Chapter 2

Membrane lipids of mesophilic anaerobic bacteria thriving in peats have typical archaeal traits

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Abstract

The 16S ribosomal DNA based distinction between the bacterial and archaeal Domains of Life is strongly supported by the membrane lipid composition of the two Domains; Bacteria generally contain dialkyl glycerol diester lipids, whereas Archaea produce isoprenoid dialkyl glycerol diether and membrane-spanning glycerol dialkyl glycerol tetraether (GDGT) lipids. Here we show that a new group of ecologically abundant membrane-spanning GDGT lipids, containing branched instead of isoprenoid carbon skeletons, are of a bacterial origin. This was revealed by examining the stereochemistry of the glycerol moieties of those branched tetraether membrane lipids, which was found to be the bacterial 1,2-di-*O*-alkyl-*sn*-glycerol stereoconfiguration and not the 2,3-di-*O*-alkyl-*sn*-glycerol stereoconfiguration as in archaeal membrane lipids. In addition, unequivocal evidence for the presence of cyclopentyl moieties in these bacterial membrane lipids was obtained by NMR. The biochemical traits of biosynthesis of tetraether membrane lipids and the formation of cyclopentyl moieties through internal cyclisation, which were thought to be specific for the archaeal lineage of descent, thus also occur in the bacterial Domain of Life.

2.1. Introduction

The phylogenetic distinction between Archaea and Bacteria (Woese et al., 1990) is supported by distinct differences in their core membrane lipid composition. Especially the chain architecture (isoprenoidal vs. *n*-alkyl) and the stereochemistry of the glycerol backbone (2,3- vs. 1,2-di-*O*-alkyl-*sn*-glycerol for Archaea and Bacteria, respectively; Kates, 1978) are strong lipid-taxonomic indicators, for which, to the best of our knowledge, no exceptions have been reported yet. Considering the stereochemistry of the glycerol backbone, it is known that only two enzymes are involved in introducing either of the stereoconfigurations (2,3- or 1,2-di-*O*-alkyl-*sn*-glycerol) in the glycerol moiety of membrane lipids. Those two stereospecific enzymes [G-1-P-dehydrogenase (Nishihara and Koga, 1995) for Archaea and G-3-P-dehydrogenase (Kito and Pizer, 1969) for Bacteria] are completely different in their amino acid sequence and are even thought to be one of the main reasons for the separate evolution of the Domains of Archaea and Bacteria (Koga et al., 1998).

Other lipid-taxonomic indicators which distinguish Archaea from Bacteria are the bond type (ether vs. ester bonds) and the membrane lipid structure (membrane spanning vs. bi-layered). However, some exceptions are known, especially in the case of thermophilic bacteria. Membrane lipids containing ether bound glycerols (e.g., **1**; see Appendix at the end of this chapter for structures) have, for example, been found in the hyperthermophilic bacteria *Thermodesulfotobacterium commune* (Langworthy et al., 1983), *Aquifex pyrophylus* (Huber et al., 1992) and *Ammonifex degensii* (Huber et al., 1996). Diether membrane lipids (e.g., **2**) were also found in yet unknown, non-thermophilic sulphate-reducing bacteria of a microbial consortium capable of anaerobic methane oxidation (Pancost et al., 2001) and recently in *Candidatus* 'K. stuttgartiensis' and *Candidatus* 'B. anammoxidans', two new members of the order *Planctomycetes* (Sinninghe Damsté et al., 2002b). These latter two strains and the sulphate-reducing bacteria *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* (Rütters et al., 2001) also produce membrane lipids (e.g., **3**) with both an ester and ether bound alkyl chain within one glycerol membrane lipid. In the bacterial Domain, membrane-spanning lipids have also been inferred for some species. The thermophilic anaerobic *Thermotoga maritima*, for example, produces 13,14-dimethyloctacosane and 15,16-dimethyltriacontane carbon chains (Carballeira et al., 1997) of which the latter one is found to be ether bound to one glycerol unit (**4**) (De Rosa et al., 1988). Tetraester membrane lipids containing α,ω-13,16-dimethyloctacosane moieties (**5**) were identified in *Thermoanaerobacter ethanolicus* (Jung et al., 1994; Lee et al., 2002). Except for the monoether found in *Thermotoga maritima*, a combination of both ether bonds and membrane-spanning carbon chains has so far never been encountered in Bacteria, specifically not in a tetraether configuration like in Archaea (e.g., **6**). In addition to those distinguishing features, the presence of cyclopentyl moieties is another characteristic which is, to the best of our knowledge, only found in the alkyl chains of archaeal membrane lipids and are absent in those of Bacteria.

Over the last years, a new group of membrane lipids, branched glycerol dialkyl glycerol tetraethers (GDGTs) (**7**, **8**, **9**), has been found in peat bogs (Schouten et al., 2000; Pancost and Sinninghe Damsté, 2003; Hopmans et al., 2004) and soils (Weijers et al., 2006b) and unambiguously identified by NMR (Sinninghe Damsté et al., 2000). Strikingly, these new GDGTs differ from known archaeal tetraether lipids (e.g., **6**) in that the alkyl moieties are not comprised of isoprenoid units but of branched carbon chains. Branched GDGTs with cyclopentyl moieties were tentatively identified as well (**10**, **11**) (Schouten et al., 2000). Hence, these branched GDGTs contain a mixture of archaeal (membrane-spanning tetraether structure and cylcopentyl moieties) and bacterial (branched carbon chains) characteristics. It is thus uncertain to which Domain of Life the organism biosynthesizing these branched GDGT lipids belongs.

Application of molecular ecological tools to ascertain the origin of these enigmatic components is hampered by the huge phylogenetic diversity of micro-organisms in peats and soils (up to an estimated 10^4 different species in one small sample; Torsvik et al., 2002) and the bias of microbiological culture techniques. Examination of the stereochemical characteristics of the membrane lipids provides a good alternative to distinguish between an archaeal and bacterial origin. To this end, the stereochemistry of the glycerol backbone of a branched GDGT (**8**) was unambiguously established. Furthermore, the structure of a branched GDGT with the putative cyclopentyl moiety (**10**) was also determined. This shows the presence of distinct archaeal traits in anaerobic bacteria thriving in soils and peats. Finally, by analysing two peat depth profiles for their branched GDGT content, information is gained about the habitat of the source organisms.

2.2. Experimental procedures

2.2.1. Sites and sampling

Peat cores for GDGT analysis were taken from the Saxnäs Mosse in Sweden and the Bolton Fell Moss in England. The Saxnäs Mosse area (SW Sweden, 56 51’ 20.78” N, 13 27’ 39.62” E) is an ombrotrophic raised bog with vegetation mainly composed of *Sphagnum* species. The upper 25 cm constitute the acrotelm layer in which the water table fluctuates. Below 25 cm the peat bog is continuously water saturated and consequently anoxic. A 50 cm long core was taken with a Wardenaar peat profile sampler and sub-samples were taken at a two cm interval.

The Bolton Fell Moss (National Grid Reference NY 495695, Cumbria, England), is a large ombrotrophic mire with a raised central dome with an average depth of 6 m and vegetation currently dominated by Sphagnum species (Xie et al., 2004). The acrotelm/catotelm boundary was determined at about 30 cm depth. A 50 cm monolith was collected alongside a 5 m Russian core covering a depth range of 25-500 cm; these were then

subsectioned in 1 cm intervals and combined. A total of 50 sub-samples were taken from the upper 175 cm.

Bulk peat samples for isolation of GDGT **10** were taken from Étang de la Gruère in the Jura Mountains of Switzerland. Étang de la Gruère is a strongly domed, raised bog with a peat accumulation of more than 6 meters (Steinmann and Shotyk, 1997). About 2.5 kg of peat (wet weight) was collected with a Belorussian peat sampler from a depth of 4.75-5.25 m.

2.2.2. Sample preparation

Peat samples were freeze dried and powdered with a mortar prior to extraction by an accelerated solvent extractor (ASE 200, DIONEX), soxhlet or by sonication, with solvent mixtures composed of dichloromethane and methanol. The obtained total extracts were prepared further for GDGT analysis as described earlier (Hopmans et al. 2000)

2.2.3. Preparative HPLC

Isolation of GDGT **10** was achieved by semi-preparative high performance liquid chromatography (HPLC) followed by flow injection analysis according to Smittenberg et al. (2002) (Smittenberg et al., 2002). The columns used are a semi-preparative and an analytical Alltech Prevail Cyano column (250 mm × 10 mm; 5 μm; flow rate 3 ml min^{-1} and 250 mm × 4,6 mm; 5 μm; flow rate 1 ml min^{-1}, respectively).

2.2.4. HPLC/APCI-MS

GDGTs were analyzed and quantified by high performance liquid chromatography/ atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI-MS), according to Hopmans et al. (2000) with minor modifications. Analyses were performed on an Agilent 1100 series/1100MSD series machine, equipped with auto-injector and HP Chemstation software. Separation was achieved on an Alltech Prevail Cyano column (150 mm × 2.1 mm; 3 μm). The flow rate of the hexane:propanol (99:1, v/v) eluent was 0.2 ml min^{-1}, isocratically for the first 5 min, thereafter with a linear gradient to 1.8% propanol in 45 min. Injection volume of the samples was 10 μl. Quantification of the GDGT compounds was achieved by integrating the peak areas in the $[M+H]^+$ and $[M+H]^+$+1 ion traces (i.e., protonated molecule and first isotope peak) and comparing these to an external calibration curve prepared with known amounts of pure crenarchaeol (**17**). A correction was made for the branched GDGTs (**7-15**), as their molecular mass is lower than that of crenarchaeol.

2.2.5. HT-GC

Bolton Fell Moss samples were analyzed by high temperature-gas chromatography (HT-GC) performed on a Hewlett Packard 5890 Series II GC, equipped with a flame ionization detector. Samples were run over an SGE HT5 (5% phenyl equivalent, polycarborane siloxane), 6 m by 0.53 mm aluminium clad column. The following temperature program was

used for most analyses: 50 °C (1 min) to 140 °C at 20 °C/min followed by 140 °C to 420 °C (10 min) at 7 °C/min. GDGT peaks were identified based on comparisons with standards (2,3,2',3'-tetra-*O*-dibiphytanyl-di-*sn*-glycerol-1'-β-gulosyl-1-phosphoryl-3''-*sn*-glycerol sodium salt standard, subsequently converted into a GDGT; Universal Biologicals Ltd.), and GDGTs identified in cold seep samples using LC-MS (Zhang et al., 2003). Interpretations were then validated by comparison to GC-MS analyses of selected HI-$LiAlH_4$ treated samples.

2.2.6. Nuclear Magnetic Resonance

^{1}H- and ^{13}C-NMR spectroscopy was performed on a Bruker DMX-600 spectrometer as described previously (Sinninghe Damsté et al., 2002a). ^{19}F-NMR spectroscopy of the Mosher derivatives of GDGTs were performed on a Varian Unity Inova 500, as described previously (Sinninghe Damsté et al., 2004).

2.3. Results

2.3.1. Structural identification of GDGTs in peats

An HPLC base peak chromatogram of a sample from the Saxnäs Mosse bog (Sweden) shows high abundances of the branched GDGTs **7** and **8** and, to a lesser extent, GDGT **9** (Fig. 2.1). Concentrations of the ring-containing GDGTs (**10**-**15**) are generally 10% or less than those of GDGTs **7**-**9**. So far, only the structures of GDGTs **7** and **8** (Sinninghe Damsté et al., 2000) have been fully elucidated by NMR. It was, therefore, decided to attempt to fully characterize a GDGT containing a cyclopentyl moiety.

Preparative HPLC on the bulk peat sample from Étang de la Gruère bog (Switzerland) resulted in ca. 3 mg of isolate in which the ring-containing GDGT **10** accounts for ca. 88%, the remainder being minor amounts of GDGT **8** (9%), GDGT **7** (1%) and GDGT **12** (1%). The ^{1}H-NMR spectrum of this isolate is extremely complex even if measured at 600 MHz. In the 3.4-3.7 ppm region, multiplets accounting for 18 protons are observed (Table 2.1). These represent the protons of the two glycerol units and the first and ultimate methylene units of the alkyl moieties bound via the ether linkages. The same signals were observed in the ^{1}H-NMR spectra of GDGT **7** and **8** in an earlier study (Sinninghe Damsté et al., 2000). At ca. 2.2 ppm a broad singlet representing the two hydroxy groups is found. At 0.85 ppm two doublets occur, representing three methyl groups, whereas the spectra of GDGT **7** and **8** showed only one doublet representing four methyl groups. Another difference with these ^{1}H-NMR spectra is the presence of a multiplet between 1.7-1.8 ppm representing four protons.

The ^{13}C-NMR spectrum of GDGT **10** reveals 3 primary, 56 secondary, and 7 tertiary atoms (Table 2.1). APT, DEPT90 and DEPT135 experiments were used to assess the multiplicity of these carbon atoms. The ^{13}C-NMR spectrum did not show 66 resolved signals

because many carbon atoms are either strictly, or effectively, equivalent. Assignments of the carbon atoms is based on comparison with the ^{13}C-NMR data of GDGT **7**, **8** and **16** (Sinninghe Damsté et al., 2000; Sinninghe Damsté et al., 2002a), in combination with HMQC and HMBC experiments. Many shifts observed in the ^{13}C-NMR spectrum are identical to those reported for GDGT **7** (Sinninghe Damsté et al., 2000), confirming their close structural relationship. The major difference is that one methyl group (i.e., carbon atom B15) is absent and is part of a cyclopentyl moiety, as suggested previously on the basis of mass spectral analyses (Schouten et al., 2000). This results in characteristic shifts for carbon atoms B7-B15 and B13'-B15' that are different from the shifts observed for these carbon atoms in GDGT **7**. Both the ^{13}C and ^{1}H shifts for the atoms of the cyclopentyl moiety are consistent with literature data (Sinninghe Damsté et al., 2002a). The ultimate proof for this structural assignment is the observed correlation of the protons of carbon atom B15 with the carbon atoms B9, B10, B11 and B12, B13, and B14 in the HMBC spectrum. The structures of the other ring-containing GDGTs (**11-15**), all possessing 1 or 2 cyclopentyl moieties, were deduced from the characteristic $[M+H]^+$, $[M+H]^+$-18 (water loss) and $[M+H]^+$-74 (glycerol loss) pattern in their mass spectra, their retention time relative to their "parent" GDGTs (**7-9**) and the structure of GDGT **10**.

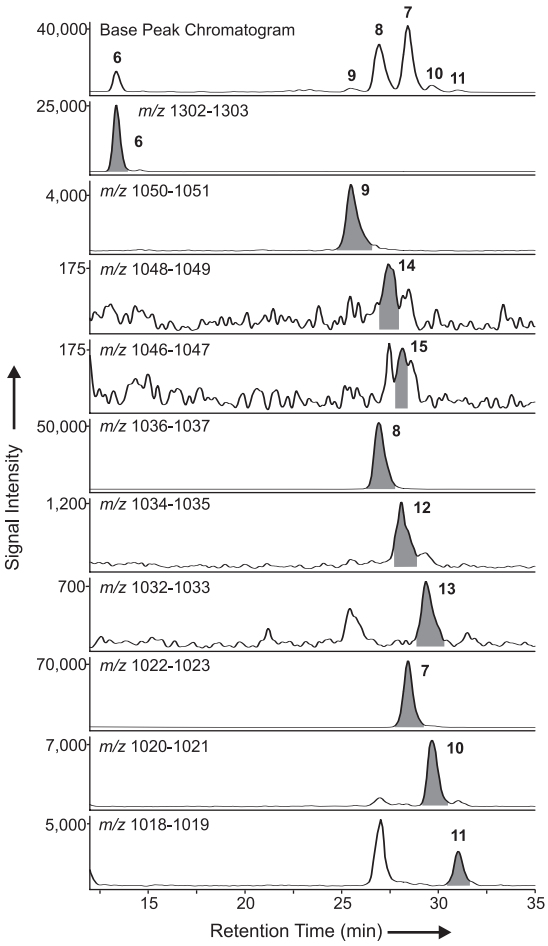

Figure 2.1: Partial HPLC/MS base peak chromatogram and mass chromatograms of the protonated molecule plus first isotope peak of the different GDGTs in a 43 cm deep sample from the Saxnäs Mosse peat bog. Numbers refer to the structures in the appendix. The highest peak at the m/z 1018 trace is a $[M+H]^+$ -18 (H_2O) fragment from m/z 1036 (GDGT **8**). Similarly, the peak in the m/z 1032 trace in front of GDGT **13** represents the $[M+H]^+$-18 fragment of GDGT **9**.

Table 2.1: ^{13}C- and ^{1}H-NMR data used for structural identification of GDGT **10**

Carbon atom[a]	Carbon shift (ppm)[b]			Proton shift (ppm)[c]
	CH	CH_2	CH_3	
A1, B1'		71.77		3.41 (4H, m)
A1', B1		70.41		3.58 (2H, m), 3.51 (2H, m)
A2, B2'		29.40		1.53 (4H, m)
A2', B2		29.98		1.55 (4H, m)
A3, B3'		26.09		1.30
A3', B3		26.06		1.30
A4, A4', B4, B4'		c. 29.6		c. 1.24
A5, A5', B5, B5'		c. 29.6		c. 1.24
A6, A6', B6, B6'		c. 29.6		c. 1.24
A7, A7', B7'		c. 29.6		c. 1.24
B7		29.86		c. 1.24
A8, A8', B8'		c. 29.6		c. 1.24
B8		28.62		1.22
A9, A9', B9'		c. 29.6		c. 1.24
B9		36.77[d]		1.23
A10, A10', B10'		30.05		1.21
B10			38.76[e]	1.80
A11, A11'		27.05		1.23
B11		33.14[f]		1.75 (eq), 1.03 (ax)
B11'		27.01		1.23
A12, A12'		37.15		1.06
B12		33.14		1.75 (eq), 1.03 (ax)
B12'		36.98		1.25
A13, A13'			33.05	1.31
B13			39.02[g]	1.76
B13'			32.85	1.32
A14, A14'		34.35		1.07, 1.22
B14		34.00[h]		1.23
B14'		35.85		1.05, 1.23
A15, A15'	19.79			0.858 (6H, d)
B15		38.67		1.30
B15'	19.86			0.854 (3H, d)
C1, C1'		71.11		3.49 (2H, m), 3.46 (2H, m)
C2, C2'			78.39	3.49 (2H, m)
C3, C3'		63.05		3.68 (2H, dd), 3.58 (2H, m)
OH				2.10 (2H, bs)

[a] numbering refers to GDGT **10** in the Appendix
[b] determined by APT, DEPT90 and DEPT135
[c] mainly obtained from the HMQC spectrum
[d] in good agreement with the δ= 36.83 calculated from data of a cyclopentane-containing isoprenoidal GDGT (Sinninghe Damsté et al., 2002a) and the additivity principle
[e] in good agreement with the δ= 38.80 calculated from data of a cyclopentane-containing isoprenoidal GDGT (Sinninghe Damsté et al., 2002a) and the additivity principle
[f] in good agreement with the δ= 33.08 calculated from data of a cyclopentane-containing isoprenoidal GDGT (Sinninghe Damsté et al., 2002a) and the additivity principle
[g] in good agreement with the δ= 39.04 calculated from the shift of carbon atom B10 and the additivity principle
[h] in agreement with the δ= 34.42 calculated from the shift of carbon atom B9 and the additivity principle

Table 2.2: Chemical shift (in ppm relative to $CFCl_3$) of the CF_3 group of the Mosher products of the reaction of the branched GDGT **8**, 1,2-di-*O*-dihexadecyl-*rac*-glycerol, 1,2-di-*O*-dihexadecyl-*sn*-glycerol and the isoprenoid GDGT **16** with the S- and R-Mosher acid chlorides. Between brackets the relative abundance of the signal is given.

	GDGT **8**	1,2-di-*O*-dihexadecyl-*rac*-glycerol	1,2-di-*O*-dihexadecyl-*sn*-glycerol	GDGT **16**
S-Mosher acid chloride	-73.212 (100%)	-73.106 (50%) -73.170 (50%)	-73.170 (100%)	-73.048 (100%)
R-Mosher acid chloride	-73.129 (100%)	n.d.	-73.106 (100%)	-73.137 (100%)

n.d., not determined

2.3.2. Stereoconfiguration of the glycerol backbone of GDGTs in peats

The isolate of GDGT **8**, obtained previously from the Holocene Bargerveen peat bog (the Netherlands) (Sinninghe Damsté et al., 2000), was used here to determine the stereochemistry of the glycerol backbone. To this end, this GDGT was reacted with the S- and R- α-methoxy-α-(trifluoromethyl)phenylacetyl chlorides to give the so called Mosher ester (Dale and Mosher, 1973) and the chemical shift of the trifluoromethyl group was investigated with ^{19}F-NMR. The Mosher esters of this branched GDGT **8** gave comparable chemical shifts for the CF_3 group as the S- and R-Mosher acid derivatives of the commercially available 1,2-di-*O*-dihexadecyl-*sn*-glycerol, but opposite of those of the Mosher esters of the isoprenoid GDGT **16** isolated from *Sulfolobus acidocaldarius* (Sinninghe Damsté et al., 2002a) which possesses the 2,3-di-*O*-alkyl-*sn*-glycerol stereoconfiguration (Table 2.2). This shows that the branched GDGT **8,** found in peats and soils, possess the bacterial 1,2-di-*O*-alkyl-*sn*-glycerol and not the archaeal 2,3-di-*O*-alkyl-*sn*-glycerol stereoconfiguration.

2.3.3. GDGT profiles in peats

Two peat cores were analyzed for their branched GDGT content and for GDGT **6**, a commonly found isoprenoid GDGT (Figs. 2.2 and 2.3). Branched GDGTs **7-15** are all detected in the Saxnäs Mosse core (see also Fig. 2.1). In the Bolton Fell Moss core, only GDGTs **7**, **8** and **10-13** could be detected, whilst GDGTs **9**, **14** and **15** were below detection limit using high temperature – gas chromatography (HT-GC). Concentrations of the branched GDGTs in the Saxnäs Mosse are very low in the upper 8 cm (0.2 μg g^{-1} dry weight (wt) peat) and slightly higher, around 3 μg g^{-1} dry wt peat, between 8 and 28 cm depth. Branched GDGT concentrations are highest below 28 cm with an average concentration of 35 μg g^{-1} dry wt peat. The Bolton Fell Moss profile shows a similar pattern; branched GDGT concentrations are below detection limit in the upper 10 cm and are about 5 μg g^{-1} dry wt peat between 10

and 28 cm. Below 28 cm the average concentration is much higher at 37 μg g^{-1} dry wt peat. In the Bolton Fell Moss core, which extends to 175 cm depth, a trend to slightly lower concentrations can be observed from ca. 120 cm downward.

The concentrations of GDGT **6** in both cores are about two times lower compared to those of the branched GDGTs. In the Saxnäs Mosse, concentrations of GDGT **6** are 0.2 μg g^{-1} dry wt peat in the upper 8 cm, 1.2 μg g^{-1} dry wt peat between 8 and 28 cm and 13 μg g^{-1} dry wt peat between 28 and 50 cm. For the Bolton Fell Moss core, those concentrations are below detection limit for the upper 10 cm and 0.7 and 16 μg g^{-1} dry wt peat for the intervals 10 to 28 and 28 to 175 cm, respectively. As is observed for the branched GDGTs, GDGT **6** decreases in concentration in the lower 50 cm of the Bolton Fell Moss core.

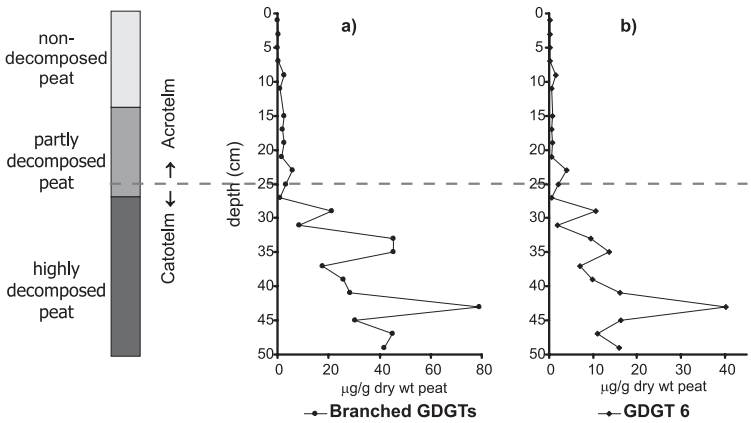

Figure 2.2: Concentrations of: **a)** the branched GDGTs **7-9** (average value) and **b)** GDGT **6** against depth in a peat core from the Saxnäs Mosse, Sweden, together with a schematic decomposition profile of the peat core. The acrotelm layer is the zone in which the water table fluctuates and the catotelm layer is the zone of permanent water saturation.

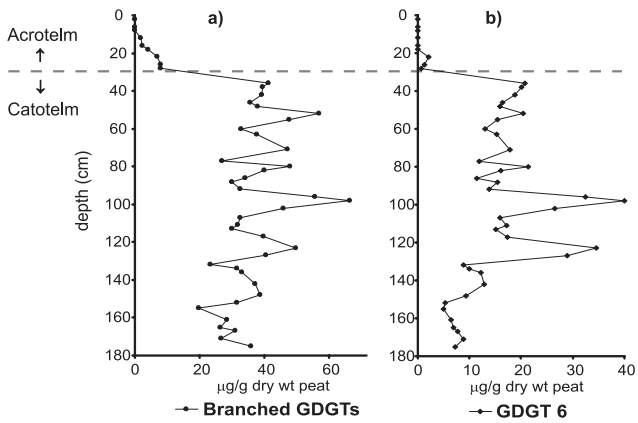

Figure 2.3: Concentrations of: **a)** the branched GDGTs **7** and **8** (average value) and **b)** GDGT **6** against depth in a peat core from the Bolton Fell Moss, Cumbria, England.

2.4. Discussion

2.4.1. A bacterial origin for branched GDGTs in peat

The elucidation of the stereochemistry of the glycerol backbone of GDGT **8** as the 1,2-di-*O*-alkyl-*sn*-glycerol stereoconfiguration strongly indicates that branched GDGTs encountered in peats are biosynthesized by members of the Domain of Bacteria. The stereochemical evidence is particularly strong since it is highly unlikely that Archaea would biosynthesize membrane lipids with a bacterial stereoconfiguration as the enzymes responsible for introducing the stereoconfiguration are thought to form the basis for the divergent evolution of the Bacteria and the Archaea (Koga et al., 1998). A possible archaeal origin would, therefore, mean a thorough reconsideration of the current ideas on early evolution and lateral gene transfer (LGT). A bacterial origin of the branched tetraethers is also consistent with the chain architecture (*n*-alkyl instead of isoprenoid). Furthermore, ether bonds as well as membrane spanning alkyl chains have previously been reported in some Bacteria (Langworthy et al., 1983; De Rosa et al., 1988; Lee et al., 2002). Considering the above stated arguments, the presence of cylcopentyl moieties in the alkyl chains, which, to the best of our knowledge, has so far never been reported before in core membrane lipids of Bacteria, should therefore be regarded as the first report of the occurrence of cyclopentyl moieties in Bacteria.

2.4.2. Biosynthetic pathway

The exact functional role of these branched GDGTs is as yet unclear, but they are likely membrane-spanning lipids just like archaeal GDGTs and may provide the cell membrane with a higher degree of stability. This assumption is supported by the calculated length of the branched GDGTs, which is ca. 34 Å versus 37 Å for the isoprenoid GDGT **6** (1 Å = 10^{-10} m; determined for an individual GDGT in a minimal energy mode between the chiral carbon atoms of the two glycerol moieties; CS ChemDraw Pro software, CambridgeSoftCorporation, MA, USA). This is of the same order of magnitude as the thickness of a black lipid monolayer membrane composed of tetraethers of the archaeon *Thermoplasma acidophilum*, which is, because stacked in a membrane and therefore smaller, determined at 25-30 Å (Stern et al., 1992). Due to the similarity in the overall molecular structure of the branched GDGTs and the archaeal isoprenoid GDGTs (e.g., **6**), a schematic biosynthetic pathway is postulated for the branched GDGTs, which to a certain extent resembles that of isoprenoid GDGTs (Boucher et al., 2004) (Fig. 2.4). The first step of this pathway involves the establishment of the *sn*-glycerol-3-phosphate backbone out of dihydroxyacetone phosphate (DHAP) by G-3-P dehydrogenase (Fig. 2.4). In this step Bacteria differentiate themselves from the Archaea, who use G-1-P dehydrogenase to form the opposite *sn*-glycerol-1-phosphate backbone out of DHAP. In step 2 and 3 two isopentadecane moieties are connected via an ether bond at the *sn*-1 and *sn*-2 positions of the glycerophosphate backbone, creating a diether. The existence of these steps seems to be plausible as similar diethers (e.g., **1**) are found in several bacteria,

such as *Thermodesulfotobacterium commune* (Langworthy et al., 1983), *Aquifex pyrophylus* (Huber et al., 1992) and *Ammonifex degensii* (Huber et al., 1996). The enzymes carrying out the ether bond formation in steps 2 and 3 are unknown and cannot be the same as those in the biosynthesis of diethers in Archaea as they have to cope with the opposite stereoconfiguration of the glycerophosphate backbone. The fourth step in the proposed pathway is similar to that of the biosynthesis of isoprenoid GDGTs and involves the head-to-head coupling of two glycerol di-alkyl diethers to form a glycerol di-alkyl glycerol tetraether. Although the details of this step as well as the enzyme(s) involved are as yet unknown for the Archaea as well, the existence of this step in the archaeal pathway has been proven indirectly by labelling experiments and by inhibiting this step with terbinafine (Nemoto et al., 2003; Eguchi et al., 2003). The responsible enzyme for this coupling process could be the same in both Archaea and Bacteria as the tails of both carbon chains are structurally identical (i.e., isoprenoid vs iso-branched). The next steps in the biosynthesis of both types of tetraethers comprise the formation of cyclopentyl moieties. In the archaeal tetraethers, formation of such a cyclopentyl moiety takes place via an internal cyclisation of a methyl branch with a δ-carbon (De Rosa et al., 1977). It is likely that the formation of cyclopentyl moieties in the branched GDGTs takes place in a similar fashion and based on the structural similarity, this cyclisation process might be carried out by similar enzymes as well. It might be possible that the bacteria producing the branched GDGTs acquired the enzymes for the head-to-head coupling and the formation of a cyclopentyl moiety via lateral gene transfer from Archaea, or vice versa, a process which has been shown to occur between thermophilic Archaea and Bacteria (Nelson et al., 1999).

2.4.3. Ecology

Analyses of two peat cores reveal that the branched GDGTs (**7-9**) are abundant in the continuously water saturated and consequently anoxic part of the peat bogs (Figs. 2.2 and 2.3). This suggests that anaerobic bacteria are the source of the branched GDGTs. The low abundances of GDGTs in the upper part of the profiles can probably be explained by alternating oxic and anoxic conditions, due to a moving water table. Concentrations of the branched GDGTs are about twice as high compared to the isoprenoid GDGT **6**, which in these settings is most likely derived from methane-producing, anaerobic archaea (Tornabene and Langworthy, 1979; Pancost et al., 2000). This is the same ratio as was found for biphytane and dimethyloctacosane in the Bargerveen peat bog (the Netherlands) after release of the alkyl moieties from the GDGTs by ether cleavage (Pancost and Sinninghe Damsté, 2003). Methanogenic archaea are known to play a major role in the carbon cycle in peat bogs and cell counts of Archaea in a Siberian peat bog showed that their relative abundance increases with depth up to 36% of the total microbial community (Kotsyurbenko et al., 2004). Therefore, the relatively high abundance of branched GDGTs indicates that the anaerobic bacteria producing them might also form an ecologically significant part of the microbial population. In both peat cores the pattern of the concentration profile of the branched GDGTs

(**7-9**) resembles to a great extent the concentration profile of the isoprenoid GDGT **6**. This suggests that both (groups of) organisms use the same substrates and/or catalyse the same processes. Until the bacteria which produce the branched GDGTs have been fully identified, however, any suggestion about their role in the biodegradation of organic matter remains speculative.

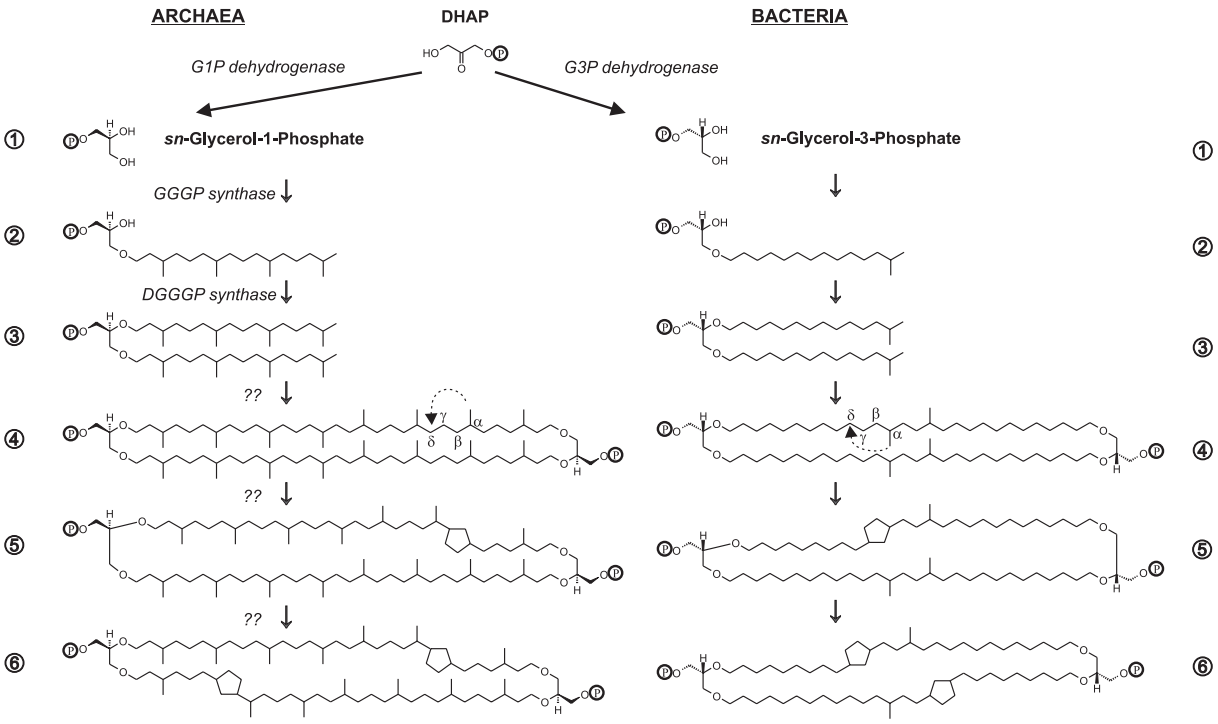

Figure 2.4: Schematic representation of the postulated biosynthetic pathway for the branched GDGTs compared with the biosynthetic pathway for isoprenoid GDGTs [modified after Boucher et al. (2004)]. P = Phosphate, DHAP = Dihydroxyacetone Phosphate, GGGP = geranylgeranylglyceryl phosphate, DGGGP = digeranylgeranylglyceryl phosphate. Double bonds, which are, at least in the isoprenoid pathway, present in all but the last stages, have been omitted for sake of simplification. For isoprenoid GDGTs the formation of cyclopentyl moieties can continue up to 8 rings.

2.4.4. Functional role of branched GDGTs

Besides being a relict from thermophilic ancestors, the presence of branched GDGTs in mesophilic bacteria could as well be an adaptation to the environment in which these organisms thrive. It is assumed that the formation of a monolayer cell membrane by membrane-spanning tetraethers is an adaptation of Archaea to more extreme conditions in order to maintain a certain degree of stability in the membrane (Van de Vossenberg et al., 1998). At first sight, the discovery of GDGTs in the ubiquitous mesophilic aquatic Crenarchaeota (Hoefs et al., 1997; DeLong et al., 1998; Schouten et al., 2000; Sinninghe

Damsté et al., 2002a) refutes this assumption. Those Crenarchaeota, however, have been shown to adapt their membrane to the cooler environment by the formation of an additional unique GDGT lipid, crenarchaeol (**17**), containing a cyclohexyl moiety (Sinninghe Damsté et al., 2002a). The presence of GDGT **6** in a peat also seems to refute the assumption that GDGTs are related to extreme environments. A peat environment, however, has pH values which are generally low, ranging from pH4-6. In *Sphagnum* moss peat those values are even known to reach as low as pH3-4. In this respect the presence in a peat bog of GDGT **6**, derived from methanogenic archaea, might be not surprising as monolayer membranes composed of isoprenoid GDGTs provide an excellent barrier against the high proton gradients present in acidic environments (Elferink et al., 1994; Van de Vossenberg et al., 1998). Indeed, Macalady et al. (2004) recently showed that there is a better correspondence between the occurrence of GDGT lipids and the degree of acidity than between the occurrence of GDGT lipids and temperature in a series of 49 archaeal species. Membrane lipids of acidophilic archaea were composed of up to 100% of GDGTs, whereas no GDGTs were detected in the three alkaliphilic archaea studied. Thus, although possessing branched instead of isoprenoid alkyl chains, the occurrence of branched tetraether lipids in anaerobic bacteria in peat bogs could well be related to the more acidic pH conditions in such an environment.

2.5. Conclusions

Branched GDGT membrane lipids (**7**-**15**) are shown to have an 1,2-di-*O*-alkyl-*sn*-glycerol stereoconfiguration. This feature, in combination with the characteristic branched alkyl chains, forms convincing evidence that branched GDGTs are biosynthesized by Bacteria. Furthermore, it has been unequivocally proven that branched GDGTs contain up to two cyclopentyl moieties. This is the first report of the occurrence of membrane-spanning tetraether lipids and the capability to form cyclopentyl moieties in core membrane lipids in the Domain of Bacteria. Although the exact source of the branched GDGT lipids remains, as yet, unknown, they are abundant in the anoxic parts of peat bogs. Since the abundance of branched GDGTs co-vary in two depth profiles with that of GDGT **6**, derived from anaerobic methanogens, a similar ecological niche for the methanogenic Archaea and the anaerobic, branched GDGT producing bacteria is expected. The biological function of branched GDGTs in the bacteria is unknown, but their presence could well be related to the acidic conditions in a peat bog.

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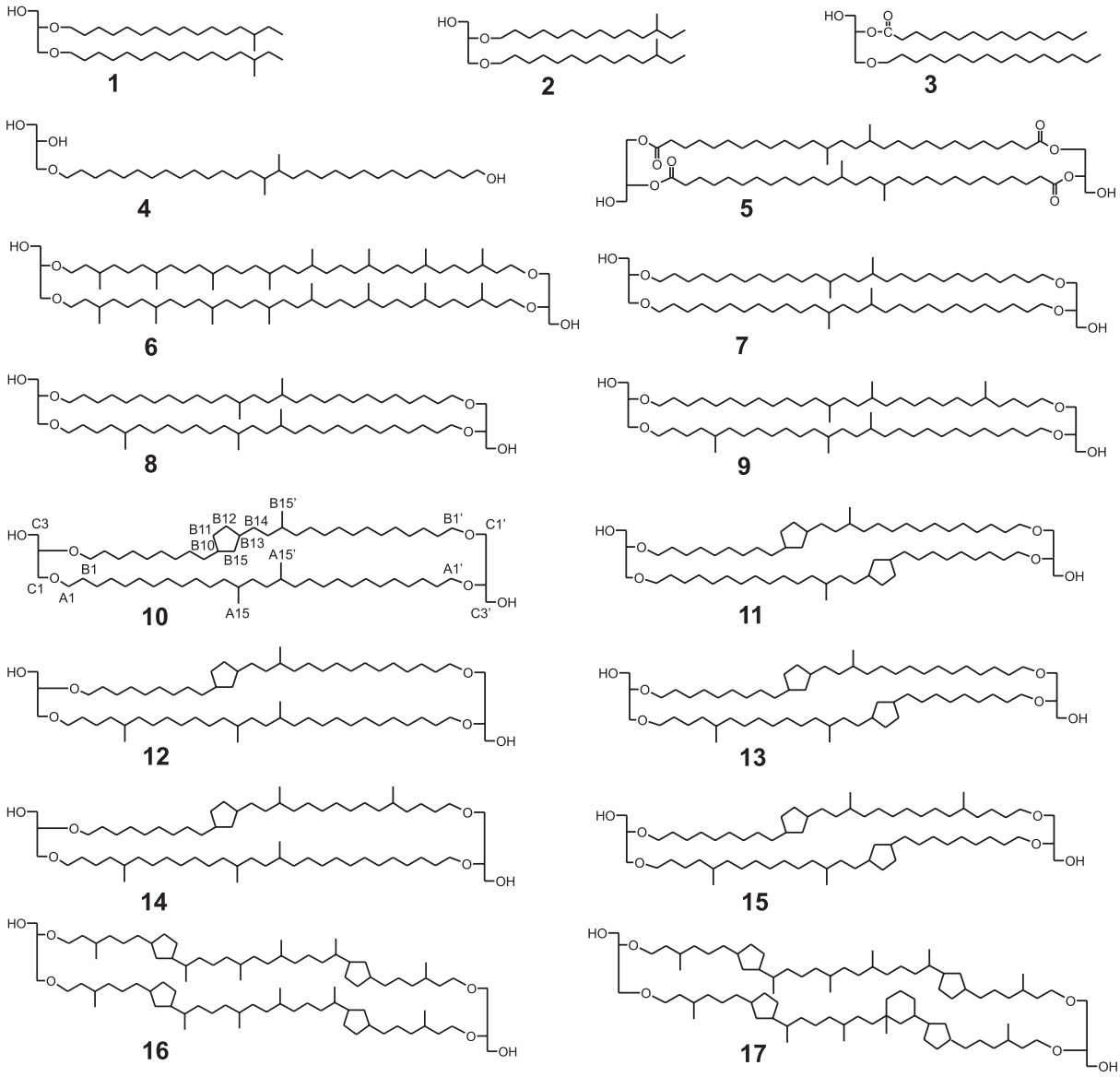
Appendix