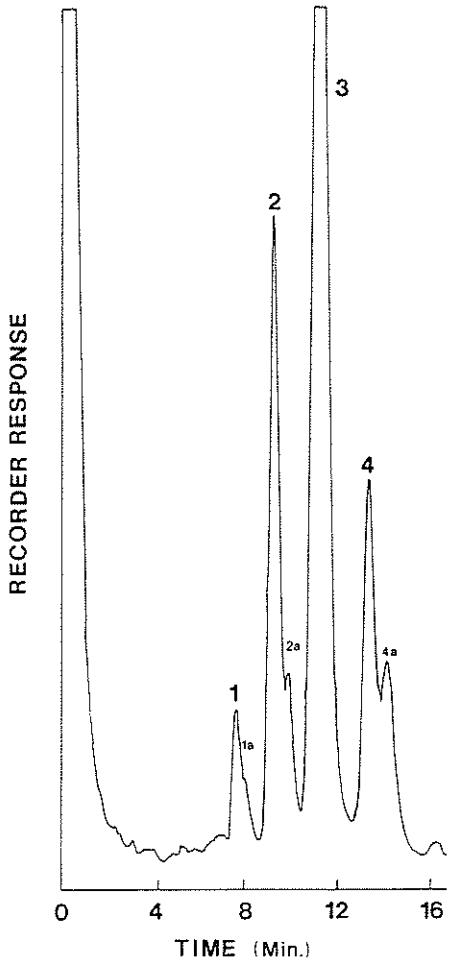

Fig. 3. Gas chromatogram of the free glycerol diethers of *Thermodesulfotobacterium*. Conditions: 5% SP-2100 (1.8 m × 4 mm column) at 320 °C. Peak 1 + 1a = C_{16}:C_{16} chain diether (4.7%); Peak 2 + 2a = C_{16}:C_{17} chain diether (19.0%); Peak 3 = C_{17}:C_{17} chain diether (58.0%); Peak 4 = C_{17}:C_{18} chain diether (12.8%); Peak 4a = C_{18}:C_{18} chain diether (5 4%) Shoulders 1a and 2a likely arise from isomeric differences in the structure and combination of the hydrocarbon chains.

The E.I. mass spectrum of the major C_{17} diether is shown in Fig. 4. The compound had a weak parent ion at m/e = 568. The peak at M-31 (m/e = 537) resulted from loss of the terminal CH_2OH group. Cleavage between the other two carbons of the glycerol molecule produced the ion at m/e = 299. The peak at m/e = 239 $(C_{17}H_{35})^+$ and the ions with lower masses were formed by the loss of the akyl side chain and its fragmentation. The other diether species showed a similar fragmentation pattern, e.g., the mixed C_{16}:C_{17} diether included peaks at m/e = 225 $(C_{16}H_{33})^+$ and 239 $(C_{17}H_{35})^+$, etc.

The alkyl side chains of the diether mixture were cleaved by hydriodic acid and the alkyl iodides reduced by zinc in acetic acid to the corresponding hydrocarbons. Analysis by routine GC on 5% SP-2100 columns revealed br-C_{16} (5.5%), *n*-C_{16} (1.4%), br-C_{17} (60.3%), br-C_{18} (15.3%), *n*-C_{18} (14.1%) and *n*-C_{19} (trace) hydrocarbons. No phytane could be detected. To further confirm identities and separate branched-chain isomers, the hydrocarbons were analysed by GC-MS on an SP-2100 fused quartz capillary column (10 m × 0.25 mm) which gave eight components. The mass spectra indicated the principal component to be the C_{17} hydrocarbon, anteisoheptadecane (M = 240) which showed characteristic ions at me/e = 211 (M-CH_2CH_3) and m/e = 183 (M-$CH_3CHCH_2CH_3$) as compared to the isoheptade-

Table 2. Relative retention times (*n*-hexatriacontane, $C_{36}H_{74}$ = 1.00) and comparison of the glycerol diethers and their derivatives from *Thermodesulfobacterium**

Diether	Free diether	TMS	TFA	Acetate
$C_{36}H_{74}$ (6.36 min)	1.00	1.00	1.00	1.00
Standard diethers				
2,3-diphytanylglycerol	2.26	1.97	1.53	2.30
1,2-dioctadecylglycerol	2.67	2.33	1.82	2.71
rac-dihexadecylglycerol	1.29	1.12	0.93	1.31
Bacterial diethers**				
diether 1	1.17	0.99	0.82	1.24
diether 2	1.41	1.29	0.99	1.50
diether 2a	1.49	1.32	1.05	1.60
diether 3	1.71	1.56	1.19	1.82
diether 4	2.00	1.83	1.40	2.13
diether 4a	2.13	1.94	1.48	2.27

* Gas chromatography on 5% SP-2100 (1.8 m x 4 mm column) at 320°C. TMS, trimethylsilyl derivative; TFA, trifluoroacetate derivative; Acetate, acetyl derivative.

** Refer to Fig. 3 for designation of diether components. Diether 1a not shown because of poor resolution from diether 1.

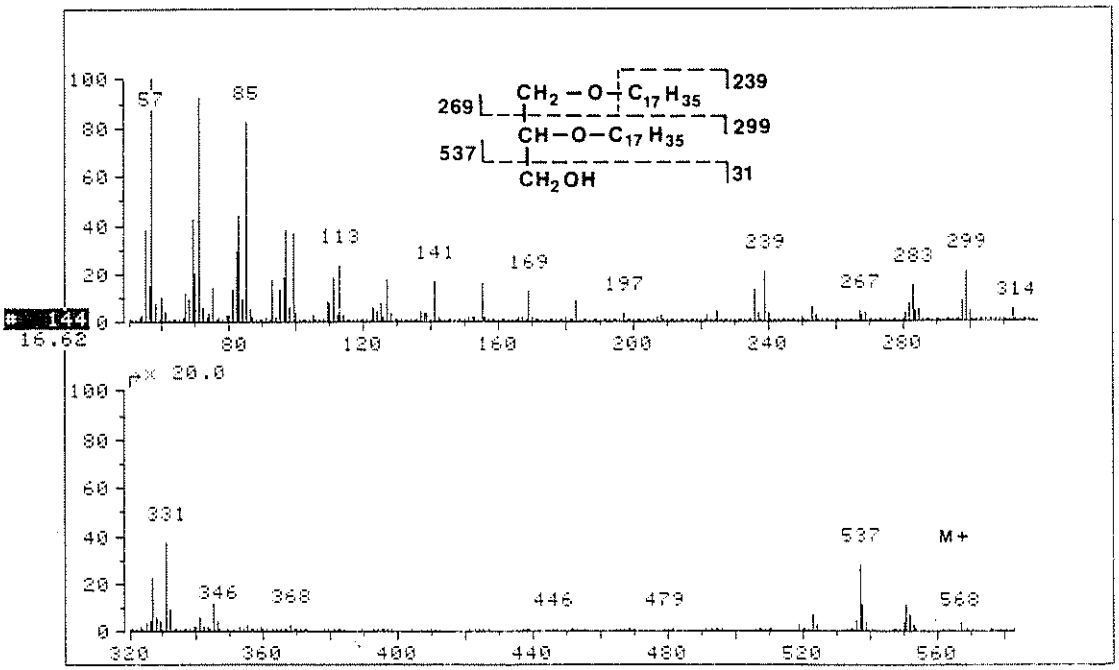

Fig. 4. Electron impact mass spectrum of the anteisoheptadecyl (br-C_{17}) glycerol diether from *Thermodesulfotobacterium*

cane (M = 240) isomer which showed a typical ion at m/e = 197 (M-CH_3CHCH_3). Similarly, the structures of the remaining hydrocarbons were assigned according to their GC retention times and mass spectral fragmentation patterns. The structures of the eight different hydrocarbon chains are shown in Fig. 5 and their distribution is given in Table 3.

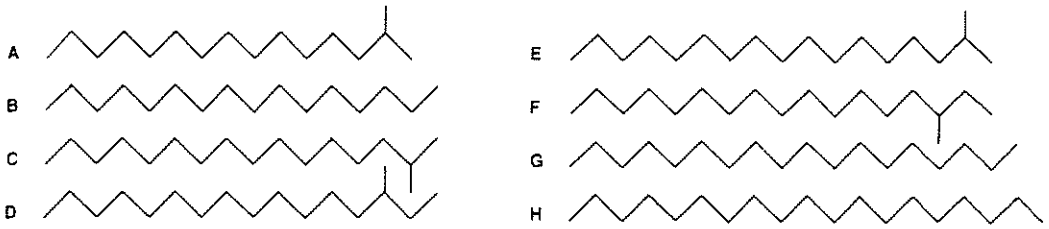

Fig. 5. Structure of the alkyl chains of the diethers from *Thermodesulfotobacterium*. (A) isohexadecane; (B) hexadecane; (C) isoheptadecane; (D) anteisoheptadecane; (E) isooctadecane; (F) anteisooctadecane; (G) octadecane; (H) nonadecane.

Table 3. Distribution of the alkyl hydrocarbon chains of the glycerol diethers from *Thermodesulfotobacterium*

Compound	Carbon number	Relative %
Isohexadecane	C_{16}	5.5
Hexadecane	C_{16}	1.9
Isoheptadecane	C_{17}	1.1
Anteisoheptadecane	C_{17}	60.2
Isooctadecane	C_{18}	7.9
Anteisooctadecane	C_{18}	8.4
Octadecane	C_{18}	14.1
Nonadecane	C_{19}	Trace

Ether-linkage of the hydrocarbon chains to glycerol was through the non-branched end of the alkyl chains as evidenced by the presence of free isopropyl groups (1380 and 1365 cm^{-1}, doublet) seen in the infrared spectrum of the diethers (Fig. 2). This was substantiated by GC-MS analysis of the alkyl side chains as the alcohol acetate derivatives prepared from the alkyl iodides by reflux in zinc and silver acetate. Eight alcohol acetates were separated by GC corresponding to the eight hydrocarbons shown in Table 3. The mass spectral fragmentation of these compounds involves a *McLafferty* rearrangement which can proceed via two different mechanisms (*Kienitz*, 1968). The principal mechanism involves the elimination of acetic acid with the positive charge remaining on the hydrocarbon chain. The resulting peak at M-60 was observed in all structures confirming the compounds to be monoalcohols, but could not be used as a diagnostic fragment. The secondary mechanism involves the elimination of acetic acid plus a second fragment, R_2C-CHR where R = CH_3 or H, with the positive charge remaining on the hydrocarbon chain.

Assuming that the ether bond did in fact occur through linkage at the branched end of the hydrocarbon chains, the second mechanism should yield (M-(60 + x) where x = 42, 56, 70, etc. However, all *iso*-structures yielded a strong fragment only at M-(60 + 28) which requires that the ether bond is made at the non-branched end of the *iso*-branched chains. A similar fragment (M-88) was seen in the mass spectra of the *anteiso*-branched compounds, but since the CH_3 group is attached to carbon 3 in these alkyl chains, the above mechanism can give an M-88 fragment whether the linkage is at either branched or non-branched end of the hydrocarbon chain. However, since the *iso*-branched chains are clearly ether-linked to glycerol via the non-branched ends, we see no reason to doubt that the *anteiso*-chains are not bound in the same manner.

Optical rotation of the diether mixture was taken in chloroform to determine the stereochemistry of the alkyl chain attachment to glycerol. The diethers were levorotary giving a specific rotation of $[\alpha]_D^{22} = -3.1°$ and a molecular rotation of $[M]_D^{22} = -16.7°$ based upon the average molecular weight distribution of the diether mixture. Although the normal and *iso*-branched side chains do not possess an asymmetric center, the *anteiso*-C_{17} and C_{18} hydrocarbons (68.6% of the total side chains) possess an optically active center through substitution of the CH_3 group to carbon 3 of the hydrocarbon chain (*Abrahamsson* et al., 1963). The contribution of the *anteiso*-chains to the apparent molecular rotation was determined by examination of the alkyl chains as the alcohol acetate derivatives in chloroform. The alcohol acetates were in fact dextrorotary $[\alpha]_D^{22} = +2.18°$ and $[M]_D^{22} = +5.83$. The diethers are therefore clearly levorotary being even more strongly negative ($M_D = -22.5$) than the apparent molecular rotation indicates. The alkyl side chains of the diethers of *Thermodesulfotobacterium* are therefore attached to the 1 and 2 carbons of glycerol (*sn*-1,2) like the naturally occurring acylglycerolipids rather than attached to the 2 and 3 carbons of glycerol (*sn*-2,3) like the archaebacterial phytanyl dieters ($[\alpha]_D = +8.7°$ and $M_D = +55°$) *Kates*, 1978).

Component 2 is therefore established to be a mixture of *sn*-1,2-dialkylglycerol diethers possessing normal (C_{16}, C_{18}, C_{19}), *iso*-branched (C_{16}, C_{17}, C_{18}) and *anteiso*-branched (C_{17}, C_{18}) hydrocarbon chains. The major species is the *sn*-1,2-dianteisoheptadecylglycerol diether.

3. *Glycerol monoethers*

Component 3 (Fig. 1) represented about 11% of the hydrophobic residues. It migrated (R_f 0.06) more slowly than dibiphytanyldiglycerol tetraethers (R_f 0.11) but similar to l-octadecylglycerol (R_f 0.06). The component was periodate-*Schiff* positive indicative of vicinal OH groups and boron trichloride released alkyl monochlorides and glycerol. The infrared spectrum was the same as for the diethers of component 2 except for the addition of a secondary OH band at 1090 cm^{-1}. These data indicated an α-alkylglycerol monoether(s).

GC analysis of component 3 on 5% SP-2100 showed 6 peaks (Fig. 6). Peak 2 (2.0%) co-chromatographed with l-hexadecylglycerol and peak 5 (12.0%) with l-octadecylglycerol. The remaining peaks (1, 3, 4, 6, respectively) had retention times corresponding to br-C_{16} (5.8%), br-C_{17} (48.4%), br-C_{18} (22.6%) and br-C_{19} (9.2%) glycerol monoethers. The relative retention times of the glycerol monoethers as the TMS, TFA and acetate derivatives are given in Table 4.

To separate isomeric structures and confirm identities, the monoethers were

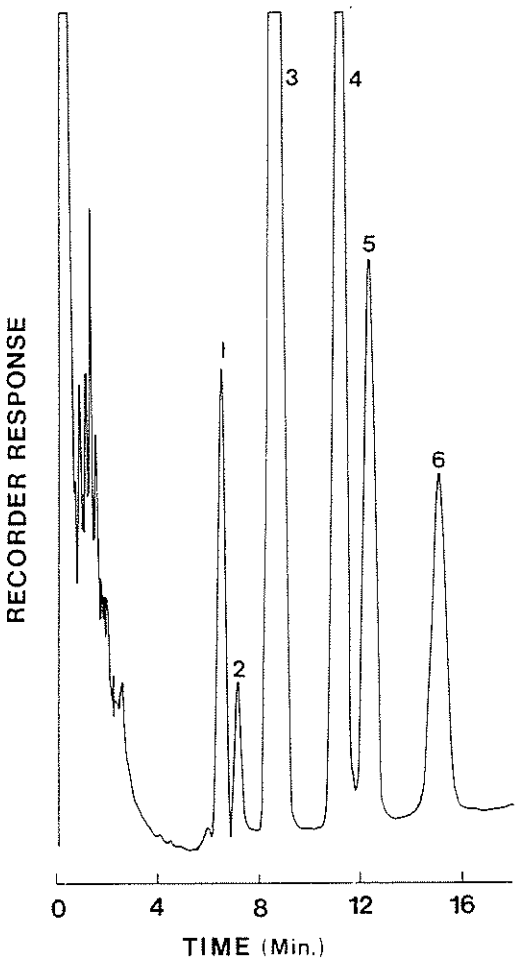

Fig. 6. Gas chromatogram of the glycerol monoethers from *Thermodesulfotobacterium*. Conditions: 5% SP-2100 (1.8 m × 4 mm column) at 245 °C. 1, br-C_{16} glycerol (5.8%); 2, *n*-C_{16} glycerol (2.0%); 3, br-C_{17} glycerol (48.4%); 4 br-C_{18} glycerol (22.6%); 5 *n*-C_{18} glycerol (12.0%); 6, br-C_{19} glycerol (9.2%).

analysed as the diacetate derivatives by GC-MS using a SP-2100 fused quartz capillary column (10 m × 0.25 mm). C.I. mass spectra with ammonia as the reactant gas showed base peaks (M + 18) at m/e = 418, 432, 446 and 460 corresponding to C_{16}, C_{17}, C_{18} and C_{19} monoalkylglycerol ether diacetates. The E.I. mass spectra of monoethers with isomeric alkyl side chains (*iso* and *anteiso*) were basically identical. Shown in Fig. 7 is the E.I. mass spectrum of the C_{17} anteisoheptadecylglycerol diacetate. No molecular ion (M = 414) was present. The peak at m/e = (-61) represented the loss of acetic acid from the M-1 ion and m/e = 341 (M-63) the loss of CH^2-OAc. The ion at m/e = 311 (M-103) was generated by loss of CH_2CO from the M-61 fragment. The peak at m/e = 269 (M-145) was produced by loss of AcOCH-CH_2OAc and the peak at m/e = 159 (M-255) by loss of $OC_{17}H_{35}$. The ion at m/e = 239 $(C_{17}H_{35})^+$ and ions with lower masses were formed by loss of the alkyl side chain and its fragmentation. The spectrum is representative of the other glycerol monoether diacetates, all of which showed no molecular ions but gave peaks at M-61, M-73, M-103, M-145 and M-OR. Importantly, all of the compounds gave a peak at M-145 (AcOCH-CH_2OAc) which can only be produced if the alkyl chain is attached to the α-carbon of glycerol.

The E.I. mass spectra of the underivatized glycerol monoethers also gave no molecular ions. The mass spectral patterns consisted of peaks at M-18 (H_2O), M-31

Table 4. Relative retention times (*n*-octacosane, $C_{28}H_{58}$ = 1.00) of the glycerol monoethers from *Thermodesulfotobacterium**

Monoether	TMS	TFA	Acetate
$C_{28}H_{58}$ (13.01 min)	1.00	1.00	1.00
Standard monoethers			
1-hexadecylglycerol (n-C_{16})	0.46	0.19	0.56
1-octadecylglycerol (n-C_{18})	0.78	0.32	0.96
Bacterial monoethers			
br-C_{16} glycerol	0.42	0.18	0.51
n-C_{16} glycerol	0.46	0.19	0.56
br-C_{17} glycerol	0.56	0.23	0.68
br-C_{18} glycerol	0.71	0.29	0.87
n-C_{18} glycerol	0.78	0.32	0.96
br-C_{19} glycerol	0.95	0.38	1.17

*Gas chromatography on 5% SP-2100 (1.8 m x 4 mm column) at 245°C. Under these conditions, branched chain isomers do not separate. Data for free monoethers not shown due to severe overlap and tailing. C = carbon number of monoalkyl chain; n = straight chain; br = branched chain. TMS, trimethylsilyl derivative; TFA, trifluoroacetate derivative; Acetate, acetyl derivative.

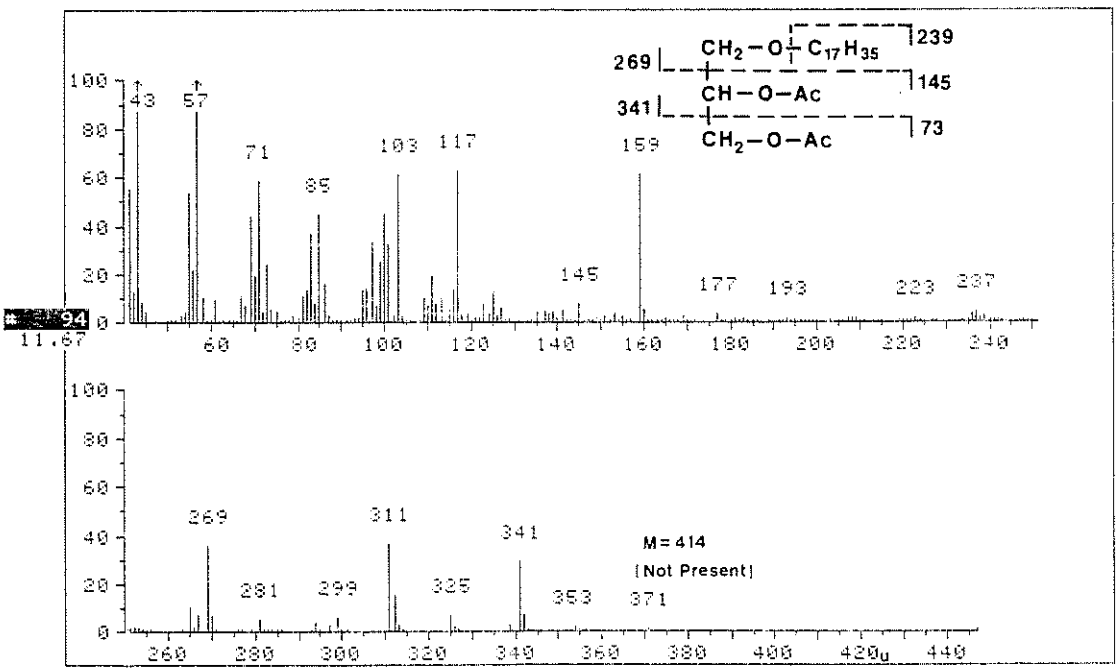

Fig. 7. Mass spectrum of the anteisoheptadecyl (br-C_{17}) glycerol monoether from *Thermodesulfotobacterium* as the diacetate derivative.

(CH_2OH), M-61 ($HOCH\text{-}CH_2OH$) and a strong peak corresponding to the alkyl side chain and the ions associated with its fragmentation.

The alkyl side chains of the diethers were cleaved by hydriodic acid, reduced to the alkanes and identified by GC-MS as already described for the glycerol diethers. The stereochemistry of the monoethers could not be determined experimentally because of insufficient sample for analysis. However, since the glycerol diethers possess the *sn*-1,2-glycerol configuration, it would be unreasonable to assume that the α-linked alkyl chains of the monoethers are attached to carbon 3 of glycerol. We therefore assign the *sn*-l-glycerol stereoconfiguration to the glycerol monoethers.

Component 3 is therefore identified as a mixture of normal (C_{16}, C_{18}) *iso*-branched C_{16}, C_{17}, C_{18}, C_{19}) and *anteiso*-branched (C_{17}, C_{18}, C_{19}) l-alkylglycerol monoethers whose distribution is given in Table 5.

Table 5. Distribution of the glycerol monoethers from *Thermodesulfotobacterium*

Compound	Carbon number alkyl chain	Relative %
1-Isohexadecylglycerol	C_{16}	5.8
1-Hexadecylglycerol	C_{16}	2.0
1-Isoheptadecylglycerol	C_{17}	15.5
1-Anteisoheptadecylglycerol	C_{17}	32.7
1-Isooctadecylglycerol	C_{18}	22.6
1-Octadecylglycerol	C_{18}	12.0
1-Isononadecylglycerol	C_{19}	1.4
1-Anteisononadecylglycerol	C_{19}	8.0

Neutral lipids

Since the neutral lipids of the archaebacteria are characteristically composed of C_{16} to C_{30} isoprenoid hydrocarbons (*Langworthy* et al., 1982), it was of interest to compare the neutral lipid composition of *Thermodesulfotobacterium*. Neutral lipids were separated by column chromatography on activated silicic acid (Unisil, 325 mesh) by elution with *n*-hexane, benzene and chloroform. Components in each eluate were identified by GC-MS using an SP-2100 fused quartz capillary column (10 × 0.25 mm).

The *n*-hexane fraction was found to consist of C_{16} to C_{35} hydrocarbons, principally straight chain compounds (Table 6). Branched hydrocarbons were only minor constituents.

The major compounds recovered from the benzene eluate consisted of free fatty acids which included: *n*-C_{14} (0.3%), *iso*-C_{16} (0.1%), *n*-C_{16} (20.4%), *n*-C_{17} (13.0%), *iso*-C_{18} (14.1%) and *n*-C_{18} (39.0%). In addition, the chloroform eluate contained several unidentified components (10.6%), but at least two isoprenoids were isolated and one of them identified as squalene (1.5%). The mass spectrum of the second component (1.1%), indicated a polyunsaturated isoprenoid ($C_{34}H_{58}$) with six double bonds. The mass spectrum of this compound showed a parent ion at m/e = 466 and peaks at M-43, M-111 and M-179. Several fragments resulting from *bis*-allylic

Table 6. Hydrocarbons from *Thermodesulfotobacterium* neutral lipids*

Compound		Relative %	Compound		Relative %
Isohexadecane	($C_{16}H_{34}$)	0.6	n-Pentacosane	($C_{25}H_{52}$)	2.3
Anteisohexadecane	($C_{16}H_{34}$)	1.2	n-Hexacosane	($C_{26}H_{54}$)	2.9
Pristane	($C_{19}H_{40}$)	0.4	n-Heptacosane	($C_{27}H_{56}$)	2.6
n-Octadecane	($C_{18}H_{38}$)	1.4	n-Octacosane	($C_{28}H_{58}$)	6.3
Phytane	($C_{20}H_{42}$)	1.2	n-Nonacosane	($C_{29}H_{60}$)	10.5
Isoeicosane	($C_{20}H_{42}$)	0.3	br-Tetratriacontane	($C_{34}H_{70}$)	1.1
n-Nonadecane	($C_{19}H_{40}$)	2.0	n-Triacontane	($C_{30}H_{62}$)	13.0
n-Eicosane	($C_{20}H_{42}$)	0.5	n-Heneitriacontane	($C_{31}H_{64}$)	15.5
n-Heneicosane	($C_{21}H_{44}$)	0.6	n-Dotriacontane	($C_{32}H_{66}$)	12.1
n-Docosane	($C_{22}H_{46}$)	1.5	br-Pentatriacontane	($C_{35}H_{72}$)	1.0
n-Tricosane	($C_{23}H_{48}$)	1.1	n-Tritriacontane	($C_{33}H_{68}$)	9.7
5-Methyltricosane	($C_{24}H_{50}$)	2.2	n-Tetratriacontane	($C_{34}H_{70}$)	5.1
n-Tetracosane	($C_{24}H_{50}$)	1.4	n-Pentatriacontane	($C_{35}H_{72}$)	2.1

*Hydrocarbons recovered from the n-hexane eluate of neutral lipids fractionated on silicic acid. Compounds listed in their order of their elution from a 10 m x 0.25 mm SP-2100 fused quartz capillary column temperature programed: 60°C-240°C at 6°C/min.

cleavage of the isoprenoid chain were noted, e.g., M-69, M-69-68, M-69-68-68. Such a pattern is consistent with a regular head to tail isoprenoid skeleton.

The components of the chloroform fraction consisted of the same free fatty acids, which in part were recovered from the benzene eluate.

Thus, in contrast to the archaebacteria, the neutral lipids of *Thermodesulfotobacterium* consist of free fatty acids accounting for about 57% of the total along with mainly non-isoprenoid hydrocarbons.

Discussion

The glycerolipids of *Thermodesulfotobacterium,* as we have shown here, consist of a new series of natural 1,2-dialkylglycerol diethers which constitute about 82% of the hydrophobic residues of the polar lipids. The alkyl chains consist of normal, *iso*- and *anteiso*-branched alkanes which give rise to C_{16}:C_{16}, mixed C_{16}:C_{17}, C_{17}:C_{17}, mixed C_{17}:C_{18} and C_{18}:C_{18} and C_{18}:C_{18} chain glycerol diethers as the principal components. The major diether present has been fully identified as 1,2-di-O-anteiso-heptadecyl-*sn*-glycerol whose structure is shown in Fig. 8. Also present in lesser quantities (11%) were a series of l-alkylglycerol monoethers whose structures have been identified and shown in Fig. 9. Although glycerol ethers represented most of the polar lipid hydrophobic residues (93%), fatty acids (7%) were also detected. Since the fatty acids and glycerol monoethers occur in fairly close proportions (0.6:1.0), it is very likely that the majority of the fatty acids are esterified to carbon 2 of glycerol in the monoether's natural form.

Up until now, the thermoacidophilic, methanogenic and halophilic archaebacteria were the only organisms known to possess exclusively etherlinked glycerolipids (*Langworthy* et al., 1982). These consists of the isopranoid-branched diphytanylglycerol diether found in all archaebacteria and dibiphytanyldiglycerol tetraethers present in the thermoacidophilic and certain methanogenic archaebacteria (Fig. 10).

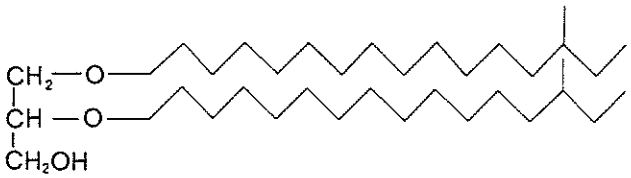

Fig. 8. Structure of the major glycerol diether from *Thermodesulfotobacterium*, 1,2-di-*O*-anteisoheptadecyl-*sn*-glycerol.

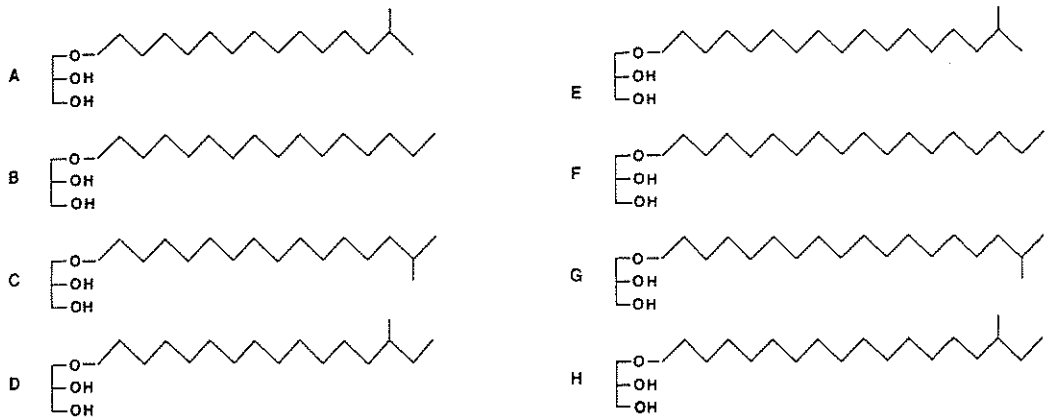

Fig. 9. Structure of the glycerol monoethers from *Thermodesulfotobacterium*. (A) 1-O-isohexadecyl-*sn*-glycerol; (B) 1-O-hexadecyl-*sn*-glycerol; (C) 1-O-isoheptadecyl-*sn*-glycerol; (D) 1-O-anteisoheptadecyl-*sn*-glycerol; (E) 1-O-isooctadecyl-*sn*-glycerol; (F) 1-O-octadecyl-*sn*-glycerol; (G) 1-O-isononadecyl-*sn*-glycerol; (H) 1-O-anteisononadecyl-*sn*-glycerol.

Although *Thermodesulfotobacterium* synthesizes glycerol diethers, they are entirely different both structurally and stereochemically from archaebacterial dieters. The archaebacterial diether exists as a single species that contains two identical alkyl side chains fixed at 20-carbon atoms as phytane and that are ether-linked to the 2 and 3 carbons of glycerol. On the other hand, the diethers of *Thermodesulfotobacterium* contain non-isopranyl side chains of variable carbon number (C_{16} to C_{19}) and are stereochemically the mirror image of the archaebacterial diethers with the side chains ether-linked to the 1 and 2 carbons of glycerol. The new diethers are in fact structurally and stereochemically identical to the *sn*-1,2-diacylglycerols of eukaryotes and eubacteria with the one important exception that the side chains are ether-linked rather than ester-linked to glycerol. This of course indicates a different route of ether lipid biosynthesis in *Thermodesulfotobacterium*.

The nature of the ether lipids suggests that *Thermodesulfotobacterium* is not an archaebacterium, but rather an organism that has evolved differently from any other known eubacterium. This is substantiated by the absence of the isoprenoid hydrocarbons that characterize the neutral lipids of archaebacteria (*Langworthy* et al., 1982), by the sensitivity of the organism to cell wall antibiotics such as penicillin and cycloserine indicating a peptidoglycan cell wall structure (*Zeikus* et al., 1982) and by 16S rRNA analysis (*C. R. Woese*, personal communication).

Thermodesulfotobacterium also represents a biological source for the contribution of branched-chain glycerol ethers and hydrocarbons to geological formations. *Chappe* et al. (1979) have identified, in addition to the archaebacterial ether-

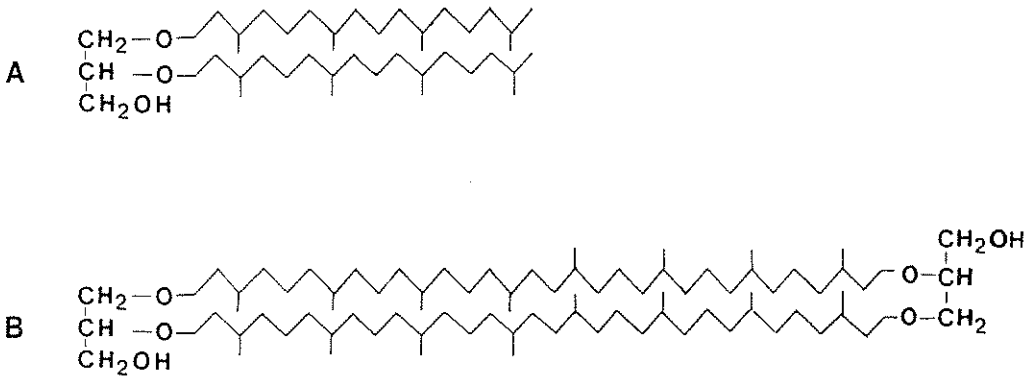

Fig. 10. Structure of the phytanyl diether and dibiphytanyldiglycerol tetraethers of archaebacteria: (A) 2,3-di-O-phytanyl-*sn*-glycerol diether; (B) 2,3,2′,3′-tetra-O-dibiphytanyl-*di-sn*-glycerol tetraether.

linked phytane and biphytane in kerogen, a mixture of ether-linked branched hydrocarbons and identified the most abundant component at isopentadecane ($C_{15}H_{32}$). Also detected was an ether-linked, $C_{30}H_{62}$, head to head condensed dimer of isopentadecane (biisopentadecane). It was predicted that such compounds arose from glycerol monoethers, diethers and tetraethers produced by a biological source. Clearly, the monoethers and diethers of *Thermodesulfotobacterium* fulfill the first part of this prediction, but we were unable to detect any head-to-head condensed *iso*-alkanes or derived diglycerol tetraethers in this organism. However, the fact that *iso*-branched diethers do exist in a living cell, and since diethers may be prerequisite to the synthesis of tetraethers (*Langworthy,* 1982b; *Langworthy* et al., 1982), other organisms found to produce the new diethers might also produce condensed *iso*-alkyl diglycerol tetraethers.

Finally, the diethers of *Thermodesulfotobacterium* have many physical properties indistinguishable from archaebacterial diethers. This indicates that a more thorough chemical analysis of the glycerolipids of new organisms is required than simply TLC mobilities and an infrared spectrum when using glycerol ethers as a chemical marker for distinguishing archaebacteria (*Ross* et al., 1981; *Langworthy* et al., 1982; *Langworthy,* 1982a, b).

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