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Membrane Lipid from Deep-Sea Hydrothermal Vent Methanogen: a New Macrocyclic Glycerol Diether

Abstract. *The membrane lipid of a new deep-sea hydrothermal vent methanogen,* Methanococcus jannaschii, *has been structurally characterized. The hydrolyzed polar lipid of this archaebacterium is primarily (95 percent) a macrocyclic glycerol diether, which has not been described previously. The structure was elucidated by a combination of chemical and spectroscopic techniques. An initial survey of selected methanogens failed to indicate the presence of this membrane lipid in any microorganism other than* Methanococcus jannaschii.

As part of an effort to determine source markers for biogenic organic matter in hydrothermal vent fluids, we have characterized the lipids of the newly discovered methanogenic bacterium *Methanococcus jannaschii* which was recently isolated from deep-sea hydrothermal vent sediment (*1*). The lipids of methanogens (*2–6*), as well as of other archaebacteria (*7–19*), consist primarily of (i) isoprenoid hydrocarbons and (ii) alkylglycerol ethers. The latter are stable ether-linked analogs of the ester-linked glycolipids and phospholipids found in other organisms, and thus fulfill our requirement of being new, source-specific markers for microbial input to organic matter at hydrothermal vent sites (*20*).

We describe here the structure of the major alkylglycerol ether, **1a** (Fig. 1), of *Methanococcus jannaschii*. This structure had been tentatively proposed for an isolated glycerol ether from another species of archaebacteria (*13, 15*), but more detailed studies have shown that the compound for which this structure had been proposed was actually the high molecular weight bis-(diphytanyl)diglycerol tetraether (**2**) (*2–6, 10–12*).

Methanococcus jannaschii was grown on hydrogen and carbon dioxide optimally at 85°C (*1*). Freeze-dried cells of the microorganism were suspended in a mixture of methylene chloride (CH_2Cl_2), methanol, and water and were sonicated. The total lipid extract (TLE) was then partitioned between CH_2Cl_2 and water, and subsequently the CH_2Cl_2 fraction was evaporated to dryness below 35°C. This resulted in 32.2 μg of TLE per milligram (dry cell weight), in approximate agreement with reported values for methanogenic bacteria (*2–6*).

The TLE was separated into individual compound classes by adsorption chromatography on silica gel. Elution with a series of solvents of increasing polarity separated the TLE into a neutral lipid fraction (NLF) and polar lipid fraction (PLF). The PLF, eluted by ethyl acetate and methanol fractions, yielded 8.4 mg/g (dry cell weight). This fraction was hydrolyzed for 5 hours at 100°C with a mixture of 4.0*M* methanolic HCl. The hydrolyzate was neutralized and extracted with hexane, and the resulting hydrolyzed PLF (HPLF) was studied spectroscopically.

Infrared spectra of the HPLF showed absorptions corresponding to a hydroxyl group (3200 to 3600 cm^{-1}, 1258 cm^{-1}), and a carbon-oxygen single bond (1060 to 1140 cm^{-1}) (*21*). The infrared spectrum is nearly identical to that of authentic bis-(phytanyl)glycerol diether (**3a**) and bis-(diphytanyl)diglycerol tetraether (**2**) (*3, 5, 9, 11, 17, 18*).

High-field proton nuclear magnetic resonance (NMR) spectra of the HPLF ethers were taken with a Bruker 250 MHz instrument (*21*). The chemical shift data show resonances consistent with a glycerol ether structure. The methyl resonances are doublets, showing coupling to one proton, as would be expected for a methyl group in an isoprenoid alkyl chain. The ^{1}H-NMR spectrum of this substance closely matched that of authentic **2** and **3a**.

Field desorption mass spectroscopy was performed on the HPLF (Varian MAT 731 double-focusing mass spectrometer). Field desorption mass spectra of authentic **3a** and the HPLF differ by two mass units with a m/z of 653 for the protonated molecular ion of **3a** and m/z of 651 for the protonated molecular ion of the HPLF molecule. Authentic tetraether **2** exhibits an ion cluster in the molecular ion region of 1292 to 1300. This ion cluster, with a maximum at 1296, is not present in the HPLF molecule, thus clearly indicating that the glycerol ether in the HPLF is not **2**.

High-resolution glass capillary gas chromatography (GC) of the acetate and trimethylsilyl derivatives of the HPLF was accomplished with the use of a deactivated, cross-linked SE-52–coated (25.5 m by 0.32 mm inside diameter) column. Two components were present in the HPLF. The minor component (5 mole percent) elutes, both as the acetate and the trimethylsilyl ether, with authentic bis-(phytanyl)glycerol diether acetate (**3b**) and trimethylsilyl ether (**3c**), respectively. GC–mass spectrometry (MS) of the HPLF shows that the minor component has identical fragmentation patterns as the authentic **3b** and **3c**. The major component (**1a**) of the HPLF (95 mole percent) has a different fragmentation pattern, both as the acetate and trimethylsilyl derivative (*21*). Quantitative data for the amounts of the alkylglycerol ethers in the NLF and PLF are displayed in Table 1.

The HPLF ethers were converted to bromides by heating with boron tribromide (BBr_3) at 90°C for 4 hours (Fig. 2). This reaction for the HPLF takes place in 95 percent yield (by weight) and results in two compounds. The major component (mole fraction 0.87) coelutes with diphytanyl dibromide (**4**), and its mass fragmentation pattern is identical to that of (**4**). The minor component (mole fraction 0.13) coelutes with phytanyl bro-

mide (**5**) and has an identical mass fragmentation pattern as (**5**) (GC-MS). The bromides were converted to hydrocarbons by reacting them with hexane-extracted lithium aluminum hydride in dry tetrahydrofuran. This reaction results in a major component (mole fraction 0.86) which coelutes and has an identical MS fragmentation pattern as diphytane (*22*) and a minor component (mole fraction 14), which coelutes and has an identical MS fragmentation pattern as phytane, confirming the identity of the isoprenoid alkyl groups that make up the glycerol ethers of the HPLF (Fig. 2).

Since phytanyl bromide (**5**) is the product from reaction of bis-(phytanyl)-glycerol diether with BBr_3, diphytanyl dibromide must be formed from the major alkylglycerol ether in the HPLF. On the basis of the stoichiometry of the bromination reaction, a mole ratio of 95:5 for ethers in the HPLF would give rise to a 90:10 mole ratio of **4** to **5**, if one molecule of diphytanyl dibromide is the product from the major glycerol ether. The ratio of **4** to **5** is 87:13, within our experimental error of the expected mole ration of 90:10. Confirmation of the bromination reaction of **3a** to give **5** was experimentally verified by reacting authentic **3a** with BBr_3; the product was exclusively phytanyl bromide. Reaction products from the glycerol portion of the molecule were not determined.

Mass balance of the bromination reaction implies a 1:1 correspondence between the major alkylglycerol ether of the HPLF and diphytanyl dibromide. Since the major alkylglycerol ether has a molecular ion *m/z* of 650 by field desorption mass spectroscopy, we propose that the structure is the macrocycle **1a** (Fig. 1). High-resolution mass spectroscopy of the trimethylsilyl ether **1c** confirms this structure. The precise mass for **1c** is 722.69526; the calculated mass for $C_{46}H_{94}O_3Si$ is 722.69729. The electron impact mass fragmentation pattern of **1c** and **1d** is entirely consistent with this structure. Strong molecular ions occur at *m/z* 722 and 731 for **1c** and **1d**, respectively. Loss of trimethylsilyl alcohol (M^+, −90 and 99) gives rise to the base peak at *m/z* 632 (*21*).

Table 1. Polar lipids and alkylglycerol ethers in *Methanococcus jannaschii*.

Fraction	Amount (μg/mg) per	
	Dry cell weight	Total lipid extract
Polar lipids (PLF)	8.44	262.4
Hydrolyzed polar lipids (HPLF)	3.83	119.1
Alkylglycerol ethers		
NLF		
1a	0.122	3.78
2a	ND	ND
3a	40.7	1.27
PLF	0.041	
1a	4.21	130.8
2a	[< 0.126]*	[$< 4 \times 10^{-3}$]*
3a	0.109	3.38

*Estimate based on bromination reaction.

We are unaware of any species of archaebacteria that contain the macrocycle **1a** other than *Methanococcus jannaschii*. We have screened selected methanogens in order to ascertain, if possible, whether the presence of **1a** is a feature of the order Methanococcales. If so, this ether lipid would give some evidence for phylogenetic or morphological relatedness among methanogens in a crude way. Two other methanogens, *M. voltae* and *M. thermolithotrophicus*, of the order Methanococcales were evaluated and neither contained **1a**.

Another possible explanation for the presence of the macrocycle **1a** is that it is synthesized by thermophiles in order to regulate membrane fluidity at high temperatures. For this reason, three thermohilic methanogens—including *M. thermolithotrophicus*, a methanogen recently isolated from geothermally heated sea sediments off the coast of Naples, Italy (*23*), *Methanobacterium thermoautotrophicum* (*24*), and *Methanosarcinae barkeri TM-1* (*25*)—were assessed. None of these thermophiles contained **1a** in the free lipid or phospholipid form.

A third order of methanogens, Methanomicrobiales, was also screened. These were *Methanospirillum hungatei JF-1*, in the family Methanomicrobiaceae, and *Methanosarcinae barkeri MS* and *TM-1*, in the family Methanosarcinaceae. None of these methanogens contained **1a**. From this limited screening, we conclude that the macrocyclic ether **1a** appears to be a cell membrane component that is characteristic of *Methanococcus jannaschii*. The apparent specificity of this membrane lipid to the hydrothermal vent microorganism, and the extreme chemical stability imparted by the isoprenyl glycerol ether structure, may potentially make this molecule useful in paleoenvironmental studies, particularly with regard to tracing biological material of this origin.

The concentration of organisms around the individual vent areas is part of a much larger biological community that extends along the axial ridge of the Galápagos Rift, the East Pacific Rise at 21°N, and Guaymas Basin. Although the hydrothermal vent sites are spatially distant and extremely localized, the biological communities appear to have low diversity and are somewhat homogeneous (*26*). For example, the Alvinellid worms,

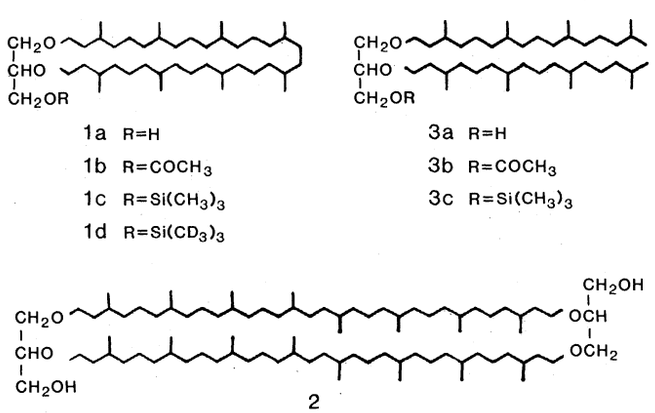
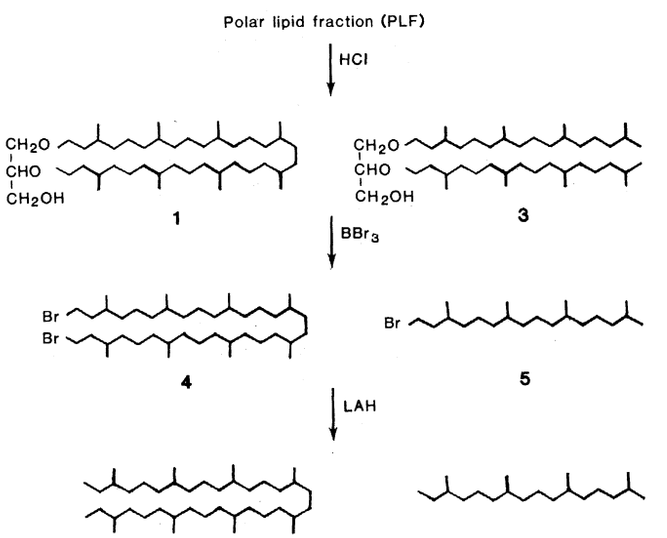


Fig. 1 (above). Structures of the macrocyclic alkylglycerol ether **1** from *Methanococcus jannaschii*, bis-(diphytanyl)diglycerol tetraether **2**, and bis-(phytanyl)glycerol diether **3**. Fig. 2 (right). Refraction scheme of the polar lipid fraction.



riftia tube worms, galatheid crabs, and *Calyptogena magnifica* clam, as well as other organisms, are present at the Galápagos site and the East Pacific Rise hydrothermal vents at 21°N. The mechanism by which these benthic organisms migrate from one isolated deep-sea hydrothermal vent site to another is likely to be in the form of a planktonic larval stage (*26*).

The most commonly observed and morphologically conspicuous microorganisms found at the Galápagos hydrothermal vent systems have been described (*27*). These microorganisms and those found at the East Pacific Rise participate in a diverse number of microbial processes; they include sulfur-oxidizing, metal-oxidizing, and methane- and methylamine-oxidizing bacteria, as well as the thermophilic methanogen *M. jannaschii* (*28*). Since the macrocycle **1a** appears to occur only in *M. jannaschii*, this molecule can potentially be used as a species-specific marker to assess this microbe's contribution to the archaebacterial community in the hydrothermal vent systems. In addition, the use of this molecular marker and the other glycerol ethers should enable us to determine whether the archaebacterial community is relatively homogeneous at geographically distant and isolated hydrothermal vent sites. Our screening does not preclude the possibility that other hydrothermal vent archaebacteria biosynthesize the macrocyclic ether **1a**, but certainly the absence of **1a** among the glycerol ethers in hydrothermal vent organic material would rule out the presence of *M. jannaschii*.

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The Exclusion of D_2O from the Hydration Sphere of $FeSO_4 \cdot 7H_2O$ Oxidized by *Thiobacillus ferrooxidans*

Abstract. *Infrared spectra demonstrate that neither $FeSO_4 \cdot 7H_2O$ nor its bacterial or abiotic hydrated oxidation products incorporate deuterium in acid D_2O solutions. Deuterium exchange occurred as bridging OD when bacterially oxidized iron was precipitated from D_2O solutions as ferric hydroxysulfates. The exclusion of deuterium depended upon the stabilization of aquated Fe(II) and Fe(III) complexes by sulfate ions in outer-sphere coordination and is consistent with the requirement and postulated role of sulfate in iron oxidation by* Thiobacillus ferrooxidans.

Despite the recognized importance of metal ions to living systems, often overlooked is the fact that all biologically important cations are present in aqueous solution as hydrated species. The chemical reactivity of a cation may depend upon molecular exchange between its primary hydration sphere and other molecules in solution (*1*), yet very little is known about how the biological activity of a metal ion relates to the coordinated water molecules. I present evidence that oxidation of iron by a chemolithotrophic bacterium is dependent upon the existence in acid solution of a Fe(II) hydrate complex stabilized by SO_4^{2-}. The organism, *Thiobacillus ferrooxidans*, is important in the natural leaching of pyritic minerals and in biohydrometallurgical technology (*2*).

Although rates for the exchange between solvent water molecules and water molecules in the hydration sphere of aquated ions differ widely, even the most tightly bound are reported to exchange at measurable rates (*1, 3*). For example, one of the slowest reported is hexaaquated Rh(III), in solution with perchlorate ions ($t_{1/2}$ = 33 hours) (*4*); more common are values like 10^{-6} second for hexaaquated Fe(II) ammonium sulfate or hexaaquated Co(II) (*5*).

A slower range of exchange rates would be expected with D_2O as solvent than with H_2O, since the energy required for breaking deuterium bonds is generally thought to be greater than for hydrogen bonds (*6*). However, isotope effects alone would not prevent the formation of deuterated complexes, at measurable rates, during incubation of soluble hydrated complexes in 95 percent D_2O solutions (*7*). It was extraordinary, therefore, to find that hydrated Fe(III) precipitates derived from $FeSO_4 \cdot 7H_2O$ in 95 percent D_2O solutions, acidified to permit oxidation by *T. ferrooxidans*, did not contain demonstrable amounts of deuterium.

Earlier investigation had shown that oxidation by the acidophilic chemolithotrophic bacterium *T. ferrooxidans* precipitated an amorphous hydrated Fe(III) sulfate from acidic Fe(II) sulfate solutions (*8*). This precipitate was similar in composition and infrared spectrum to an amorphous Fe(III) sulfate ($2Fe_2O_3 \cdot SO_3 \cdot mH_2O$) described by Margulis *et al.* (*9*). In an effort to characterize this material and determine if the