Crenarchaeol: the characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota

Jaap S. Sinninghe Damsté,[1,*] Stefan Schouten,* Ellen C. Hopmans,* Adri C. T. van Duin,[†] and Jan A. J. Geenevasen[§]

Royal Netherlands Institute for Sea Research (NIOZ),* Department of Marine Biogeochemistry and Toxicology, PO Box 59, 1790 AB Den Burg, The Netherlands; Newcastle University,[†] Department of Fossil Fuels and Environmental Geochemistry, Drummond Building, Newcastle upon Tyne NE1 7RU, UK; University of Amsterdam,[§] Faculty of Chemistry, Department of Organic Chemistry, Nieuwe Achtergracht 129, 1018 WS Amsterdam, The Netherlands

Abstract The basic structure and stereochemistry of the characteristic glycerol dibiphytanyl glycerol tetraether (GDGT) membrane lipid of cosmopolitan pelagic crenarchaeota has been identified by high field two-dimensional (2D)-NMR techniques. It contains one cyclohexane and four cyclopentane rings formed by internal cyclisation of the biphytanyl chains. Its structure is similar to that of GDGTs biosynthesized by (hyper)thermophilic crenarchaeota apart from the cyclohexane ring. These findings are consistent with the close phylogenetic relationship of (hyper)thermophilic and pelagic crenarchaeota based 16S rRNA. The latter group inherited the biosynthetic capabilities for a membrane composed of cyclopentane ring-containing GDGTs from the (hyper)thermophilic crenarchaeota. However, to cope with the much lower temperature of the ocean, a small but key step in their evolution was the adjustment of the membrane fluidity by making a kink in one of the bicyclic biphytanyl chains by the formation of a cyclohexane ring. This prevents the dense packing characteristic for the cyclopentane ring-containing GDGTs membrane lipids used by hyperthermophilic crenarchaeota to adjust their membrane fluidity to high temperatures.—Sinninghe Damsté, J. S., S. Schouten, E. C. Hopmans, A. C. T. van Duin, and J. A. J. Geenevasen. **Crenarchaeol: The characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota. *J. Lipid Res.* 2002.** 43: **1641–1651.**

Supplementary key words archaea • ether-bound biphytanes • ocean • ether lipids

Archaea form one of the three domains of life on Earth and are subdivided based on 16S rRNA in two major kingdoms (Euryarchaeota and Crenarchaeota) and one smaller kingdom (Korarchaeota) (1). Traditionally, archaea are viewed as organisms that especially thrive under extreme conditions, such as high salinity, high temperatures, strong acid, and anoxic conditions. However, this view is changing rapidly based on the occurrence of characteristic archaeal gene sequences (2) and archaeal membrane lipids (3) in non-extreme environments. Recently, for example, cultivation-independent rRNA surveys have shown that archaea belonging to the kingdom Crenarchaeota, traditionally only thought to be comprised of thermophiles (i.e., growth temperatures >40°C), thrive in the ocean (4). These so-called pelagic crenarchaeota are probably the most abundant group of archaea on Earth; the global oceans are estimated to comprise 1.3×10^{28} cells (4).

Apart from their characteristic rRNA sequences, archaea are also biochemically distinct from bacteria and eukaryotes since they use biphytanyl glycerol diethers or (glycerol dibiphytanyl glycerol tetraethers) GDGTs instead of diacyl membrane lipids. This has been interpreted to be an adaptation to the extreme environments in which archaea thrive, as ether linkages are more stable than ester linkages (5). The use of membrane-spanning GDGTs by (hyper)thermophilic crenarchaeota is thought to be a further adaptation of their membranes to cope with high temperatures (>60°C). In addition, (hyper) thermophilic archaea form cyclopentane rings by internal

Abbreviations: APCI, atmospheric pressure chemical ionization; APT, attached proton test; COSY, correlated spectroscopy; DCM, dichloromethane; DEPT, distorsionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; GDGT, glycerol dibiphytanyl glycerol tetraether; MS, mass spectrometry; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; 2D, two-dimensional; TOCSY, total correlation spectroscopy.

[1] To whom correspondence should be addressed.
e-mail: damste@nioz.nl

Manuscript received 3 April 2002 and in revised form 5 June 2002.
DOI 10.1194/jlr.M200148-JLR200

cyclization of the dibiphytane moieties, resulting in a more densely packed and consequently thermally more stable membrane (6). The number of cyclopentane rings in GDGTs of (hyper)thermophilic crenarchaeota indeed increases with growth temperature (7). Hence, GDGTs seem to be specifically designed to cope with extremely high temperatures.

Remarkably, however, cyclopentane ring-containing GDGTs have recently also been identified in non-thermophilic crenarchaeota and in the marine environment. Ether cleavage studies on water column particulate matter (8) and *Cenarchaeum symbiosum* (9), the only uni-archaeal culture available from the group of pelagic crenarchaeota (10), and studies of intact GDGTs in marine sediments (3) have all indicated that the GDGTs of pelagic crenarchaeota comprise dibiphytanes with predominantly no, two, or three cyclopentane rings. Detailed mass spectrometry (MS) studies indicated that the dibiphytane comprising three cyclopentane rings is different from the isomer found in hyperthermophilic crenarchaeota since the position of one of the cyclopentane rings is different (11). These studies confirmed the phylogenetically close relationship between the thermophilic and pelagic crenarchaeota; both biosynthesize cyclopentane-containing GDGTs. It remains, however, unclear how pelagic crenarchaeotea can thrive in the relatively cold ocean waters with a set of membrane lipids specifically designed to cope with high temperatures.

Here we report a detailed account on the structural identification of the core GDGT membrane lipid of pelagic crenarchaeota using high-field two-dimensional (2D)-NMR techniques. Our results reveal that these organisms have adjusted their membrane lipids to cope with the much colder conditions in the ocean by the formation of an internal cyclohexane moiety, an unprecedented biochemical reaction for the archaea.

MATERIALS AND METHODS

GDGT isolation

For isolation of GDGT-0[2] and the core tetraether lipid of pelagic crenarchaeota, 700 g of surface sediments of the Arabian Sea (Netherlands Indian Ocean Program Site 311, off Yemen; 16°02'N, 52°46'E; water depth 1,087 m) was extracted by Soxhlet using dichloromethane (DCM)/methanol (9:1) as solvent. From this extract, apolar and polar fractions were obtained by column chromatography over aluminum oxide using hexane-DCM (9:1; v/v) and DCM-methanol (1:1, v/v) as eluents, respectively. Solvent was removed from the polar fraction by rotary evaporation under vacuum. The remaining solvent was removed under a stream of nitrogen, and the residue dissolved by sonication (10 min) in hexane-propanol (99:1, v/v). The resulting suspension was centrifuged (1 min, 3,500 rpm) and the supernatant filtered through a 0.45 μm, 4 mm diameter PTFE filter prior to injection.

GDGT-4 was isolated from a GDGT fraction of the thermophilic archaeon *Sulfolobus solfataricus*, prepared as previously described by Nicolaus et al. (12) and a kind gift of Dr. A. Gambacorta, Instituto di Chimica di Molecole di Interesse Biologico, Napoli, Italy. The GDGT fraction was dried, re-dissolved, and filtered as described above before injection.

GDGTs were isolated using an HP (Palo Alto, CA) 1100 series LC equipped with an auto-injector, and a fraction collector (Foxy Jr., Isco, Inc., Lincoln, NE). A first isolation was achieved on a semi-preparative Econosphere NH_2 column (10 × 250 mm, 10 μm; Alltech, Deerfield, IL), maintained at 30°C. Typical injection volume was 100 μl containing up to 10 mg material. GDGTs were eluted isocratically with 99% hexane and 1% propanol for 5 min, followed by a linear gradient to 1.8% propanol in 45 min. The flow rate was set at 2.5 ml/min. After each run the column was cleaned by back-flushing hexane-propanol (9:1; v/v) at 2.5 ml/min for 10 min. Column effluent was collected in 1 min fractions, which were screened for the presence of the target GDGT by HPLC/atmospheric pressure chemical ionization (APCI)-MS as described by Hopmans et al. (13). Fractions containing the target GDGT were pooled and further purified on an Econosphere NH_2 column (4.6 × 250 mm, 5 μm; Alltech, Deerfield, IL). Typical injection volumes were 50 μl containing up to 0.5 mg of material. GDGTs were eluted using an identical gradient and conditions as described above, but at a flow rate of 1 ml/min. After each run the column was cleaned by back-flushing hexane-propanol (95:5, v/v) at 1 ml/min for 10 min. Column effluent was collected in 0.5 min fractions and screened as described above.

GDGT extraction and analyses

In case of the sponge *Axinella mexicana* containing *Cenarchaeum symbiosum* (kind gift of Dr. E. DeLong, Monterey Bay Aquarium Research Institute, CA) direct solvent extraction did not yield significant amounts of GDGTs. The sponge was therefore extracted by refluxing with 2 N HCl in methanol for 8 h, followed by liquid/liquid extraction with DCM. Released GDGTs were subsequently analyzed by HPLC-APCI/MS as described previously (13).

Nuclear magnetic resonance

All GDGTs were solved in $CDCl_3$ at a concentration of 3–6 μmol/ml. NMR spectroscopy was performed on a Varian Unity Inova 500, a Bruker DRX600, and a Bruker AV-750 spectrometer equipped with an SWBB probe, an inverse TBI-Z probe with a pulsed field gradient (PFG) accessory, and a BBI-zGRAD probe, respectively. All experiments were recorded at 300 K in $CDCl_3$. Proton and carbon chemical shifts were referenced to internal $CDCl_3$ (7.24/77.0 ppm). In the 2D ^1H-^{13}C correlated spectroscopy (COSY), the number of complex points and sweep widths were 2K points/6 ppm for ^1H and 512 points/150 ppm for ^{13}C. In the 2D ^1H-^1H COSY the number of complex points and sweep widths were 2 K points/5.5 ppm. Quadrature detection in the indirect dimension was achieved with the time-proportional-phase-incrementation method. The data were processed with Varian or NMRSuite software packages. After apodization with a 90 shifted sinebell, zero filling to 512 real points were applied for the indirect dimensions. For the direct dimensions zero filling to 4 K real points, Lorentz transformations were used.

Torsion angles in GDGT-4

To determine the average torsion angles in the cyclopentane rings of GDGT-4, a constant volume, constant temperature molecular dynamics (MD) simulation (14, 15) was performed at a system temperature of 298 K on a single GDGT-4 lipid. After

[2] A number after GDGT indicates the total number of cyclopentane rings.

equilibration at this temperature, a 25,000-iteration MD-simulation was performed. Atom positions were saved every 50 iterations, thus generating 500 conformational snapshots. Subsequently, average torsion angles in the five-membered rings and associated standard deviations were obtained from the analysis of these snapshots. The ReaxFF-force field (16) was used in these simulations.

Membrane volume determination

To determine the influence of molecular structure on membrane volume we performed constant pressure, constant temperature MD simulations (14, 15) on systems containing 3 × 3 GDGT-4 or crenarchaeol-lipid monomers. In accordance with work by Gabriel and Chong (6) we used β-D-glucopyranose and *myo*-inositolphosphate as the polar groups in both the GDGT-4 and crenarchaeol-lipids. Periodic images of the 3 × 3-lipid system were used in the y- and z-direction (parallel to the membrane), no periodicity was used in the x-direction (perpendicular to the membrane). The system was allowed to expand and contract in the y- and z-directions according to the intermolecular forces. By this means the initial membrane structure, containing the 3 × 3 lipid monomers evenly and symmetrically distributed in a periodic box of dimensions 21 × 30 Å in y- and z-directions, was allowed to relax until it reached a steady-state configuration, after which the periodic box dimensions (the yz-surface area) could be used as a direct measure for the membrane volume. The system temperature (298 K) and pressure (10 bar) were controlled using the algorithm described by Berendsen et al. (17), with temperature and pressure damping constants of 1,000 femtoseconds and a MD time-step of 1 femtosecond.

A modified version of the AMBER-protein force field (18), as described by van Duin and Larter (19), was used to evaluate the interatomic forces in these simulations. To test for potential biases associated with this choice of force field all simulations were repeated with the ReaxFF-potential (16).

RESULTS

HPLC-APCI/MS

Cenarchaeum symbiosum is an archaeon that lives in symbiosis with the sponge *Axinella mexicana*, originally in the Gulf of Mexico (10). Detailed molecular biological work has documented that this "culture" is uni-archaeal. It is the only archaeon available in culture belonging to the phylogenetic group of pelagic crenarchaeota (Marine Group 1) (2). Analysis of the H^+-extract with an HPLC-MS technique, recently developed to analyze intact core GDGT membrane lipids (13), showed a base peak ion chromatogram dominated by GDGT-0 (structure I; see **Fig. 1** for structures), the GDGT comprised of two biphytane chains containing no cyclopentane rings, and an unknown component, representing ~60% of total GDGTs (**Fig. 2A**). This latter component showed a mass spectrum

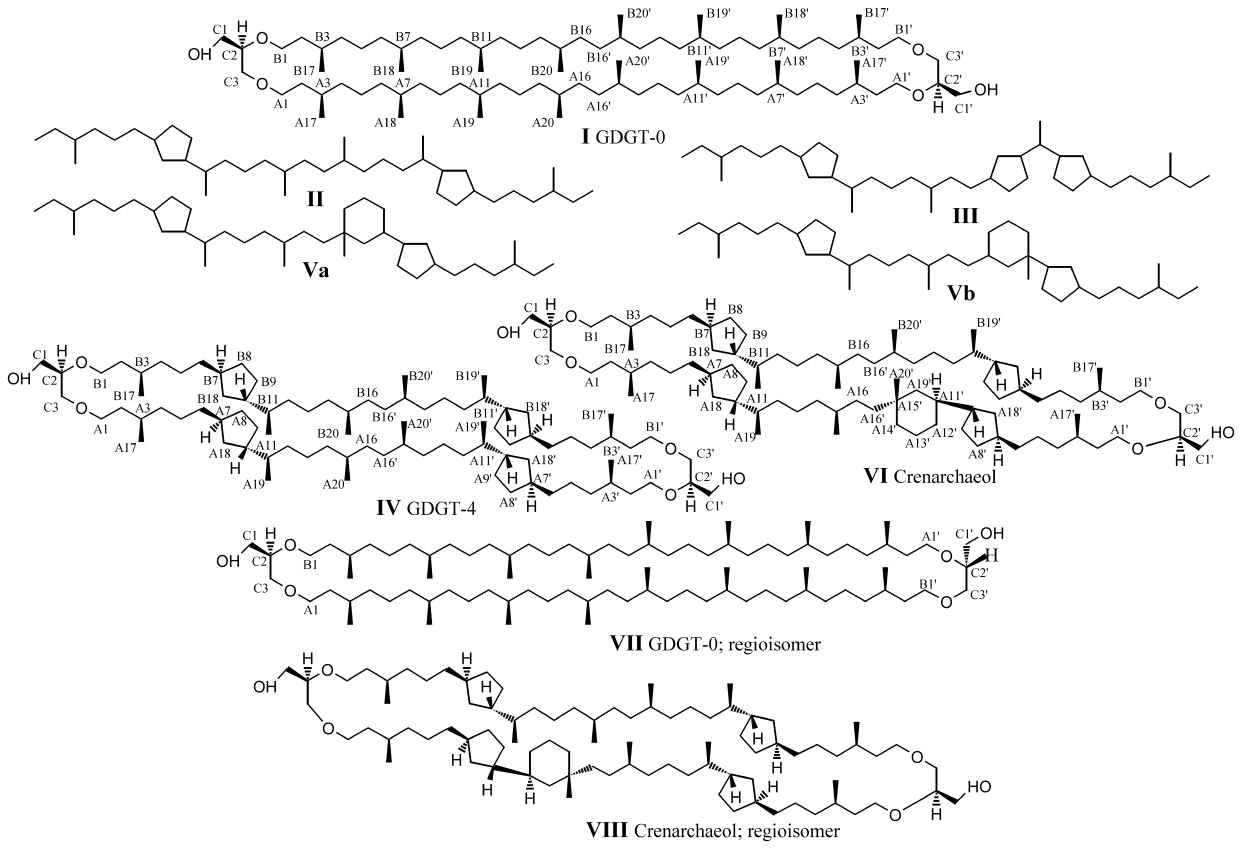

Fig. 1. Structures of components listed in the text. For glycerol dibiphytanyl glycerol tetraether (GDGT)-0, GDGT-4, and crenarchaeol, the numbering of carbon atoms is indicated. The arabic numbering of carbon atoms was done after DeRosa et al. (22). The numbering with numerals was applied to make as much use as possible of the C_2 symmetry of the biphytanyl chains in the GDGT molecules to describe the NMR signals (Table 1–3) as efficiently as possible.

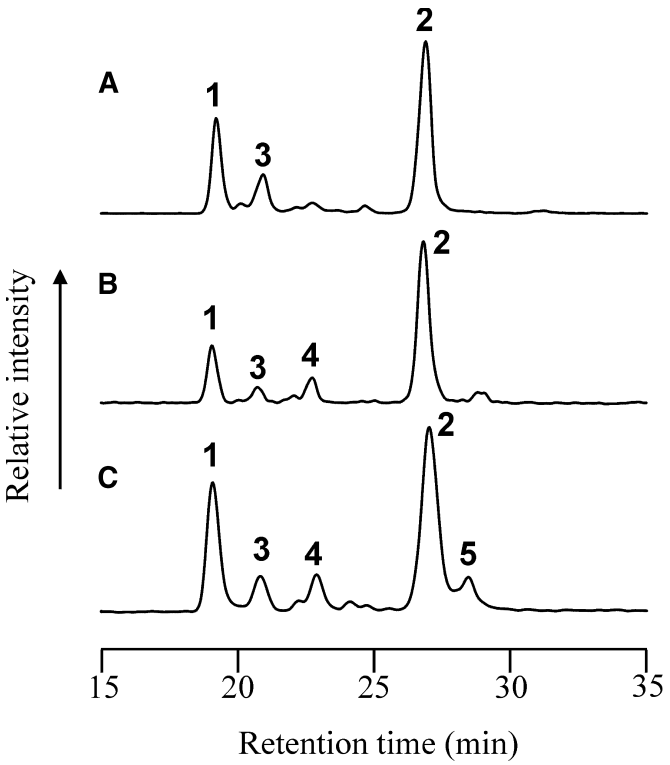

Fig. 2. Partial base peak chromatogram obtained by HPLC-atmospheric pressure chemical ionization (APCI)/mass spectrometry (MS) showing the distribution of GDGTs of (A) the H^+-extract of the non-(hyper)thermophilic archaeon *Cenarchaeum symbiosum*, (B) the extract of water column suspended particulate matter obtained from a station (17°42'N, 57°51'E; 1,000 m water depth) in the Arabian Sea (20), and (C) the polar fraction of the solvent extract of surface sediment (Netherlands Indian Ocean Program Site 311, 16°02'N, 52°46'E; water depth 1,087 m) from the Arabian Sea used to isolate crenarchaeol. Key: 1, GDGT-0; 2, crenarchaeol; 3, GDGT-1, 4, GDGT-2; 5, isomer of crenarchaeol.

typical for a GDGT (i.e., loss of water and glycerol) (13) but with a protonated molecular ion 10 daltons lower than that of GDGT-0, establishing the molecular formula as $C_{86}H_{162}O_6$ and indicating that this GDGT contained five rings. These results are in good agreement with ether cleavage studies of GDGTs of *C. symbiosum* (9), which revealed biphytanes with two and three cyclopentane rings (structures II and III) as major components.

This major unknown GDGT is also the major GDGT in water column particulate organic matter and marine surface sediments (Fig. 2B, C) (3, 20), indicating that it is probably also the dominant GDGT of the pelagic crenarchaeaota, which represent 20% of the picoplankton in the ocean (4). This is in good agreement with earlier suggestions based on ether cleavage products of GDGTs (i.e., II and III) in the marine water column (8).

Isolation of the unknown GDGT

To fully elucidate the structure of this major unknown GDGT, it was isolated and its structure was determined by high-field 2D-NMR studies. As the source for isolation, surface sediments of the Arabian Sea were chosen. These sediments have a GDGT composition very similar to that of *C. symbiosum* (Fig. 2), contain high amounts of this GDGT, and are thus a good source for isolation. By solvent extraction, column chromatography over silica, and preparative HPLC a fraction significantly enriched in the unknown GDGT core membrane lipid was obtained. From this fraction, the unknown GDGT was isolated by repetitive analytical HPLC, resulting in ~4 mg of isolate. HPLC-APCI/MS of this fraction indicated that the unknown GDGT was the only GDGT present in this fraction and did not reveal any other impurities. Consequently, this fraction was used for NMR studies.

For comparison of NMR data, GDGT-0 (I) and GDGT-4 (IV) were also isolated in high purity by HPLC from Arabian Sea sediments and cells of the hyperthermophilic crenarchaeon *Sulfolobus acidocaldarius*, respectively. Both GDGTs (but especially GDGT-4) share structural similarities with the unknown GDGT of pelagic crenarchaeota and ^{13}C-NMR data have been reported for the biphytane carbon skeletons of these components (21–23), thus assisting in the identification of the unknown GDGT. However, no high-resolution ^{1}H, ^{13}C-NMR, and 2D-NMR correlation techniques have been applied to the intact GDGTs.

Basic skeleton

The ^{1}H-NMR spectrum is extremely complex even if measured at 750 MHz. In the 3.4–3.7 ppm region multiplets representing 18 protons are observed (**Table 1**). These represent the protons of the two glycerol units and the first and ultimate methylene units of the biphytane moieties bound via the ether linkages. The same signals are observed in the ^{1}H-NMR spectra of GDGT-0 and GDGT-4 (**Table 2**). At ~2.2 ppm a broad singlet representing the two hydroxy groups is found. Between 0.82 and 0.88 ppm a complicated pattern of signals (mainly doublets) occurs in total representing eleven methyl groups. At 750 MHz, the resolution is high enough to separate a singlet at $\delta = 0.836$ ppm, representing one methyl group, from a doublet at $\delta = 0.844$ ppm, representing three methyl groups. In the 0.7–0.8 ppm region two "high-field" protons are observed; the remaining protons are all found in the 1.0–1.8 ppm region.

The ^{13}C-NMR spectrum of the unknown GDGT shows 11 primary, 53 secondary, 21 tertiary, and 1 quaternary carbon atoms (Table 1). Attached proton test (APT), distorsionless enhancement by polarization transfer (DEPT)90, and DEPT135 experiments were used to assess the multiplicity of carbon atoms. The ^{13}C-NMR spectrum did not show 86 resolved signals because many carbon atoms are either strictly, or effectively, equivalent. Assignments of the carbon atoms is partially based on literature data (22, 23) and the ^{13}C-NMR data of GDGT-0 and GDGT-4, in combination with an heteronuclear multiple quantum correlation (HMQC) experiment. This established one of the diether-bound biphytane moieties as structure II, well known from the membrane lipids of hyperthermophilic crenarchaeota. The other proposed dibiphytanyl moiety (structure III) (8, 11) is, however, inconsistent with

TABLE 1. ^{13}C- and ^{1}H-NMR data of crenarchaeol (VI)

Carbon Number[a]	Carbon Shift CH_3	CH_2	CH	C	Proton Shift
A1, B1′		70.09			3.48 (4H, t, J = 6.9 Hz)
A1′, B1		68.56			3.55 (2H, m); 3.67 (2H, m)
A2, B2′		36.58			1.35 (2H, m); 1.60 (2H, m)
A2′, B2		37.03			1.39 (2H, m); 1.61 (2H, m)
A3, A3′, B3, B3′			29.71		1.53 (4H, m)
A4, A4′, B4, B4′		37.23			1.1 (4H, m); 1.24 (4H, m)
A5, A5′, B5, B5′		25.86			1.22 (4H, m); 1.29 (4H, m)
A6, A6′, B6, B6′		37.13			1.23 (8H, m)
A7, B7, B7′			39.08		1.79 (3H, m)
A7′			38.85		1.79 (1H, m)
A8, B8, B8′		33.36			ax: 1.05 (3H, m); eq: 1.77 (3H, dd, J = ∼12, ∼7 Hz)
A8′		33.30			ax: 1.05 (1H, m); eq: 1.77 (1H, dd, J = ∼12, ∼7 Hz)
A9, B9, B9′		31.18			ax: 1.12 (3H, m); eq: 1.74 (3H, dd, J=12.0, 6.7 Hz)
A9′		31.23			ax: 1.08 (1H, m); eq: 1.74 (1H, dd, J = 12.0, 6.7 Hz)
A10, B10, B10′			44.74		1.69 (3H, qh, J = ∼8 Hz)
A10′			45.66		1.47 (1H, qh, J = ∼8 Hz)
A11, B11, B11′			38.18		1.23 (3H, m)
A11′			39.08		1.17 (1H, m)
A12, B12, B12′		35.68			1.02 (3H, m); 1.36 (3H, m)
A12′		32.11			ax: 0.72 [1H, dddd, J = 13.0 (3×), 4.0 Hz]; eq: 1.74 (1H, m)
A13, B13, B13′		24.39			1.16 (3H, m); 1.36 (3H, m)
A13′		22.24			ax: 1.02 (1H, m); eq: 1.52 (1H, t)
A14		37.39			1.06 (1H, m); 1.25 (1H, m)
A14′		43.97			ax: 1.06 (1H, m); eq: 1.18 (1H, m)
B14, B14′		37.56			1.07 (2H, m); 1.30 (2H, m)
A15			33.54		1.32 (1H, m)
A15′				33.04	—
B15, B15′			33.07		1.34 (2H, m)
A16		29.97			1.08 (1H, m); 1.18 (1H, m)
A16′		37.64			1.07 (1H, m); 1.30 (1H, m)
B16, B16′		34.22			1.10 (2H, m); 1.26 (2H, m)
A17′, B17	19.74				0.886 (6H, d, J = 6.6 Hz)
A17, B17′	19.74				0.879 (6H, d, J = 6.6 Hz)
A18, B18, B18′		35.93			1.28 (3H, m); 1.39 (3H, m)
A18′		36.43			1.33 (2H, m)
A19, B19, B19′	17.73				0.843 (9H, d, J = 7.0 Hz)
A19′		43.94			ax: 0.70 (1H, dd, J = 12.5, 12.5 Hz); eq: 1.39 (1H, m)
A20	19.93				0.857 (3H, d, J = 6.5 Hz)
A20′	22.39				0.836 (3H, s)
B20, B20′	19.93				0.853 (6H, d, J = 6.6 Hz)
C1, C1′		63.06			3.61 (2H, bdd, J = ∼6, ∼11 Hz); 3.72 (2H, bdd, J = ∼11, ∼4 Hz)
C2, C2′			78.36		3.52 (2H, quasi p, J ∼5 Hz)
C3, C3′		71.11			3.47 (2H, dd, J = 9.2, 5.0 Hz); 3.54 (2H, dd, J = 9.2, 5.0 Hz)

[a] Numbering refers to Fig. 1.

the NMR data as it does not contain a quaternary carbon atom. The ^{13}C-NMR data, however, do match with the position of the two cyclopentane rings as in II (Table 1). The presence of the quaternary carbon atom suggests that the third ring in the second biphytanyl moiety is not a cyclopentane (as in III) but a cyclohexane ring if we infer that the additional ring is biochemically formed through ring closure of a biphytanyl skeleton. This would also be consistent with the observed methyl group at δ = 0.836 ppm as a singlet in the ^{1}H-NMR spectrum, which is absent in the ^{13}C-NMR spectrum of GDGT-4 (**Fig. 3**). There are two possible structures (Va and Vb) for the second moiety through ring closure of the biphytanyl chain. Both are consistent with mass spectrometry data (11). Inverse long-range heteronuclear multiple bond correlation (HMBC) experiments enabled discrimination between these two possibilities since the singlet at δ = 0.836 ppm did not show correlation with the neighboring carbon atom (i.e., A10′) of the cyclopentane ring, as would be expected for skeleton Vb, but instead with carbon atom A16′ (Fig. 3). The remaining NMR data (Table 1) are also in agreement with this assignment. Furthermore, this structure is also in better agreement with published MS data of the biphytane moiety with three rings released after ether bond cleavage (11) because cleavage of the C-C bond between A15′ and A16′ explains why the fragment at *m/z* 263 is relatively abundant. This established that the abundant unknown GDGT membrane lipid in pelagic crenarchaeota is VI. We propose to call this component crenarchaeol, in analogy to the nomenclature of other archaeal ether lipids (24). Of the 86 carbon atoms of crenarchaeol, 23 are chiral and below we will explore literature and our NMR data to determine their stereochemistry.

Stereochemistry of the glycerol moieties and the acyclic chiral centers

A significant feature of archaeal ether lipids is that glycerol is *sn*-2,3-di-*O*-alkylated but not *sn*-1,2-diacylated as in

TABLE 2. ^{13}C- and ^{1}H-NMR data of glycerol dibiphytanyl glycerol tetraether-4 (IV)

Carbon Number[a]	Carbon Shift CH_3	Carbon Shift CH_2	Carbon Shift CH	Proton Shift(s)
A1, B1′		70.07		3.47 (4H, t, J = 6.8 Hz)
A1′, B1		68.58		3.55 (2H, ddd, J = 9.3, 7.0, 7.0 Hz); 3.67 (2H, ddd, J = 9.3, 7.5, 6.0 Hz)
A2, B2′		36.57		1.35 (2H, m); 1.61 (2H, m)
A2′, B2		37.05		1.39 (2H, m); 1.61 (2H, m)
A3, A3′, B3, B3′			29.74[b]	1.53 (4H, m)
A4, A4′, B4, B4′		37.22[b]		1.10 (4H, m); 1.23 (4H, m)
A5, A5′, B5, B5′		25.86[b]		1.22 (4H, m); 1.29 (4H, m)
A6, A6′, B6, B6′		37.11[b]		1.23 (8H, m)
A7, A7′, B7, B7′			39.08	1.79 (4H, m)
A8, A8′, B8, B8′		33.36		ax: 1.06 (4H, m); eq: 1.77 (4H, dd, J = ∼12, 7.8 Hz)
A9, A9′, B9, B9′		31.19		ax: 1.12 (4H, m); eq: 1.73 (4H, dd, J = 12.2, 6.8 Hz)
A10, A10′, B10, B10′			44.76	1.68 (4H, qh, J = ∼8 Hz)
A11, A11′, B11, B11′			38.18	1.24 (4H, m)
A12, A12′, B12, B12′		35.67		1.02 (4H, m); 1.37 (4H, m)
A13, A13′, B13, B13′		24.39		1.14 (4H, m); 1.36 (4H, m)
A14, A14′, B14, B14′		37.58		1.05 (4H, m); 1.26 (4H, m)
A15, A15′, B15, B15′			33.08	1.34 (4H, m)
A16, A16′, B16, B16′		34.23		1.09 (4H, m); 1.24 (4H, m)
A17′, B17	19.75			0.89 (6H, d, J = 6.7 Hz)
A17, B17′	19.75			0.88 (6H, d, J = 6.6 Hz)
A18, A18′, B18, B18′		35.94		1.30 (4H, m); 1.40 (4H, m)
A19, A19′, B19, B19′	17.72			0.836 (12H, d, J = 6.8 Hz)
A20, A20′, B20, B20′	19.89			0.845 (12H, d, J = 6.7 Hz)
C1, C1′		63.08		3.61 (2H, ddd, J = 11.2, 6.5, 6.5 Hz); 3.71 (2H, ddd, J = 11.2, 7.0, 4.0 Hz)
C2, C2′			78.37	3.51 (2H, pseudo p, J ∼5 Hz)
C3, C3′		71.13		3.47 (2H, dd, J = 9.3, 4.8 Hz); 3.54 (2H, dd, J = 9.3, 4.8 Hz)

[a] Numbering refers to Fig. 1.
[b] Average of two signals (see Table 3).

bacteria and eukaryotes. The unusual (*R*) configuration at the *sn*-2 position has been confirmed in case of the GDGTs of *Sulfolobus acidocaldarius* by appropriate incorporation experiments (25). The NMR data (both chemical shift and splitting pattern) of crenarchaeol, GDGT-0, and GDGT-4 (**Table 3**) of the protons and carbon atoms of the glycerol units and the ultimate and penultimate carbon atoms of the biphytanyl moieties and their attached protons are identical. This indicates that the stereochemistry of the glycerol units of crenarchaeol is the same as in GDGT-4 of *Sulfolobus acidocaldarius* and, thus, (*R*), as all other archaeal diethers and GDGTs.

Heathcock et al. (26) have established the full stereostructure of GDGT-0 [2,3,2′,3′-tetra-*O*-di-(3R,7R,11S,15S, 18S,22S,26R,30R-3,7,11,15,18,22,26,30-octamethyldotriacontanyl)-di-*sn*-glycerol; I]. Since all cyclopentane ring-containing GDGTs are biosynthesized by internal cyclization reactions of GDGT-0 (I), it is assumed that the remaining acyclic stereocentres in cyclopentane-containing GDGTs are also as in I (23). This assumption is also likely for crenarchaeol. This establishes the stereocentres of A3, A11, A3′, A15′, B3, B11, B3′, B11′ as (*R*) and A15, B15, and B15′ as (*S*). Note that due to changes in the priorities of the groups on chiral carbon atoms according to the Cahn-Ingold-Prelog convention, the naming of the configuration may be different although the absolute stereochemistry remains the same.

Stereochemistry of the cyclopentane rings

The absolute stereochemistry of the cyclopentane-ring has not yet been established. De Rosa et al. (22) reported, on basis of the chemical shifts of the carbon atoms of the cyclopentane rings in comparison with ^{13}C-NMR data of dimethylcyclopentanes (27), that the 1,3-substitution pattern of the cyclopentane ring in archaeal GDGTs is probably *trans*.

To determine the full stereochemistry of the cyclopentane rings, it was decided to first concentrate on the symmetrical GDGT-4 (IV), where no interference of signals from the cyclohexane ring occurs. HMQC, HMBC, COSY, and total correlation spectroscopy (TOCSY) experiments resulted in the assignment of all protons of the cyclopentane rings (Table 2). The two protons of both the A8 and A9 methylene groups showed a large difference (0.6–0.7 ppm) in chemical shift, whereas this difference for the two protons of the A18 methylene unit was only small (∼0.1 ppm) (Table 2). This suggested for the protons at A8 and A9 a situation comparable to that of cyclohexane rings, where the chemical shifts of protons strongly depend on their axial or equatorial position: the axial protons often resonate at much higher field than their equatorial counterparts. On the other hand, the protons at A18 seem to be more in eclipsed than in staggered positions. This assignment is, however, complicated by the fact that much conformational freedom exists in the cyclopentane compared with the cyclohexane ring. Therefore, we simulated the conformation of GDGT-4 using molecular dynamics and determined the average torsion angle of the protons and alkyl substituents of the cyclopentane ring. The results show that *i*) there is indeed a significant degree of conformational freedom in the cyclopentane ring and *ii*) the protons at A8 and A9 have a pronounced ax-

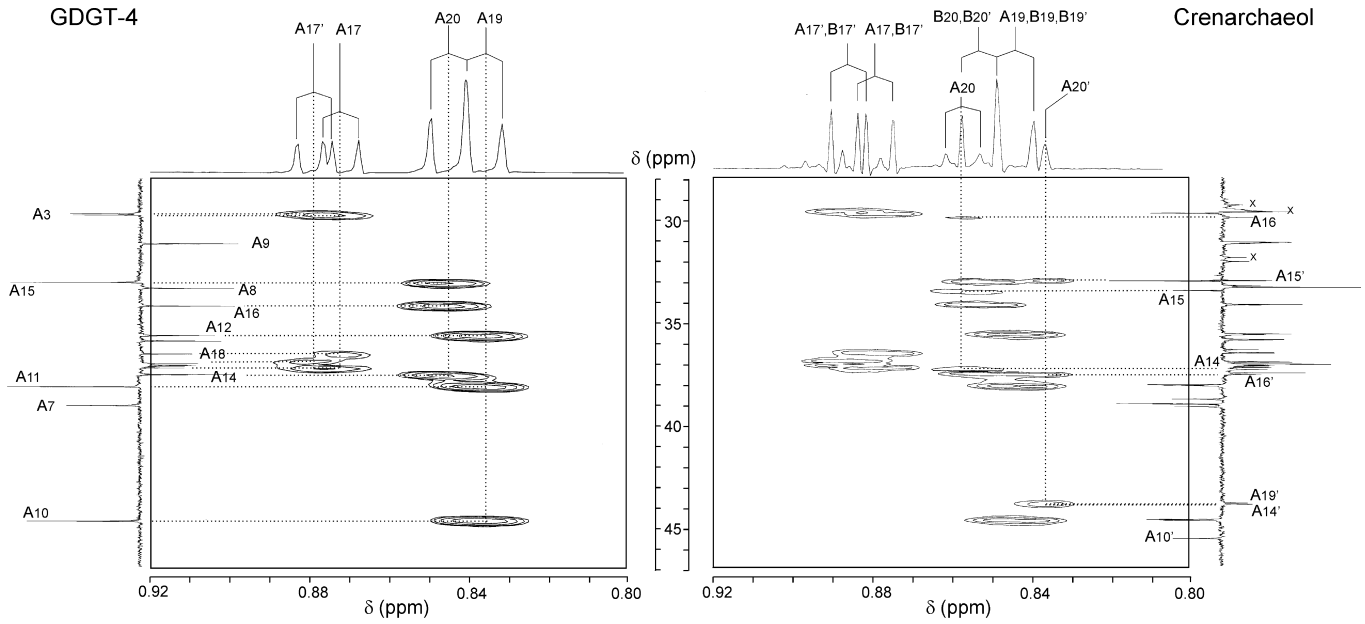

Fig. 3. Heteronuclear multiple bond correlation (HMBC) experiments (at 750 MHz) for GDGT-4 (left panel) and crenarchaeol (right panel). A selected range of the spectrum is displayed to show the correlations between the methyl groups and specific carbon atoms. Partial proton spectra (750 MHz) and attached proton test (APT) (125 MHz) spectra are plotted above and beside, respectively, the contour plot. Peak labeling refers to carbon numbering indicated in Fig. 1. Correlations between methyl groups and carbon atoms are indicated by stippled lines. In the HMBC spectrum of crenarchaeol, only the correlations of the methyl groups different from those in GDGT-4 (A20 and A20′) are indicated.

ial/equatorial character, whereas the torsion angles of the two protons at A18 with the ring are similar (**Fig. 4A**), consistent with our assignments of the NMR signals. The shifts of protons at the more substituted, tertiary carbon atoms A7 and A10 are at 1.79 and 1.68 ppm, respectively, not allowing any conclusion on whether they are axially or equatorially substituted.

A definite stereochemical assignment was revealed by a nuclear Overhauser effect spectroscopy (NOESY) experiment, which showed nuclear Overhauser effect (NOE) interactions of the proton at A10 with the axial proton at A9 and one of the protons at A18, and NOE interaction of the proton at A7 with the axial proton at A8 and the other proton at A18 (Fig. 4B). This proved that the stereochemistry of the cyclopentane ring is indeed *trans*, as suggested by DeRosa et al. (22). The full stereochemistry is subsequently determined by the original stereochemistry of GDGT-0 (26), in combination with the fact that biosynthesis of cyclopentane moieties in GDGTs occurs through internal cyclization (22): only one of the two possible ring closures results in an 1,3-alkyl *trans* substituted cyclopentane ring. This stereochemical assignment is confirmed by the observed coupling constants for the equatorial protons at A8, A9, and the proton at A10 (Table 2). This establishes the stereochemistry at the chiral centers A7 and A10 to be (*S*).

Based on these assignments, the stereochemistry of the cyclopentane rings of crenarchaeol was assessed to be identical to those in GDGT-4. All the protons and carbon atoms resonate at identical field strength (Tables 1 and 2), except those of the cyclopentane ring attached to the cyclohexane ring. In this cyclopentane ring, most chemical

TABLE 3. ^{13}C shifts (125 MHz) of selected carbon atoms of the four isolated GDGTs

Carbon Atom	GDGT-0	GDGT-4	GDGT-4′	Crenarchaeol
A1, B1′	70.09	70.07	70.06	70.07; 70.11
A1′, B1	68.60	68.58	68.58	68.56
A2, B2′	36.58	36.57	36.55	36.57; 36.59
A2′, B2	37.06	37.05		37.03
A3, A3′, B3, B3′	29.80; 29.84	29.71; 29.77	29.75	29.67; 29.68; 29.75
A4, A4′, B4, B4′	37.34	37.19. 37.25	37.25	37.18; 37.21; 37.26; 37.29
A5, A5′, B5, B5′	24.37	25.85; 25.87	25.87	25.84; 25.88
A6, A6′, B6, B6′	37.37	37.12; 37.14	37.13	37.13
A17, A17′, B17, B17′	19.76	19.75	19.75	19.71; 19.75; 19.76;19.78
C1, C1′	63.09	63.08	63.06	63.06
C2, C2′	78.35	78.37	78.34	78.35; 78.36
C3, C3′	71.07	71.13	71.11	71.11; 71.12

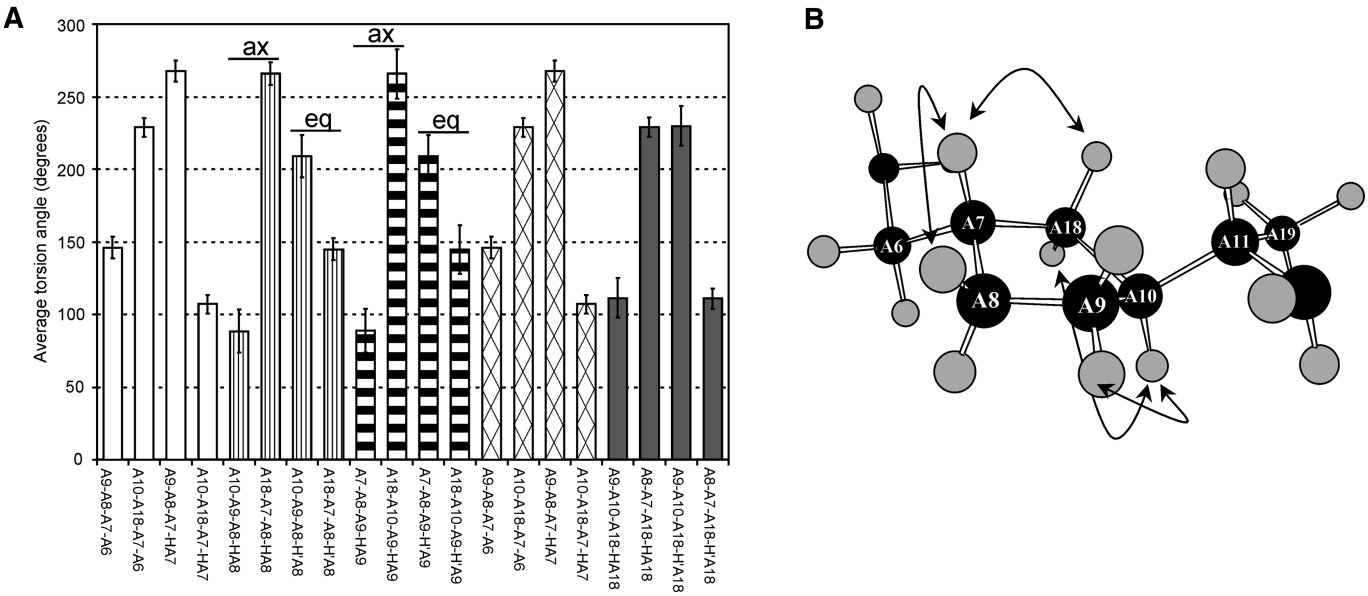

Fig. 4. A: Average (over four rings) torsion angles of substituents of the cyclopentane rings in GDGT-4 calculated by molecular dynamics. The standard deviation is indicated. Because the cyclopentane ring is not planar but is in an "envelope" form, two torsion angles have to be taken into account. The calculations indicate that the protons of carbon atoms A8 and A9 are in equatorial-like and axial-like positions. B: The calculated 3D-structure of the "average" cyclopentane partial stucture in GDGT-4. Indicated are the nuclear Overhauser effect spectroscopy interactions which determine the *trans* substitution of the alkyl side-chains.

shifts are also identical except for the shift of the proton at carbon atom A10′, which is at slightly higher field (1.47 ppm vs. 1.68 ppm in GDGT-4). This is attributed to the attached cyclohexane ring, which forces the proton in a slightly more "axial" position.

Stereochemistry of the cyclohexane ring

A prominent feature in the ^{1}H-NMR data of crenarchaeol are the two high-field protons, which are absent in the spectrum of GDGT-4, and obviously related to the presence of the cyclohexyl moiety. The assignment of these high-field protons is based on COSY, TOCSY, HMQC, and HMBC correlations. The proton at $\delta = 0.70$ represents a quasi triplet with a coupling constant of $J = 12.5$ Hz. This signal must be assigned to the axial proton at A19′; in addition to the relatively large geminal coupling there must be an equally large, axial-axial coupling with the proton at carbon atom A11′. The other high field proton absorbs at $\delta = 0.72$ and forms a quasi-double quartet with coupling constants $J = 13.0$ and 4.5 Hz. This is the axial proton at carbon atom A12′, which couples with the axial protons at A11′ and A13′, the geminal proton (all with large coupling constants of ~13 Hz), and with the equatorial proton at carbon atom A13′ with a much smaller coupling constant. The proton at carbon atom A11′ absorbs at $\delta = 1.17$ and is not well resolved from other signals. However, in 2D-NMR spectra it shows up as a double doublet with two relatively large coupling constants, in agreement with this assignment. Coupling with the proton at carbon atom A10′ is only weak, indicating that the dihedral angle is probably close to 90°C. These results indicate that the cyclopentane ring is equatorially substituted at carbon atom A11′ and that the stereochemistry at position A11′ is thus (*S*).

The stereochemistry at position A15′ follows from two observations. First, the chemical shift of the methyl group at A15′ (A20′) in the ^{13}C-NMR spectrum is at relatively low field (22.39 ppm), indicative for equatorially substituted methyl groups of cyclohexane rings (28). Second, remarkably, both axial protons at carbon atoms A14′ and A19′ show a strong long-range (four bonds) correlation with methyl group A20′ in the COSY spectrum. This established the (*R*) stereochemistry at A15′.

The stereochemistry of the cyclohexane ring is consistent with its presumed biosynthetic formation through ring closure via A15′ and A19′; the resulting stereochemistry is "inherited" from the stereochemistry of the GDGT-0 (I) precursor. This also determines the equatorial/axial positions of the alkyl substituents of the cyclohexane ring. If the cyclohexane ring is in the more stable chair configuration, this fits with the stereochemical configuration of the cyclohexane ring in crenarchael as determined by NMR.

Regioisomerism

It has been assumed for a long time that archaeal GDGTs were characterized by an antiparallel arrangement of glycerol units as in I (29). However, Gräther and Arigoni (30) showed by selective chemical degradation for three archaeal species that GDGT-0 is in fact a 1:1 mixture of the regioisomeric components I and VII.

During isolation of GDGT-4 from *S. solfataricus*, a fraction enriched in a less abundant, slightly later eluting (13) (~35% of GDGT-4) isomer (GDGT-4′) was also isolated.

This isomer had virtually identical ^{1}H- and ^{13}C-NMR spectra, indicating that the four cyclopentane rings must be in the same position and have the same stereochemistry. Detailed comparison of the ^{13}C data indicated, however, a subtle difference; the carbon atoms A3, A4, A5, and A6 showed two signals in case of GDGT-4 but only one in case of GDGT-4′ (Table 3). This observation led us to the conclusion that GDGT-4 is the antiparallel isomer whereas GDGT-4′ is the parallel isomer. This latter isomer has a plane of symmetry and only one stereoisomer exists. For GDGT-4 there is no plane of symmetry and its mirror image is therefore different. This explains why for some carbon atoms two close but not identical signals are observed.

For crenarchaeol an even more complicated situation exists since for some carbon atoms even four different signals are observed (Table 3). This indicates that the isolated isomer probably has the antiparallel configuration of glycerol units like in GDGT-4. Indeed, a minor isomer of crenarchaeol (presumably the parallel regioisomer) elutes later on the HPLC column, just as with GDGT-4 and GDGT-4′. In case of crenarchaeol there are, however, two additional regioisomers (VI and VIII) both with the antiparallel configuration of glycerol units, since the two biphytanyl chains in crenarchaeol are not the same, resulting in four stereoisomers. This explains the even more complex ^{13}C-NMR spectrum.

DISCUSSION

Our results establish, for the first time, the presence of a cyclohexyl ring in archaeal membrane lipids. Like the cyclopentane rings, this cyclohexane ring is also formed by internal cyclization of one of the biphytane chains. We have hypothesized that the formation of the cyclohexane ring is an adaption of the membrane lipids of hyperthermophilic archaea to relatively cold conditions in the open ocean (3, 31). It is well known that the presence of cyclopentane rings in GDGTs has a pronounced effect on the thermal transition points of cell membranes composed of GDGTs (7, 32). Consequently, hyperthermophilic archaea adjust the physical characteristics of their membranes to higher temperatures by increasing the number of cyclopentane rings. Our assessment of the stereochemistry at the 23 chiral centers of crenarchaeol now enables us to determine the influence of the additional cyclohexane ring on the 3D-structure and thus the physical properties of crenarchaeol. The 3D-structure of the energy-minimized crenarchaeol (**Fig. 5A**) shows that the cyclohexane moiety is some sort of bulge of one of the alkyl side chains. This bulge seems to prevent dense packing biphytanyl chains in the GDGT membranes of marine crenarchaeota.

To confirm this idea, we simulated GDGT membranes

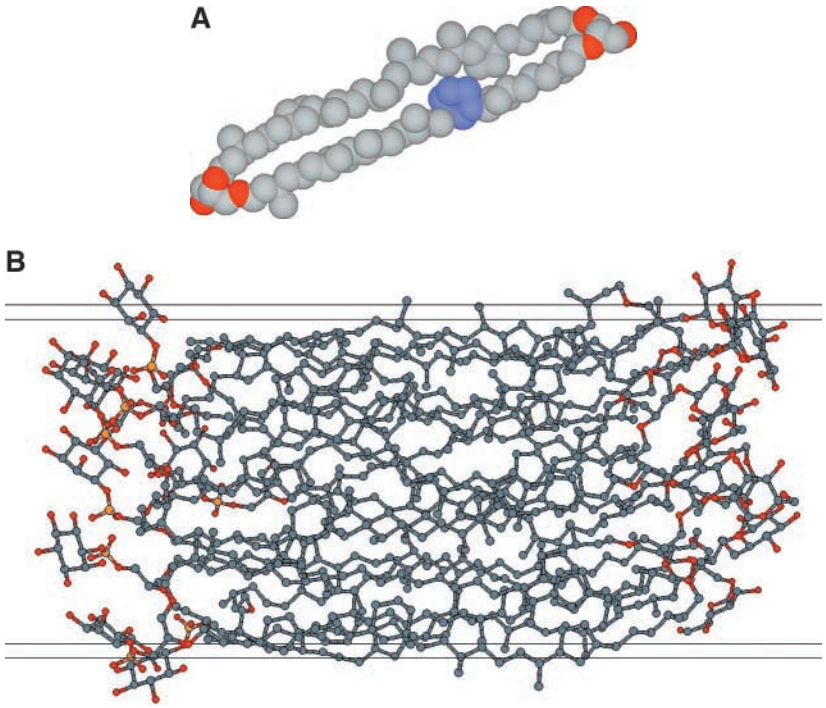

Fig. 5. A: Energy-minimized 3D-structure of a crenarchaeol lipid monomer showing the misalignment of the cyclohexane ring. The molecular dynamics simulations indicate that introduction of this cyclohexane ring causes a decrease in membrane density, which could aid the non-thermophilic crenarcheaota in surviving at lower temperatures. Oxygen atoms are depicted in red, the cyclohexane ring in blue. B: Snapshot from the molecular dynamics simulation on the crenarchaeol lipid membrane. The snapshot shows the periodic cell, containing 3 × 3 lipid monomers, and the cell boundaries in the y- and z-directions (parallel to the membrane). Hydrogen atoms were removed from the figure to enhance its clarity. Carbon atoms are depicted in dark gray, oxygen in red and phosphorus atoms in orange.

using molecular dynamics and calculated the average GDGT volume. Indeed, the calculated yz-surface area of the 3 × 3 GDGT cell (see experimental for details of method; Fig. 5B) for crenarchaeol (526.6 ± 0.9 Å^2) is larger than the corresponding area of a membrane comprised of GDGT-4 (515.9 ± 0.3 Å^2) and the membrane volume is thus higher. The less dense packing of biphytanyl chains in the GDGT membranes of marine crenarchaeota likely results in a lower thermal transition point of the membrane. Such a membrane would indeed be more suitable for archaea living at relatively cold temperatures. Therefore, the stereochemical structure of crenarchaeol is consistent with the idea that marine crenarchaeota evolved from (hyper) thermophilic archaea in the mid-Cretaceous (31). They inherited the biosynthetic capability to produce a membrane composed of cyclopentane ring-containing GDGTs produced from the (hyper)thermophilic archaea. However, to cope with the much lower temperature of the ocean, a small but key step in their evolution may have been the adjustment of the membrane fluidity by making a kink in one of the bicyclic biphytanyl chains by the formation of a cyclohexane ring.

This inferred evolutionary adaptation of membrane fluidity has not only resulted in the development of a dominant group of archaea but of microorganisms in general. Marine pelagic crenarchaeota probably represent one of the most abundant clades of microorganisms on earth. Their estimated total cell number in the oceans ($1.3{\cdot}10^{28}$) is ~40% of the estimated total number of all bacteria in the ocean (4). This indicates that the oceans also contain a massive amount of crenarchaeol. We have recently estimated that one crenarchaeal cell contains $1{\cdot}10^{-3}$pg GDGT (20). This indicates that the oceans contain 13 Mt GDGT, of which ~50% (6.5 Mt) is comprised of crenarchaeol. Together with GDGT-0, crenarchaeol is by far the most abundant GDGT in the biosphere, much more abundant than the cyclopentane-containing GDGT's derived from (hyper) thermophilic archaea.

The authors gratefully thank Dr. E. F. DeLong, Dr. A. Gambacorta, and Dr. F. G. Prahl, and Dr. S.G. Wakeham for their generous gift of cell material of *C. symbiosum*, the GDGT fraction of *S. solfataricus*, and suspended particulate matter from the Arabian Sea, respectively. Dr. E. Koning and Dr. Tj. van Weering are thanked for the Arabian Sea sediment. Mr. C. Erkelens (University of Leiden) is thanked for running samples on the 600 and 750 MHz instruments. Mrs. W. I. C. Rijpstra and Mr. S. Rampen provided analytical assistance. This research was partially supported by a Royal Society Fellowship for ACTvD.

REFERENCES

1. Barns, S. M., C. F. Delwiche, J. D. Palmer, and N. R. Pace. 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proc. Natl. Acad. Sci. USA.* **93:** 9188–9193.
2. DeLong, E. F. 1998. Everything in moderation: Archaea as "non-extremophiles". *Curr. Opinion Genet. Dev.* **8:** 649–654.
3. Schouten, S., E. C. Hopmans, R. D. Pancost, and J. S. Sinninghe Damsté. 2000. Widespread occurrence of structurally diverse tetraether membrane lipids: Evidence for the ubiquitous presence of low-temperature relatives of hyperthermophiles. *Proc. Natl. Acad. Sci. USA.* **26:** 14421–14426.
4. Karner, M. B., E. F. DeLong, and D. M. Karl. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature.* **409:** 507–510.
5. Madigan, M. T., J. M. Martinko, and J. Parker. 2000. Brock Biology of Microorganisms, Prentice Hall International, London.
6. Gabriel, J. L., and P. K. L. Chong. 2000. Molecular modeling of archaebacterial bipolar tetraether lipid membranes. *Chem. Phys. Lipids.* **105:** 193–200.
7. Gliozzi, A., G. Paoli, M. De Rosa, and A. Gambacorta. 1983. Effect of isoprenoid cyclization on the transition temperature of lipids in thermophilic archaebacteria. *Biochim. Biophys. Acta.* **735:** 234–242.
8. Hoefs, M. J. L., S. Schouten, J. W. De Leeuw, L. L. King, S. G. Wakeham, and J. S. Sinninghe Damsté. 1997. Ether lipids of planktonic Archaea in the marine water column. *Appl. Environ. Microbiol.* **63:** 3090–3095.
9. DeLong, E. F., L. L. King, R. Massana, H. Cittone, A. Murray, C. Schleper, and S. G. Wakeham. 1998. Dibiphytanyl ether lipids in nonthermophilic Crenarchaeotes. *Appl. Environ. Microbiol.* **64:** 1133–1138.
10. Preston, M. P., K. Y. Wu, T. F. Molinski, and E. F. DeLong. 1996. A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. *Proc. Natl. Acad. Sci. USA.* **93:** 6241–6246.
11. Schouten, S., M. J. L. Hoefs, M. P. Koopmans, H. J. Bosch, and J. S. Sinninghe Damsté. 1998. Structural characterization, occurrence and fate of archaeal etherbound acyclic and cyclic biphytanes and corresponding diols in sediments. *Org. Geochem.* **29:** 1305–1319.
12. Nicolaus, B., A. Trincone, E. Esposito, M. R. Vaccaro, A. Gambacorta, and M. DeRosa. 1990. Calditol tetraether lipids of the archaebacterium *Sulfolobus solfataricus*. *Biochem. J.* **266:** 785–791.
13. Hopmans, E. C., S. Schouten, R. D. Pancost, M. T. J. Van der Meer, and J. S. Sinninghe Damsté. 2000. Analysis of intact tetraether lipids in archaeal cell material and sediments by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **14:** 585–589.
14. Allen, M. P., and D. J. Tildesley. 1987. Computer Simulation of Liquids, Clarendon Press, Oxford.
15. Frenkel, D., and B. Smit. 1995. Understanding Molecular Simulation, Academic Press, San Diego.
16. van Duin, A. C. T., S. Dasgupta, F. Lorant, and W. A. Goddard, III. 2002. ReaxFF: A reactive force field for hydrocarbons. *J. Phys. Chem.* **105:** 9396–9409.
17. Berendsen, H. J. C., J. P. M. Postma, W. F. van Gunsteren, A. DiNola, and J. R. Haak. 1984. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* **81:** 3684–3690.
18. Cornell, W. D., P. Cieplak, C. Bayly, I. R. Gould, K. M. Merz, Jr., D. M. Ferguson, D. C. Spellmeyer, T. Fox, J. W. Caldwell, and P. A. Kollman. 1995. A second generation force field for the simulation of proteins, nucleic acids and organic molecules. *J. Am. Chem. Soc.* **117:** 5179–5197.
19. van Duin, A. C. T., and S. Larter. 2001. A computational chemical study of penetration and displacement of water films near mineral surfaces. *Geochem. Trans.* Paper number 006.
20. Sinninghe Damsté, J. S., W. I. C. Rijpstra, E. C. Hopmans, S. G. Wakeham, F. G. Prahl, and S. Schouten. 2002. Distribution of intact core ether lipids of planktonic crenarchaeota in the Arabian Sea. *Appl. Environ. Microbiol.* **68:** 2997–3002.
21. De Rosa, M., S. de Rosa, A. Gambacorta, L. Minale, and J. D. Bu'lock. 1977. Chemical structure of the ether lipids of thermophilic acidophilic bacteria of the *Caldariella* group. *Phytochemistry.* **16:** 1961–1965.
22. De Rosa, M., S. de Rosa, and A. Gambacorta. 1977. ^{13}C-NMR assignments and biosynthetic data for the ether lipids of *Caldariella*. *Phytochemistry.* **16:** 1909–1912.
23. De Rosa, M., and A. Gambacorta. 1988. The lipids of Archaebacteria. *Prog. Lipid Res.* **27:** 153–175.
24. Nishihara, M., H. Morii, and Y. Koga. 1987. Structure determina-

tion of a quartet of novel tetraether lipids from *Methanobacterium thermoautotrophicum*. *J. Biochem.* **101:** 1007–1015.
25. Kakinuma, K., Y. Obata, T. Matsuzawa, T. Uzawa, and T. Oshima. 1990. The stereochemical fate of glycerol during the biosynthesis of membrane lipids in thermoacidophilic archaebacteria *Sulfolobus acidocaldarius*. *J. Chem. Soc., Chem. Commun*. 925–927.
26. Heathcock, C. H., B. L. Finkelstein, E. T. Jarvi, P. A. Radel, and C. R. Hadley. 1988. 1,4- and 1,5- stereoselection by sequential aldol addition to α,β- unsaturated aldehydes followed by Claisen rearrangement. application tot total synthesis of the vitamin E side chain and the archaebacterial C_{40} diol. *J. Org. Chem.* **53:** 1922–1942.
27. Christl, M., H. J. Reich, and J. D. Roberts. 1971. Nuclear magnetic resonance spectroscopy. Carbon-13 chemical shifts of methylcyclopentanes, cyclopentanols, and cyclopentyl acetates. *J. Am. Chem. Soc.* **93:** 3463–3468.
28. Kruk, C., H. C. Cox, and J. W. De Leeuw. 1988. Assignments of the ^{1}H and ^{13}C NMR resonances of cadinanes and bicadinane by means of two-dimensional shift-correlated NMR techniques. *Magn. Res. Chem.* **26:** 228–235.
29. Langworthy, T. A. 1977. Long-chain diglycerol tetraethers from *Thermoplasma acidophilum*. *Biochim. Biophys. Acta.* **487:** 37–50.
30. Gräther, O., and D. Arigoni. 1995. Detection of regioisomeric macrocyclic tetraethers in the lipids of *Methanobacterium thermoautotrophicum* and other archaeal organisms. *J. Chem. Soc. Chem. Commun*. 405–406.
31. Kuypers, M. M. M., P. Blokker, J. Erbacher, H. Kinkel, R. D. Pancost, S. Schouten, and J. S. Sinninghe Damsté. 2001. Massive Expansion of Marine Archaea During a Mid-Cretaceous Oceanic Anoxic Event. *Science.* **293:** 92–94.
32. Uda, I., I. Sugai, I. H. Itoh, and T. Itoh. 2001. Variation on molecular species of polar lipids from *Thermoplasma acidophilum* depends on growth temperature. *Lipids.* **35:** 103–105.