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Novel archaeal macrocyclic diether core membrane lipids in a methane-derived carbonate crust from a mud volcano in the Sorokin Trough, NE Black Sea

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Summary A methane-derived carbonate crust was collected from the recently discovered NIOZ mud volcano in the Sorokin Trough, NE Black Sea during the 11th Training-through-Research cruise of the R/V Professor Logachev. Among several specific bacterial and archaeal membrane lipids present in this crust, two novel macrocyclic diphytanyl glycerol diethers, containing one or two cyclopentane rings, were detected. Their structures were tentatively identified based on the interpretation of mass spectra, comparison with previously reported mass spectral data, and a hydrogenation experiment. This macrocyclic type of archaeal core membrane diether lipid has so far been identified only in the deep-sea hydrothermal vent methanogen *Methanococcus jannaschii*. Here, we provide the first evidence that these macrocyclic diethers can also contain internal cyclopentane rings. The molecular structure of the novel diethers resembles that of dibiphytanyl tetraethers in which biphytane chains, containing one and two pentacyclic rings, also occur. Such tetraethers were abundant in the crust. Compound-specific isotope measurements revealed $\delta^{13}C$ values of –104 to –111‰ for these new archaeal lipids, indicating that they are derived from methanotrophic archaea acting within anaerobic methane-oxidizing consortia, which subsequently induce authigenic carbonate formation.

Keywords: anaerobic oxidation of methane, archaeal membrane lipids, fluid venting, microbial processes.

Introduction

Geological exploration of the ocean floor has revealed widely occurring fields of submarine fluid discharge, so-called "cold seeps" or "methane seeps," which create specific structures on the sea floor such as mud volcanoes and pockmarks (Hovland and Judd 1988, Limonov et al. 1994, 1997, Ivanov et al. 1996*a*, 1996*b*). In these settings, constant and focused fluid supply induces marked biological activity depending on chemosynthetic nutrition (Hovland and Judd 1988, Sibuet et al. 1988, Corselli and Basso 1996, Olu et al. 1996, 1997, Sibuet and Olu 1998). Specifically, it is manifested in the appearance and diversity of chemoautotrophic microorganisms (bacteria and archaea) thriving in the unique environments created by strong advection of altered methane-rich fluids (Sassen et al. 1993, Cragg et al. 1996, Lanoil et al. 2001). The most important processes fueling these light-independent biological communities are the aerobic and anaerobic oxidation of methane.

Numerous field and laboratory observations have indicated that, in areas of intensive hydrocarbon gas venting, anaerobic oxidation of methane (AOM) is the dominant pathway for methane consumption. It has been proposed that AOM is performed by methanogenic archaea, acting in reverse, in tandem with sulfate-reducing bacteria consuming the formed hydrogen, which makes the reaction thermodynamically feasible (Reeburgh 1976, Zender and Brock 1979, Alperin and Reeburgh 1985, Hoehler et al. 1994). Recently, multiple lines of investigation have demonstrated the ability of archaea to use methane as a carbon source, i.e., to act as methanotrophic organisms. The light carbon isotopic composition of archaeal membrane lipids revealed that archaea incorporated isotopically light, methane-derived carbon into their cell carbon (Hinrichs et al. 1999, Elvert et al. 2000, Hinrichs et al. 2000*b*, Pancost et al. 2000, 2001*a*, 2001*b*, Thiel et al. 2001, Zhang et al. 2002, Teske et al. 2002). In association with isotopically depleted archaeal membrane lipids, lipids derived from sulfate-reducing bacteria have been found in varying distributions (Pancost et al. 2000, 2001*a*). These lipids are also substantially depleted in $\delta^{13}C$, albeit slightly less so than the archaeal lipids, indicating involvement of sulfate-reducing bacteria in the microbial community engaged in AOM. The best evidence of a consortium of archaea and sulfate-reducing bacteria was obtained by comparison of 16S ribosomal RNA gene sequences and fluorescence in situ hybridization analysis, which showed that methane-oxidizing archaea are phylogenetically associated with the methanogenic orders Methan-

omicrobiales and Methanosarcinales (Hinrichs et al. 1999, 2000*b*, Boetius et al. 2000, Orphan et al. 2001, Teske et al. 2002), and that their cell aggregates are surrounded by exosymbiotic, nutritionally versatile sulfate-reducing bacteria (Boetius et al. 2000, Orphan et al. 2001).

The most distinct chemotaxonomic markers of archaea are the presence of ether core membrane lipids composed of isoprenoidal units linked to a glycerol moiety (Langworthy et al. 1982, De Rosa et al. 1983, 1991, Comita et al. 1984, Langworthy 1985, Hoefs et al. 1997, Schouten et al. 1998, Hopmans et al. 2000, Pancost et al. 2000, 2001*a*, 2001*b*). Glycerol dibiphytanyl glycerol tetraethers (GDGTs; see Appendix I for structures) are known to occur in archaeal membranes and may contain from zero to eight cyclopentane rings (De Rosa et al. 1983, De Rosa and Gambacorta 1988). The presence of cyclopentane rings within biphytanyl chains is well known in (hyper)thermophilic archaea, whereas methanogenic archaea contain predominantly GDGTs with no cyclopentane rings. Recently, crenarchaeol (Compound XII, Appendix I), a GDGT with four cyclopentane rings and one distinctive cyclohexane ring, has been suggested as a specific core membrane lipid for non-thermophilic planktonic Crenarchaeota widely observed in low-temperature marine environments (Schouten et al. 2000, Sinninghe Damsté et al. 2002*a*). Glycerol dibiphytanyl glycerol tetraethers with zero to three cyclopentane rings are derived from methanotrophic archaea in the Eastern Mediterranean cold seep areas (Pancost et al. 2001*b*, Aloisi et al. 2002). Diphytanyl glycerol diethers (DGDs; see Appendix I for structures) are also common archaeal core membrane lipids, which could be considered archaeal counterparts of the conventional diglycerides of eukaryotic origin. They are characterized by unusual 2,3-*sn* glycerol stereochemistry (De Rosa et al. 1991). Archaeol (Compound IV, Appendix I) (2,3-bis-*O*-phytanyl glycerol diether) and hydroxyarchaeols (*sn*-2 and *sn*-3 isomers; Compounds V and VI, respectively (Appendix I)) are the universal archaeal core membrane lipids formed by condensation via ether linkage between two C_{20} isoprenoid alcohols and a glycerol molecule (De Rosa and Gambacorta 1988, De Rosa et al. 1991). The *sn*-2 and *sn*-3 isomers of hydroxyarchaeol have been identified in cultured members of Methanosarcinales (Sprott et al. 1990, Nishihara and Koga 1991, Koga et al. 1993). The major producers of *sn*-2-hydroxyarchaeol are *Methanosarcina* spp., whereas the *sn*-3 isomer has been observed in *Methanosaeta concilii* (Sprott et al. 1993). Based on the wide occurrence of these lipids in methane seepage areas (Hinrichs et al. 1999, 2000*a*, Boetius et al. 2000, Elvert et al. 2000, Pancost et al. 2001*b*) and their extremely depleted $\delta^{13}C$ values, which indicates methane consumption, these archaeal lipids are widely used as indicators of AOM.

Here we report a new type of archaeal core membrane ether lipid in a methane-derived carbonate crust collected from the NIOZ mud volcano recently discovered in the Sorokin Trough, NE Black Sea. The novel macrocyclic isoprenoidal glycerol diethers contain one or two cyclopentyl rings in their biphytanyl chain. The distinct molecular structure of these lipids, together with compound-specific carbon isotopic data, indicate that they are derived from a new group of archaea capable of oxidizing methane anaerobically.

Geological setting

The Sorokin Trough is one of the large depressions in the deep part of the Black Sea, extending along the south-eastern margin of the Crimea Peninsula with a length of 150 km, a width of 45–50 km and a water depth of 600–2100 m (Tugolesov et al. 1985) (Figure 1). The trough is well known for the occurrence of mud diapiric structures of Oligocene-Early Miocene age. Some of these diapirs are topped by mud volcanoes (Ivanov et al. 1998). A high gas content was noted in the uppermost part of the sedimentary sequence in the form of both free gas and gas hydrate accumulations (Bouriak and Akhmetzhanov 1998). Hydrocarbon gas profiles show a characteristic decrease in methane concentration in the uppermost part of the sedimentary column (Stadnitskaia 1997). This effect is well documented as a distinctive imprint on the sedimentary geochemical record caused by methane consumption (Barners and Goldberg 1976, Bernard 1979).

During the 11th Training-through-Research expedition (TTR-11) of the Russian R/V Professor Logachev in July–September 2001, a mud volcano (44°19′ N, 35°04′ E) was discovered on the basis of MAK-1, high-resolution, deep-towed, side-scan sonar data and named "NIOZ" mud volcano. A total of five sediment cores were subsequently taken from the crater of the NIOZ mud volcano at a water depth of 2020 m. Four of the cores (TTR-11 BS-325G, TTR-11 BS-326G, TTR-11 BS-327G and TTR-11 BS-328G) recovered carbonate crusts. The carbonate crust that we examined and which we describe here was collected from sampling site TTR-11 BS-328G (Figure 1). The crust (17 × 9 × 1.5 cm) was associated with spherical, drop-sized, brownish microbial mats, which filled the pores and cavities on the surface and interior of the carbonate crust.

Methods

Sample collection

Bottom sampling was performed according to standard TTR procedures, as described in Ivanov et al. (1992). The crust TTR-11 BS-328G-2 was collected from the NIOZ mud volcano with a gravity corer. The sample was described and photographed and then stored at –20 °C until lipid extraction.

Extraction and separation

About 100 g of crust was freeze-dried, crushed to a fine powder, and extracted with dichloromethane:methanol (9:1, v/v) using the Accelerated Solvent Extractor (ASE 200, Dionex, Sunnyvale, CA) at 6895 kPa and 100 °C. Elemental sulfur was removed from the total extract by flushing the extract over a small pipette filled with activated copper. An aliquot of the total extract was chromatographically separated into apolar and

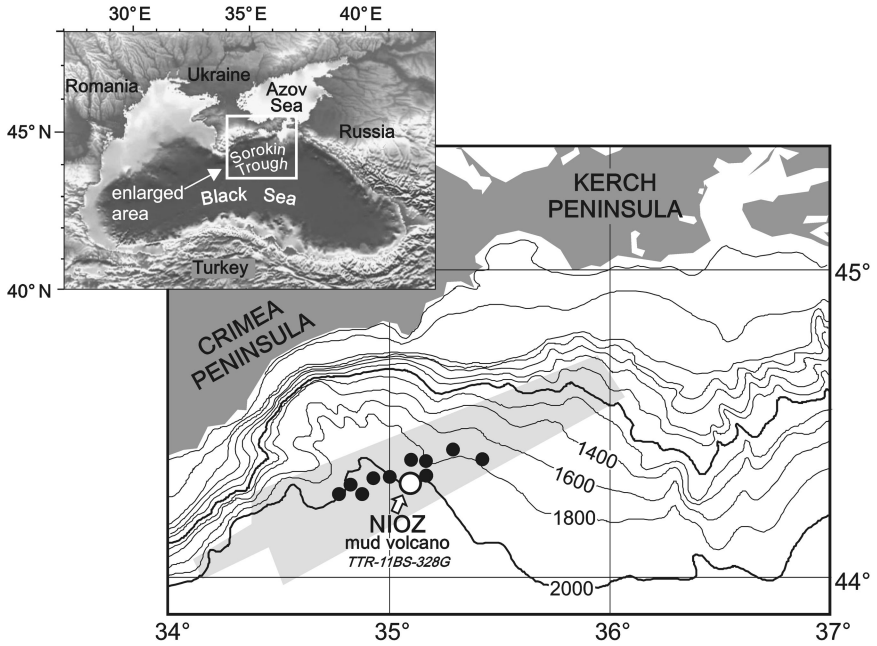

Figure 1. Location map of the study area (light gray) within the Sorokin Trough, NE Black Sea. Filled circles indicate the mud volcanoes investigated during the 11th Training-through-Research cruise (July–September 2001); the open circle indicates the position of the NIOZ mud volcano.

polar fractions using a column with activated (2 h at 150 °C) Al_2O_3 as the stationary phase. Apolar compounds were obtained using hexane:dichloromethane (9:1, v/v) as eluent, and polar compounds, including glycerol ether core membrane lipids, were obtained with the eluent dichloromethane:methanol (1:1, v/v). A known amount of 2,3-dimethyl-5-(1,1-dideuterohexadecyl)-thiophene ($C_{22}H_{38}SD_2$) was added to each fraction as an internal standard. Alcohols were transformed to trimethylsilyl derivatives by adding 25 µl of pyridine and 25 µl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heating at 60 °C for 20 min.

Hydrogenation

Hydrogenation was performed in ethyl acetate with H_2, a few drops of acetic acid and PtO_2 for 1 h. The sample was then stirred for 8 h at room temperature.

Gas chromatography

Gas chromatography (GC) was performed with a Hewlett Packard 6890 gas chromatograph equipped with an on-column injector and a flame ionization detector. A fused silica capillary column (CPsil5 25 m × 0.32 mm, df = 0.12 µm) with helium as the carrier gas was used. The samples were injected at 70 °C; thereafter, the temperature was increased to 130 °C at a rate of 20 °C min^{-1}, and then to 320 °C at a rate of 4 °C min^{-1}, where it was held constant for 15 min.

Gas chromatography–mass spectrometry

The polar fraction was analyzed by gas chromatography–mass spectrometry (GC–MS) for compound identification. Gas chromatography–mass spectrometry was conducted with a Hewlett Packard 5890 gas chromatograph interfaced to a VG Autospec Ultima Q (Micromass, Manchester, U.K.) mass spectrometer operated at 70 eV with a mass range of *m*/*z* 50–800 and a cycle time of 1.8 s (resolution 1000). The gas chromatograph was equipped with a fused silica capillary column (CPsil5 25 m × 0.32 mm, df = 0.12 µm) and helium as the carrier gas. The temperature program used for GC–MS was the same as for GC.

Isotope ratio monitoring–gas chromatography–mass spectrometry

Isotope ratio monitoring–gas chromatography–mass spectrometry was performed on a Finnigan MAT DELTA plus XL (Finnigan MAT, Bremen, Germany) instrument to determine compound-specific $\delta^{13}C$ values. The GC was a Hewlett Packard 6890A series and the analytical conditions were the same as those used for GC and GC–MS. For carbon isotopic correction of the added trimethylsilyl groups, we used BSTFA with a known carbon isotopic composition (–49.29 ± 0.5‰). Values are reported in ‰ relative to the PeeDee belemnite standard, and have been corrected for the addition of an $Si(CH_3)_3$ group during the derivatization procedure. To monitor the accuracy of the measurements, the analyses were carried out with co-injection of two standards, C_{20} and C_{24} *n*-alkanes, with known isotopic composition.

High performance liquid chromatography–atmospheric pressure chemical ionization/mass spectrometry

To determine the distribution and composition of intact glycerol dibiphytanyl glycerol tetraethers (GDGTs), the underivatized polar fraction of the carbonate crust was analyzed by high performance liquid chromatography–atmospheric pressure chemical ionization/mass spectrometry according to Hopmans et al. (2000).

Results

Diphytanyl glycerol diethers in the crust

Analysis of the polar fraction of the carbonate crust TTR-11 BS-328G-2 by GC–MS revealed a suite of ether lipids diagnostic of the non-thermophilic group of the euryarchaeota and sulfate-reducing bacteria (Figure 2). Two isoprenoidal diphytanyl glycerol diethers (DGDs) distinctive for methanotrophic archaea (Hinrichs et al. 1999, 2000*a*) were identified. Archaeol (Compound IV, Appendix I) is the most abundant ether lipid. The *sn*-3 isomer of hydroxyarchaeol (Compound VI) was also detected in relatively high abundance. Series of non-isoprenoidal DGDs (Compounds I, II and III), previously reported as markers of sulfate-reducing bacteria (Pancost et al. 2001*a*), were also identified in the crust. Diphytanyl glycerol diethers with anteiso alkyl chains (Compound I), and with a cyclopropane ring in the alkyl chain attached to the glycerol group at position 2 (Compound II), were most abundant. Dicyclic biphytane diol (Compound VII; Schouten et al. 1998) was also present in the carbonate crust, but in small amounts relative to the other archaeal and bacterial lipids. It is probably also derived from archaea involved in anaerobic oxidation of methane (AOM) (Pancost et al. 2001*b*, Teske et al. 2002).

Novel macrocyclic diphytanyl glycerol diethers in the crust

In addition to these well-known archaeal and bacterial lipids, two relatively abundant, unknown compounds (A and B) were observed (Figure 2). Their mass spectra (Figure 3) are similar to that of the macrocyclic biphytanyl glycerol diether, the core membrane lipid of the archaeon *Methanococcus jannaschii* (Comita et al. 1984). The mass spectra of compounds A and B show intense fragment ions at *m*/*z* 145, which arise from loss of the entire biphytanyl moiety and a hydroxy group, $C_3H_4O_2Si(CH_3)_3$ (Comita et al. 1984). The presence of relatively abundant fragment ions at $M_\bullet^+ - 43$, $M_\bullet^+ - 57$ and $M_\bullet^+ - 90$ is the result of the loss of C_2H_3O, C_3H_5O and $HOSi(CH_3)_3$, respectively. The fragment ions at *m*/*z* 103 and $M_\bullet^+ - 103$ are related to the derivatized alcohol group $CH_2OSi(CH_3)_3$ (Wood 1980), and those at *m*/*z* 130, 131 and 132 are related to the $C_2H_3O_2Si(CH_3)_3$ moiety, indicating that the $Si(CH_3)_3$ fragment is attached to the glycerol group at the 1 or 3 position (Pancost et al. 2001*a*). These mass spectral fragmentation characteristics suggested that A and B are also macrocyclic biphytanyl glycerol diethers. However, compared with the macrocyclic diphytanyl glycerol diether with a molecular ion at *m*/*z* 722 (Comita et al. 1984), the molecular ions of the novel lipids are shifted by 2 Da for A ($M_\bullet^+ = 720$) and by 4 Da for B ($M_\bullet^+ = 718$) (Figure 3). Therefore, it was hypothesized that the biphytanyl chains of A and B contain either one or two rings or double bonds, respectively. To eliminate one of these possibilities, the polar fraction was hydrogenated and reanalyzed by GC–MS. This experiment showed no changes in the molecular weight of compounds A and B, establishing the absence of double bonds within the biphytanyl skeleton. Consequently, it was concluded that A and B contain one and two rings, respectively.

Archaeal core membrane lipids have been reported to contain a cyclopentane ring (De Rosa et al. 1983, 1991, De Rosa and Gambacorta 1988) and, recently, a cyclohexane ring (Sinninghe Damsté et al. 2002*a*) in their biphytanyl moieties. The presence and location of one and two cyclopentane rings in the biphytanyl chains of A and B, respectively, is revealed by the distinctive fragment ion at *m*/*z* 165 present in both mass spectra (Figure 3). The *m*/*z* 165 fragment corresponds to fragmentation associated with the cyclopentyl ring of biphytane derivatives (Schouten et al. 1998). The position of the cyclopentane ring in biphytane moieties has been established by nuclear magnetic resonance spectroscopy (De Rosa et al. 1977, Sinninghe Damsté et al. 2002*b*). The mass spectrum of B exhibits an enhanced fragment ion of *m*/*z* 165 compared with that of A, consistent with the presence of two cyclopentane rings.

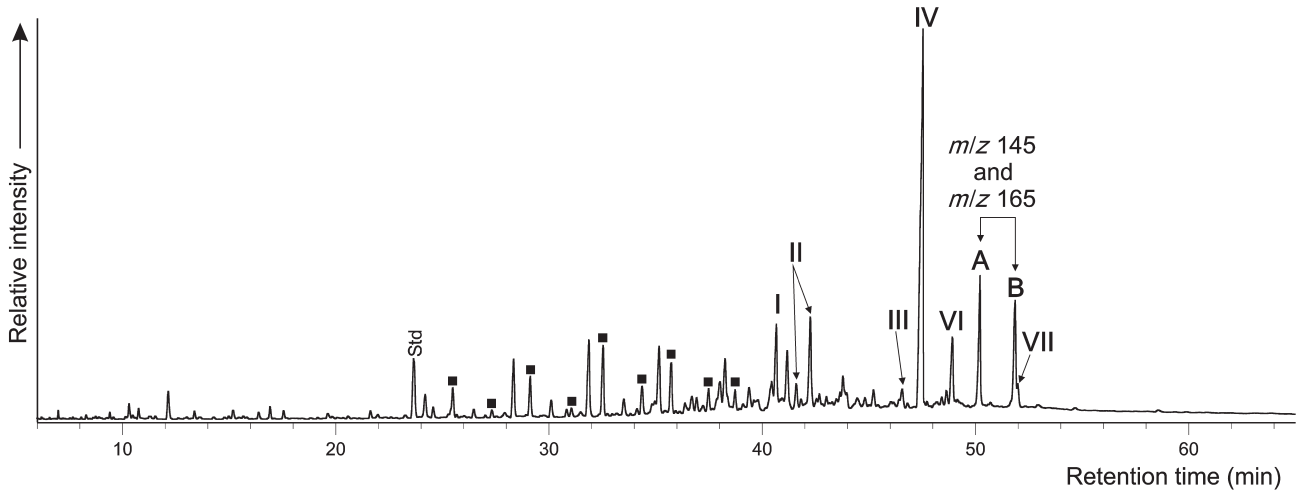

Figure 2. Gas chromatogram of the polar fraction extracted from the carbonate crust TTR-11 BS-328G-2. All alcohols were analyzed as their trimethylsilyl derivatives. Black squares indicate straight chain alcohols. Roman numbers and letters refer to Compounds shown in Appendix I.

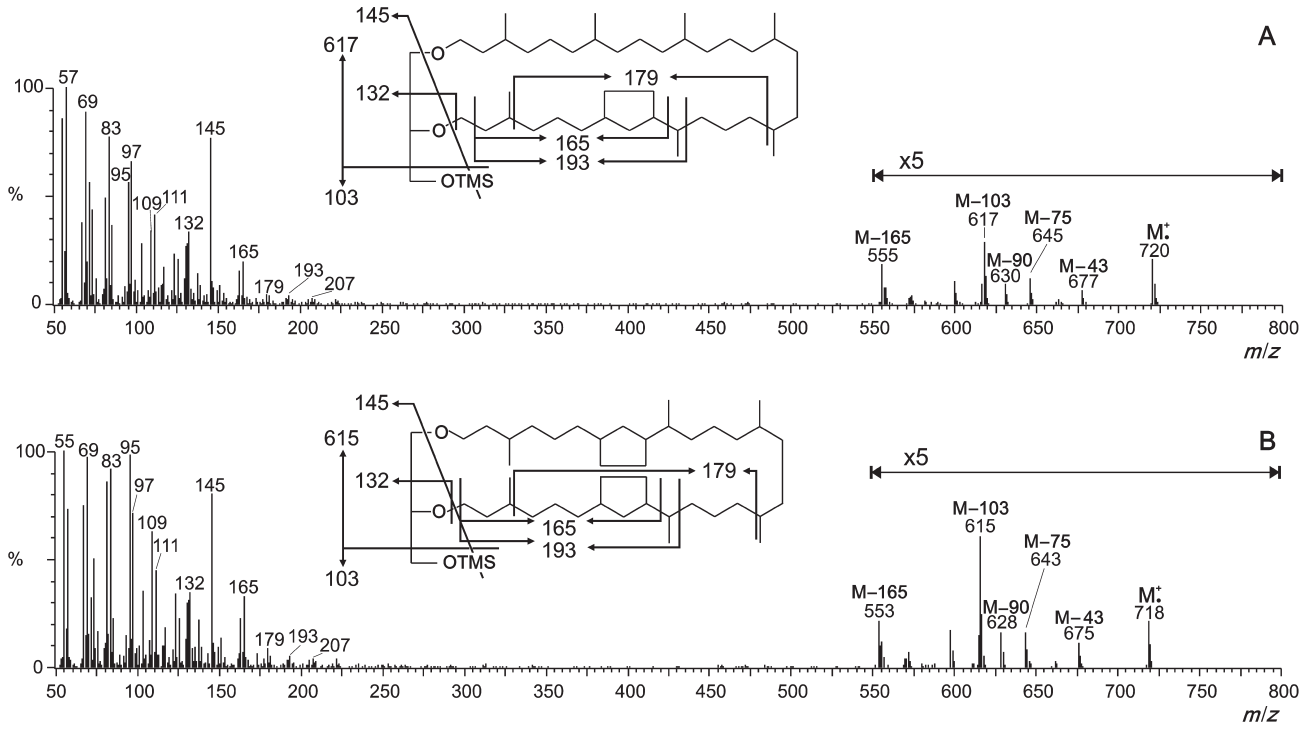

Figure 3. Mass spectra (subtracted for background) of novel macrocyclic diethers A and B.

Glycerol dibiphytanyl glycerol tetraethers in the crust

High performance liquid chromatography–atmospheric pressure chemical ionization/mass spectrometry revealed that intact GDGTs in the TTR-11 BS-328G-2 carbonate crust are dominated by GDGTs containing zero to two cyclopentane rings (Compounds VIII, IX and X; Figure 4). This distribution has been reported previously in cold seep sediments and crusts of the Eastern Mediterranean (Pancost et al. 2001*b*, Aloisi et al. 2002) and in the anaerobic water column of the Black Sea (Schouten et al. 2001, Wakeham et al. 2002), where extensive AOM takes place. Crenarchaeol (Compound XII), the GDGT of ubiquitous planktonic crenarchaeota (Hoefs et al. 1997, Schouten et al. 1998, 2000, 2001, Sinninghe Damsté et al. 2002*a*), is nearly absent, indicating only a minor contribution of pelagic archaeal lipids to the crust.

Carbon isotopic composition of glycerol diethers

Determination of the carbon isotopic composition of A and B gave $\delta^{13}C$ values of –104 and –111‰, respectively. Archaeol and *sn*-3 hydroxyarchaeol are also characterized by extremely depleted $\delta^{13}C$ values (–102 and –112‰, respectively). The $\delta^{13}C$ values of non-isoprenoidal dialkyl diethers (Compounds I and II) are 10–20‰ heavier than A, B, archaeol and *sn*-3 hydroxyarchaeol. The carbon isotopic composition of Compound I is –95‰ and that of Compound II is –91‰.

Discussion

To the best of our knowledge, the macrocyclic biphytanyl glycerol diethers with one and two cyclopentane rings identified here have not been reported previously. Their molecular structure unites two characteristics reported for ecologically contrasting archaeal groups. The thermophilic methanogen *Methanococcus jannaschii* (Comita et al. 1984) is the only archaeon known to contain the macrocyclic diether as its core membrane lipid. On the other hand, cyclopentane-containing GDGTs are well known in (hyper)thermophilic crenarchaeota (Langworthy et al. 1982, De Rosa and Gambacorta 1988), (hyper)thermophilic euryarchaeota (De Rosa and Gambacorta 1988, De Rosa et al. 1991), marine mesophilic crenarchaeota (Sinninghe Damsté et al. 2002*a*) and mesophilic euryarchaeota capable of AOM (Pancost et al. 2001*b*, Aloisi et al. 2002), but so far have not been detected in DGDs. This structural information leaves little doubt that the new lipids are derived from archaea. Since archaeal anaerobic methanotrophy results in highly ^{13}C-depleted lipids, we infer that membrane lipids A and B are derived from methanotrophic archaea. This inference is confirmed by the fact that these types of archaea biosynthesize structurally related GDGTs containing biphytane chains with one or two cyclopentane rings (Pancost et al. 2001*b*, Aloisi et al. 2002, Wakeham et al. 2002). It is unclear why some archaeal species capable of AOM present in the NIOZ mud volcano biosynthesize cyclopentane ring-containing DGDs instead of GDGTs. This may be related to adaptation of the physical properties of their cell membranes. It is, however, consistent with the observed archaeal diversity with respect to lipid composition in cold seep areas (Elvert et al. 2000, Pancost et al. 2001*b*, Teske et al. 2002). In any case, our

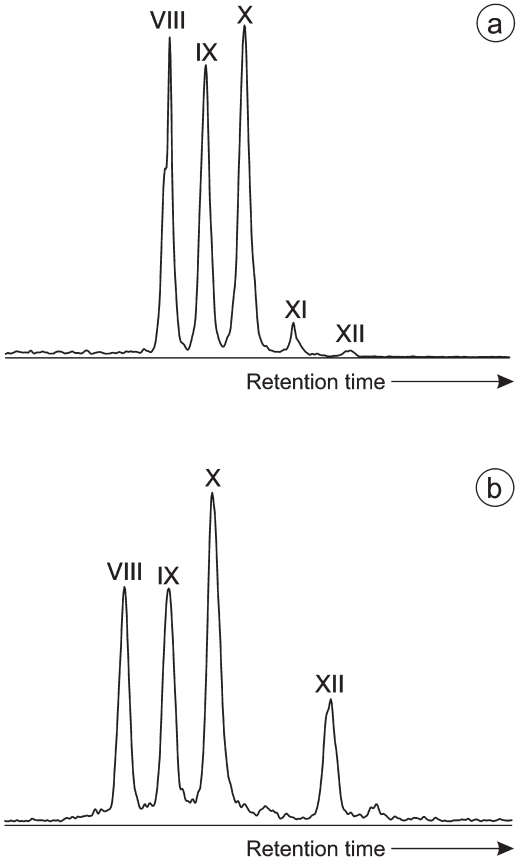

Figure 4. Base peak chromatograms of GDGT distribution: (a) carbonate crust TTR-11 BS-328G-2 and (b) Napoli mud volcano, Eastern Mediterranean (Pancost et al. 2001*b*). Roman numbers refer to GDGT Compounds shown in Appendix I.

data show that archaeal macrocyclic DGDs are not restricted to thermophilic methanogens but are also used by euryarchaeota involved in AOM.

Conclusions

Two novel macrocyclic archaeal diether core membrane lipids containing cyclopentane rings were identified in a methane-derived carbonate crust found in the NIOZ mud volcano in the Black Sea. The unprecedented molecular structure and isotopic composition of the novel glycerol diethers A and B reveal their biosynthesis by archaea performing anaerobic methanotrophy in cold-water environments. The identification of these biomarkers will enable better understanding of AOM-related microorganisms and their metabolic behavior.

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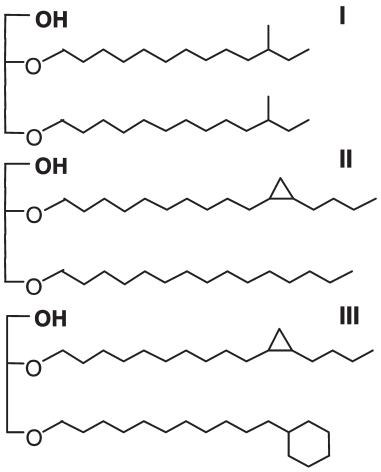
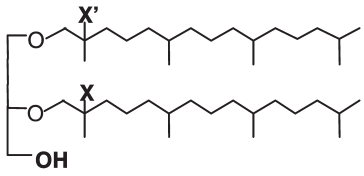
Appendix I

Dialkyl Glycerol Diethers



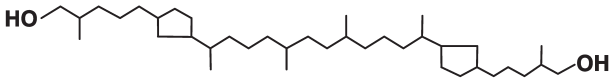


IV: X = H, X' = H (Archaeol)

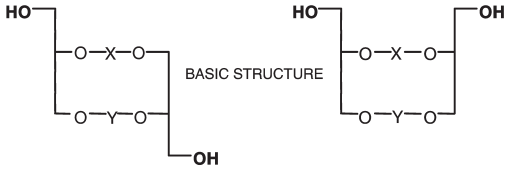
V: X = OH, X' = H (*sn*-2-hydroxyarchaeol)

VI: X = H, X' = OH (*sn*-3-hydroxyarchaeol)

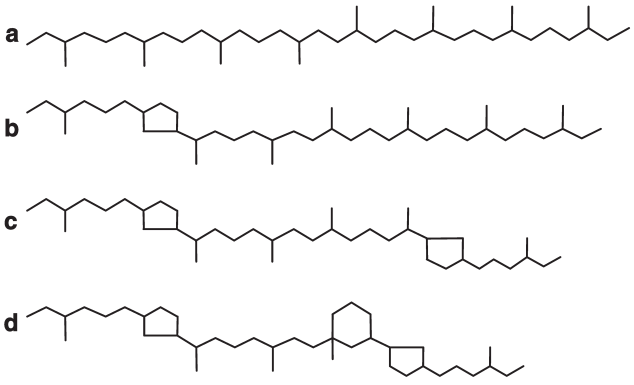
Biphytane Diol **VII**



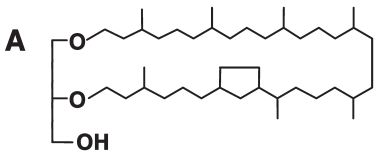
Glycerol Dialkyl Glycerol Tetraethers

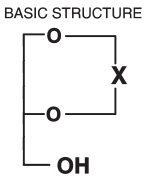


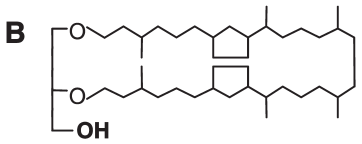
No	X	Y
VIII	a	a
IX	a	b
X	b	b
XI	b	c
XII	c	d



Macrocyclic Diethers







X = a

A: X = b

B: X = VII (-2OH) or **c**