Glycerol ether lipids in sediments: sources, diversity and implications

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‘I know that nothing good lives in me, that is, in my sinful nature.’

--- Romans 7:18

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Abstract

Glycerol ether lipids are prominent membrane constituents in Archaea and Bacteria that are characterized by high potential for preservation in geological settings. During the past decade they were increasingly used in molecular proxies. For example, selected glycerol dialkyl glycerol tetraethers (GDGT) are used in ratios such as the TEX_{86} and BIT index for reconstructing past sea surface temperature (SST) and terrestrial input, respectively. However, the distribution and structural diversity of glycerol ether lipids in marine sediments has not yet been fully explored. In order to obtain a better understanding of the origin and fate of these lipids and to evaluate the potential impact on molecular proxies, a globally distributed set of samples was analyzed in this PhD thesis project. More than forty novel compounds were revealed and it could be shown that the diversity of glycerol ether lipids in marine sediments is much higher than previously recognized.

Among the studied lipids, isoprenoid GDGTs were shown as the most dominant component in all analyzed sediments with over 70% of total ether lipids. A comparison of the lipid composition of core and intact polar isoprenoid GDGTs pointed to a potential impact of live benthic archaea and their intact lipids on the application of the TEX_{86} SST proxy. The same study suggested that recycling of fossil GDGTs from planktonic archaea by the benthic archaeal community could be an important process. In a set of peat sediments, two intact polar lipids of the orphan branched GDGTs were for the first time observed. These branched GDGTs that constitute the BIT index have in their intact form glucuronosyl and glucosyl headgroups. The two compounds accounted for 4-7% of total IPLs, suggesting that their producers represent a sizeable but not dominant component of the microbial community. The identification of these intact polar precursors of these orphan lipids provides important constraints for the search of their microbial sources.

The presence of newly identified glycerol ether lipids with distinctive structures and ubiquitous distribution in all analyzed marine sediments provided potential phylogenetic biomarkers and a large reservoir for novel molecular proxies to be developed. A series of novel ether core lipids coexisting with corresponding isoprenoid GDGTs was identified in all analyzed sediments. The first series of compounds,

accounting on average for 7% of total ether lipids, was identified as glycerol dibiphytanol diethers (GDDs) and is considered to represent either biosynthetic intermediates or degradation products of GDGTs. A second series was identified as hydroxylated GDGTs based on nuclear magnetic resonance (NMR) and mass spectral interpretation. Accordingly, a series of unknown IPLs that had been previously reported as a major component in samples of archaeal cultures, sediments, and the water column were then recognized to be glycosidic hydroxy-GDGTs. The widespread occurrence of IPLs of hydroxy-GDGTs suggests an important contribution of specific archaeal species with high activity in a wide range of geological settings. Several other groups of novel compounds were also tentatively identified based on interpretation of mass spectra. Specifically, extended H-shaped GDGTs, hybrid isoprenoid/branched GDGTs, and overly and sparsely branched GDGTs are characterized with a common feature of methylation series, containing compounds with one -CH_2- unit difference between each other. It is expected that the information encoded in the distribution of the novel lipids can be used to develop molecular proxies indicating past environmental factors such as temperature and salinity and/or information pertaining to the composition and activity of extant microbial communities.

An overall distribution of detected glycerol ether lipids in marine subsurface sediments was shown by the estimated relative abundance of 11 structural groups, and therefore reflects a general composition of ether lipid producing microbes contributing to the sedimentary record.

Zusammenfassung

Glyzerolbasierte Etherlipide sind bedeutende Membranbestandteile von Archaeen und Bakterien und zeichnen sich durch ein hohes Erhaltungspotential im geologischen Kontext aus. In den letzten zehn Jahren erhielten sie eine zunehmende Bedeutung für molekulare Proxies. Bestimmte Glyzerol-dialkyl-glyzerol-tetraetherlipide (GDGT) kommen z.B. zur Anwendung im TEX_{86} und BIT Index welche zur Rekonstruktion von Oberflächenwassertemperaturen beziehungsweise terrestrischem Eintrag in Sedimente in der Vergangenheit benutzt werden können. Allerdings ist die Verteilung und strukturelle Vielfalt von glyzerolbasierten Etherlipiden in marinen Sedimenten immernoch nicht vollständig erforscht. Um einen besseren Überblick über die Herkunft und das Schicksal dieser Lipide zu bekommen und um einen potentiellen Einfluss auf molekulare Proxies abschätzen zu können, wurde in der vorliegenden Doktorarbeit ein global verteilter Probensatz analysiert. Mehr als vierzig neue Komponenten wurden entdeckt und es konnte gezeigt werden dass die Diversität von glyzerolbasierten Etherlipiden in marinen Sedimenten sehr viel höher ist als bisher angenommen.

Den grössten Anteil an den erforschten Lipiden haben die isoprenoiden GDGTs, welche die dominante Komponente mit einem relativen Anteil von mehr als 70% von allen Etherlipiden ausmachten. Ein Vergleich der Lipidzusammensetzung von GDGT Kernlipiden und intakten polaren isoprenoiden GDGTs deutete auf einen potentiellen Einfluss von lebenden benthischen Archaeen und deren intakten Lipiden auf den TEX_{86} Oberflächenwassertemperatur Proxy hin. Gleichzeitig gab es Hinweise darauf, dass Recycling von fossilen GDGTs von planktonischen Archaeen durch die benthische Archaeengemeinschaft ein wichtiger Prozess sein könnte. In mehreren Proben von Torfsedimenten konnten zum ersten Mal zwei intakte polare Lipide der bisher nur als Kernlipide bekannten verzweigten GDGTs identifiziert werden. Diese verzweigten GDGTs, welche auch im BIT index verwendet werden, haben als intakte Kopfgruppen Glucuronsäure und Glucose. Die beiden Komponenten machen zusammen 4-7% der gesamten intakten Lipide aus. Dies deutet darauf hin, dass ihre Produzenten eine beträchtliche, aber nicht unbedingt die dominante Komponente der mikrobiellen Gemeinschaft ausmachen. Die Identifizierung dieser intakten polaren Vorläufermoleküle

der verzweigten GDGTs ist ein wichtiger Schritt in der Suche nach ihren mikrobiellen Produzenten.

Das Vorhandensein von neu identifizierten glyzerolbasierten Etherlipiden mit prägnanten Strukturen und allgegenwärtiger Verteilung in allen analysierten marinen Sedimenten birgt viel Potential um neue phylogenetische Biomarker und neue molekulare Proxies zu entwickeln. Es konnte eine Serie von neuen Etherkernlipiden in Kombination mit zugehörigen isoprenoiden GDGTs in allen analysierten Sedimenten gefunden werden. Die erste Reihe von Komponenten, welche im Durchschnitt 7% von allen Etherlipiden ausmachen, konnte als glyzerolbasierte Dibiphytanoldiether (GDD) identifiziert werden. Diese Komponenten sind entweder Zwischenprodukte der Biosynthese oder Abbauprodukte von GDGTs. Die zweite Reihe von Lipiden konnte basierend auf Kernspinresonanzspektroskopie (NMR) und massenspektrometrischer Untersuchung als hydroxylierte GDGTs identifiziert werden. Gleichzeitig konnte eine Reihe von bisher unbekannten IPLs, welche schon vorher als Hauptkomponenten in Proben von Archaeenreinkulturen, Sedimenten und Proben aus der Wassersäule beobachtet worden waren, als glykosidische Hydroxy-GDGTs erkannt werden. Das weitverbreitete Vorkommen von intakten polaren hydroxylierten GDGTs deutet auf einen wichtigen Beitrag von bestimmten Spezies von Archaeen mit hoher mikrobieller Aktivität unter breitgefächerten Umweltbedingungen hin. Mehrere andere Gruppen von neuen Verbindungen wurden basierend auf massenspektrometrischen Untersuchungen vorläufig identifiziert. Alle Verbindungen, erweitertes H-förmiges GDGT, hybrides isoprenoid/verzweigte GDGTs und andere mehr oder weniger verzweigte GDGTs haben als gemeinsames Merkmal eine Serie an Methylierungen, wobei immer eine $–CH_2$-Gruppe den Unterschied zwischen den Verbindungen ausmacht. Es ist zu erwarten dass die Information die in der Verteilung dieser neuen Lipide steckt benutzt werden kann um molekulare Proxies zu entwickeln für Umweltbedingungen wie Temperatur oder Salinität, und/oder um die Zusammensetzung und Aktivität von mikrobiellen Gemeinschaften zu untersuchen.

Die Gesamtverteilung der entdeckten Etherlipide in tief versenkten marinen Sedimenten konnte anhand der abgeschätzten relativen Verteilung von 11 strukturellen Gruppen gezeigt werden. Das sich ergebende Bild zeigt die generelle Zusammensetzung

von Mikroorganismen welche Etherlipide produzieren und welche in der Sedimentabfolge wiedergefunden werden können.

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Chapter 1

Introduction

1. Molecular proxies

Molecular proxies applied for paleoenvironmental studies are based on the abundance and isotopic composition of specific organic compounds found in terrestrial or marine deposition environments, and are therefore completely independent of the conventional proxies based on inorganic or isotopic compositions or on assemblages of microfossils (Eglinton and Eglinton, 2008). These molecular compounds, or so-called biomarkers, can be divided into two major groups, terrigenous and aquatic, according to the habitats of their source organisms. One example of terrigenous biomarkers found in marine sediments is the saturated long chain n-alkanes, such as C_{27}, C_{29} and C_{31} n-alkanes. They are derived from the leaf waxes of higher plants on land (e.g. Eglinton and Hamilton, 1967) and are the foundation of many proxies reflecting continental vegetation and climate changes (e.g. Bird et al., 1995; Schefuss et al., 2003). Marine sediment also receives biomarkers contributed from the dwellers in the overlying water column. For instance, dinosterol, a unique molecular biomarker for dinoflagellates (De Leeuw et al., 1983; Robinson et al., 1984), was used to reconstruct paleoproductivity in the ocean (e.g. Prahl et al., 1989). Its saturated hydrocarbon counterpart, dinosterane, has even been identified in ancient sediments and crude oils and was taken as the evidence of marine organic matter input (Summons et al., 1987, 1992).

A classical example showing the independence of organic molecular proxies from inorganic and micropaleontological proxies comes from the studies of past sea surface temperature (SST). $U_{37}^{K'}$ index defined by the ratio of $C_{37:2}$ and $C_{37:3}$ alkenones, is the

first and one of the most prevailing molecular proxies (Brassell et al., 1986; Prahl and Wakeham, 1987).

$U_{37}^{K'}$=[$C_{37:2}$]/([$C_{37:2}$]+[$C_{37:3}$]);

SST=($U_{37}^{K'}$ -0.044)/0.033 (Müller et al., 1998)

The correlation between $U_{37}^{K'}$ and SST is attributed to the fact that these long straight-chain ketones are synthesized by haptophyte algae living in the marine photic zone (Brassell et al., 1986). In certain circumstances these molecular proxies can be more robust, but their application also relies on both the quality of samples and geological distribution of biomarkers.

Analytical techniques play an essential role in determining the discovery of new biomarkers and the subsequent development of proxies. While earlier generation of biomarker studies depended mostly on gas chromatography, the recent employment of liquid chromatography (LC) in organic geochemistry, particularly in combination with mass spectrometry (MS), extended the range of detectable molecules to higher molecular weight and high polarity (e.g. Hopmans et al., 2000; Sturt et al., 2004). Among those polar compounds whose detection facilitated by LC-MS are glycerol dialkyl glycerol tetraethers (GDGTs), including isoprenoid GDGTs and non-isoprenoid GDGTs (branched GDGTs). New proxies were soon developed and widely applied in many researches. Studies on the GDGTs composition in environmental samples have led to establishment of several proxies. The most prominent is TEX_{86}, an SST molecular proxy (Schouten et al., 2002) defined by the ratio of isoprenoid GDGTs with different number of rings (see the equation in Fig 1-6). The applicability of TEX_{86} is built on the assumption that planktonic archaea in surface water column is the primary source and the composition of isoprenoid GDGTs synthesized by the organism is temperature dependent (Schouten et al., 2002). Branched GDGTs are much more abundant in soil than in marine sediment. Accordingly, the BIT index was developed on branched GDGTs for tracing soil input into marine environments (equation in Fig 1-2; Hopmans et al., 2004). The exact source organisms for branched GDGTs remain unclear but are likely to be soil Bacteria (Weijers et al., 2006).

Like the other proxies, these organic molecular proxies also have their constraints and problems. Multiple factors controlling the input, transportation, and preservation of these molecular compounds set up the barrier of their application. Even for the well-established $U_{37}^{K'}$ index, the biochemical function of alkenones in the source organisms of haptophytes is still unknown. Furthermore, changes in haptophytes communities under varied nutrient conditions can affect the alkenone input (Herbert, 2003; Prahl et al., 2005), and aerobic degradation has also an impact on the preservation of alkenone (Hoefs et al., 1998). The newborn TEX_{86} index faced more unsolved problems, particularly with respect to the complex sources of GDGTs used in TEX_{86} index. GDGTs in marine surface sediment are believed to originate from the photic zone (Wuchter et al., 2005; Menzel et al., 2006). Algal grazing and repackaging of materials in the upper water column appears to play an important role in the transmission of GDGTs from surface waters to sediments (Wakeham et al., 2003; Huguet et al., 2006; Wuchter et al., 2005). However, it is found that significant amounts of GDGTs can be contributed by archaeal species living below the mixed layer in the water column (Pearson et al., 2001; Ingalls et al., 2006). Additionally, within some anoxic basins, a contribution from the Archaea thriving in deeper water has been inferred (Huguet et al., 2007). Recent studies also discuss the varied contributions of two major marine archaeal groups, Crenarchaeota and Euryarchaeota, which occupy different water depths and nutrient regimes (Turich et al., 2007; Schouten et al., 2008; Turich et al., 2008). The problem of various planktonic sources can be further compounded by the contribution of GDGTs of benthic origin, a possibility supported by the suspiciously ^{14}C depleted GDGTs in Santa Monica Basin (Shah et al., 2008), and the discovery of intact polar GDGTs in sediment (Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009), as discussed below.

Sedimentary input from benthic species is a big problem for the SST molecular proxies, but, it is also conceivable that there may be microbial biomarkers produced by benthic heterotrophic bacteria or other bottom-dwelling organisms which would provide paleoproxies for bottom-water temperature, extent of oxygenation etc. (Eglinton and Eglinton, 2008).

2. Deep biosphere in marine subsurface sediments: another source of organic molecule

Marine subsurface sediments hosting the deeply buried microbial ecosystem are one of the most mysterious habitats on Earth. The cell count-derived prokaryotic biomass in marine subsurface biosphere is almost one-tenth (Parkes et al., 2000) or even one-third (Whitman et al., 1998) of the total living biomass on Earth. Quantification using fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (Q-PCR) on targeted ribosomal RNA (rRNA) and DNA showed that a large fraction of the sub-seafloor bacteria is alive (Schippers et al., 2005). Other studies employing the same rRNA-based technique analysis showed that another predominant fraction of this deeply buried microbial community, Archaea, is also active (Biddle et al., 2006).

The presence of active Archaea was supported by the results of intact polar lipid (IPL) analysis, an LC-MS technique for detection of intact membrane lipid molecules in environmental samples (e.g. Sturt et al., 2004; Biddle et al., 2006; Lipp et al., 2008). IPLs, especially, phospho-IPLs were believed to degrade rapidly upon death of the source organism (White et al., 1979; Harvey et al., 1986; Lipp and Hinrichs, 2009), and are therefore an indicator of living organism activities. IPL analysis can provide simultaneous detection of both bacterial and archaeal lipids (Sturt et al., 2004). A survey of extractable IPLs in a set of globally distributed samples showed that IPL contents decrease with subseafloor depth, and the IPL pool is dominated by archaeal lipids with glycosyl headgroups (Fig. 1-1, Lipp et al., 2008). Taken together, marine deep biosphere is another source of organic molecules, particularly archaeal lipids.

The discovery of marine deep biosphere with detectable IPLs has several important applications. On the one hand, IPL can be used as a tool to address the question "who is doing what". The numerous novel phylogenetic lineages of Bacteria and Archaea sustained by subsurface sediment are mostly uncultivated (Parkes et al., 2000; Teske and Sørensen, 2008), and their activities are presumably much lower than those in the surface (D'Hondt et al., 2002 and 2004; Biddle et al., 2006). In this case, carbon isotopic analysis of IPLs can provide information regarding the metabolism of the in situ communities. For example, the $\delta^{13}C$ values of archaeal IPLs suggest heterotrophy using buried organic

carbon as a possible trophic style of benthic archaea (Biddle et al., 2006). On the other hand, deep biosphere can also represent a negative factor for proxy users, such as the complexity brought by mediating degradation of organic matter and contributing lipids to the pool of biomarkers.

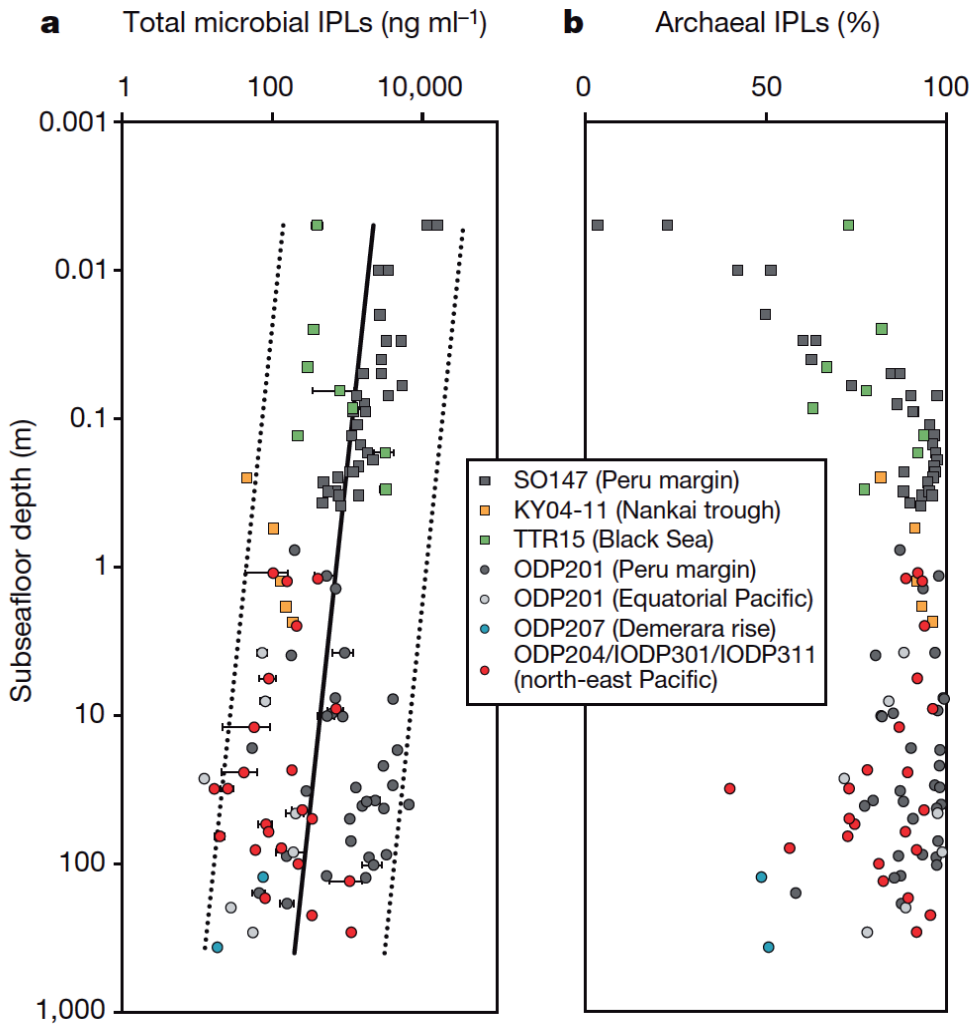

Figure 1-1. Depth profile of IPLs in marine sediments. a, Concentration of total IPLs vs. Depth. b, relative contribution of archaeal IPLs to total microbial IPLs. Figure taken from Lipp et al. (2008)

However, the fact that the IPLs in marine subsurface sediment contain mostly sugar instead of phospho-headgroups (Biddle et al., 2006; Lipp et al., 2008) poses a question that is currently being intensively investigated: Are glyco-IPLs good proxies for live cells? Phospho-IPLs have been shown to degrade rapidly upon death of the source organism (White et al., 1979; Harvey et al., 1986), whereas the degradation kinetics of the more stable glyco-IPLs remains to be constrained. Recently, a laboratory experiment of IPL degradation test indicated that the bacterial glycerol ester lipids were faster degraded than archaeal ethers, but the bonding type of the head groups had no influence on the degradation rate (Logemann et al., 2011). Nevertheless, as discussed in Lipp et al. (2008) and Lipp and Hinrichs (2009), glyco-GDGTs turn over at much shorter time scales than the GDGT core lipids and can be used, to some extent, as an indicator of sedimentary biomass. In this thesis, this conclusion of Lipp and Hinrichs (2009) has been employed as a basic assumption for the work presented in Chapter 3.

3. Glycerol ethers as distinctive membrane lipids

The membrane lipids of Bacteria and Eucarya are usually composed of glycerol dialkyl diesters (Fig. 1-2), although there are exceptions that some thermophilic bacteria are able to synthesize glycerol diethers (Langworthy et al., 1983; Huber et al., 1992), glycerol tetraethers, or even glycerol with both ether- and ester-bound hydrocarbons (Sinninghe Damsté et al., 2007). As the third domains of life (Fig. 1-2; Woese et al., 1990; Pace, 1997), Archaea differ from Bacteria and Eucarya also in terms of membrane lipids: the major constituents of archaeal membranes are isoprenoid glycerol ethers. Since the first isolation and characterization of archaeol, a glycerol diphytanyl diether, from the culture of *Halobacterium cutirubrum* (Kates et al., 1963, 1965), a lot of studies have been carried out to investigate archaeal lipids. Archaeol remains the most common archaeal diether. Other kinds of diethers include hydroxyl archaeol (Ferrante et al. 1988), extended C_{20}, C_{25} and C_{25}, C_{25} archaeol (DeRosa et al., 1982, 1983a), and macrocyclic archaeol (Comita et al., 1983). Isoprenoid GDGTs were discovered about one decade later than archaeol. The first described tetraether is a fully saturated GDGT extracted from a thermoacidophile (Langworthy et al., 1977), and later the structure of tetraethers

was also proved to be highly diverse. The identified varieties include GDGTs with cyclopentane ring structures (DeRosa et al., 1977, 1983b), glycerol trialkyl glycerol tetraethers (GTGT, DeRosa et al., 1983b), and H-shaped GDGTs (Morii et al., 1998).

Archaea were first detected and isolated from extreme environments. As a result, the earliest discoveries of novel archaeal lipids were made on cultivated extremophiles (Kates et al., 1963; DeRosa et al., 1976; Langworthy, 1977) and methanogens (Makyla and Singer, 1978; Tornabene et al., 1979). Nevertheless, with the finding that Archaea, mainly the uncultivated mesophilic marine Crenarchaeota, are also widely distributed in marine water column and sediments (DeLong et al., 1992; Fuhrman et al., 1992; Vetriani et al., 1998, 1999), these non-extreme environments became another research highlight for exploration of archaeal lipids, particularly isoprenoid GDGTs (Hoefs et al., 1997; DeLong et al., 1998; Schouten et al., 1998, 2000).

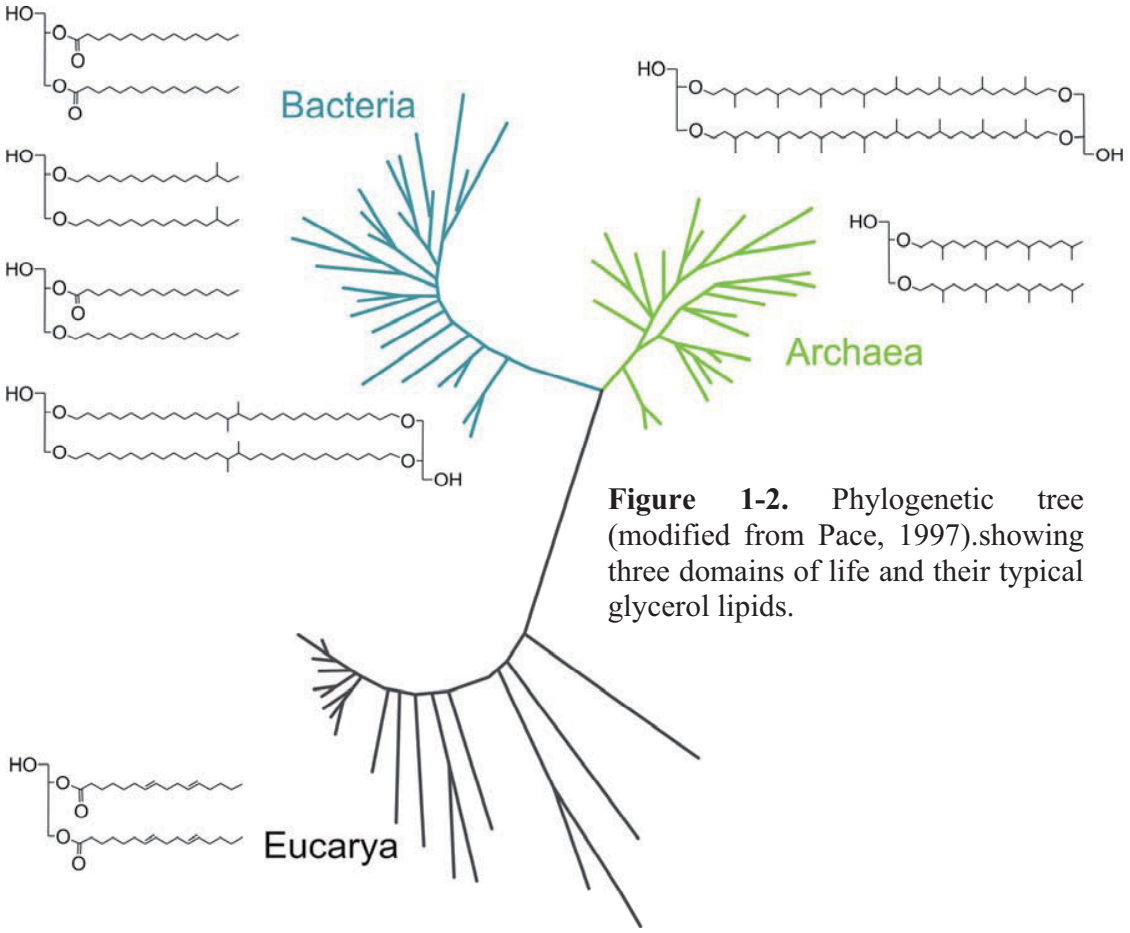

Figure 1-2. Phylogenetic tree (modified from Pace, 1997).showing three domains of life and their typical glycerol lipids.

4. Glycerol tetraethers in marine sediment

There are two types of glycerol tetraethers found in marine sediment: isoprenoid GDGTs and branched GDGTs. Isoprenoid GDGTs usually occur as the predominant components and consist of four isoprenoid tetraethers with 0-3 pentacyclic rings and crenarchaeol with its regioisomer, while branched GDGTs are relatively less abundant and consist of non-isoprenoid tetraethers with different number of methyl groups (Fig. 1-3). As mentioned above (cf. Section 1.1), TEX_{86} and BIT indices were developed on the basis of these two groups of glycerol tetraethers for reconstructing past SST and terrestrial input, respectively (Schouten et al., 2002; Hopmans et al., 2004).

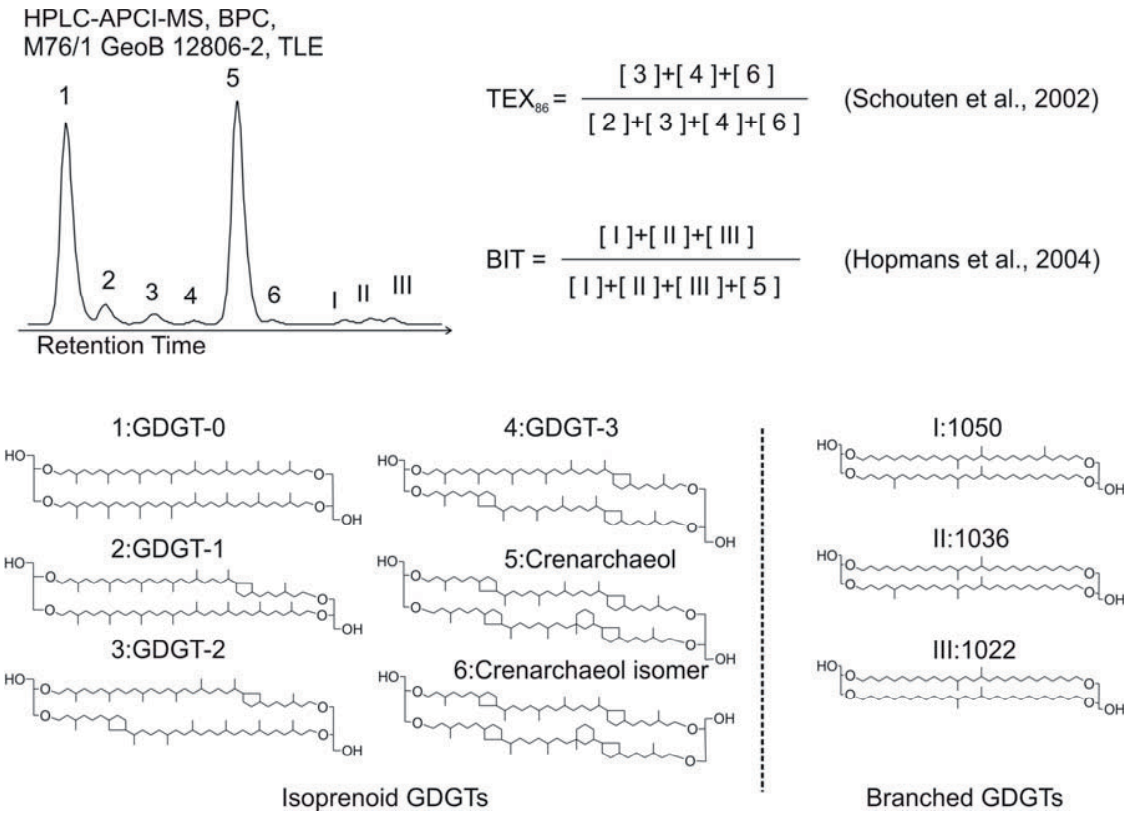

Figure 1-3. BPC (basic peak chromatogram) of the TLE of M76/1 GeoB12806-2 showing the distribution of isoprenoid and branched GDGTs. Molecular structures and the equations of TEX_{86} and BIT were also given above.

While branched GDGTs seem to be derived mainly from terrestrial sources (Hopmans et al., 2004; Weijers et al., 2006), isoprenoid GDGTs are known to have multiple sources. They are: input from planktonic archaea in water column, input from benthic archaea in sediment, and terrestrial input (Fig. 1-4). Planktonic archaea in the photic zone are believed to be the main source of sedimentary isoprenoid GDGTs (Wuchter et al., 2005; Menzel et al., 2006). Contribution from benthic archaea in sediment might be small but not necessarily negligible, especially for sediments that are up to tens of millions of years (e.g., Kuypers et al., 2001) and may inherit GDGTs from many generations of benthic archaea. In Chapter 3, we compare the distribution of intact and core isoprenoid GDGTs in marine sediments and discuss the different inputs of archaeal lipids from water column and sediment.

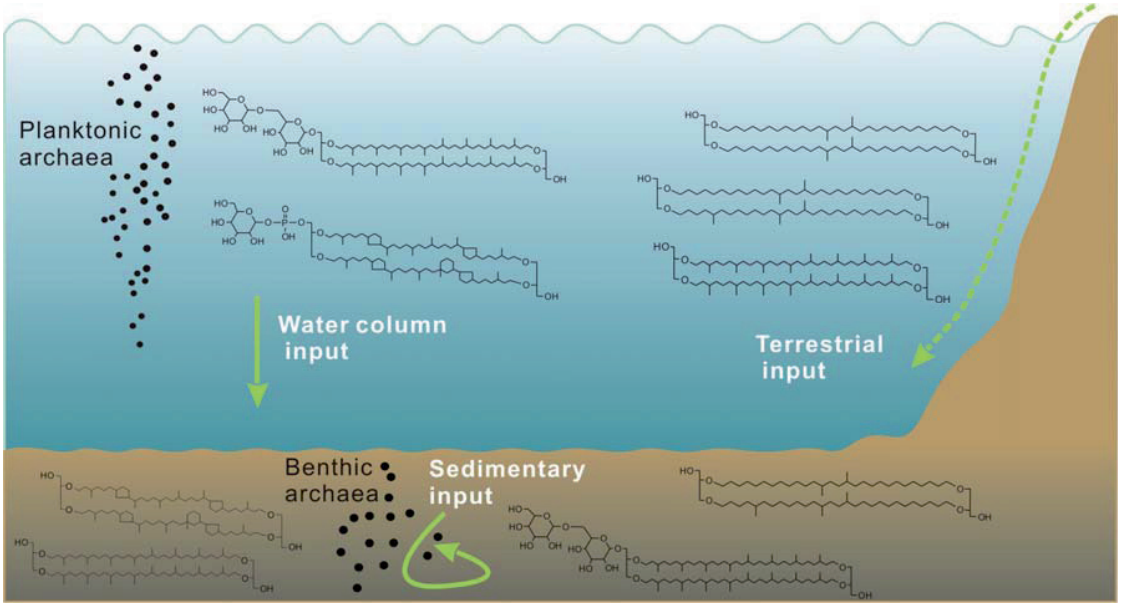

Figure 1-4. Major sources contributing to the glycerol tetraethers in marine sediments, namely, water column input from planktonic archaea, terrestrial input from sources on land and sedimentary input from benthic archaea.

Based on the extent of structural alteration, glycerol ether lipids in marine sediments can either exist in the form of IPLs or be modified into core lipids or degraded fossil derivatives. As an example, the three forms of selected archaeal lipids were shown

in Fig. 1-5. The hydrocarbon skeleton provides the basis for establishing parent-daughter relationships. In the case of isoprenoid GDGTs, their IPL and core lipid forms have been identified in marine sediments (Sturt et al., 2004; Rossel et al., 2008; Lipp and Hinrichs, 2009; Liu et al., 2011). Hence, the biphytane derivatives in the 2.7 Ga old metasedimentary rocks were believed to be the molecular evidence of Late Archean archaea (Ventura, et al., 2007). Biphytanediols (Schouten et al., 1998; Saito and Suzuki, 2010) and biphytanic diacids (Meunier-Christmann, 1988; Birgel et al., 2008) found in both recent sediments and rock samples were also reasonably considered as archaeal biomarkers. However, it is not clear whether these biphytane derivatives are products of GDGT degradation or biosynthesis. In this thesis, we described another series of lipids (GDD in Chapter 4), which is likely to be either degradation products of isoprenoid GDGTs or intermediates of biosynthesis.

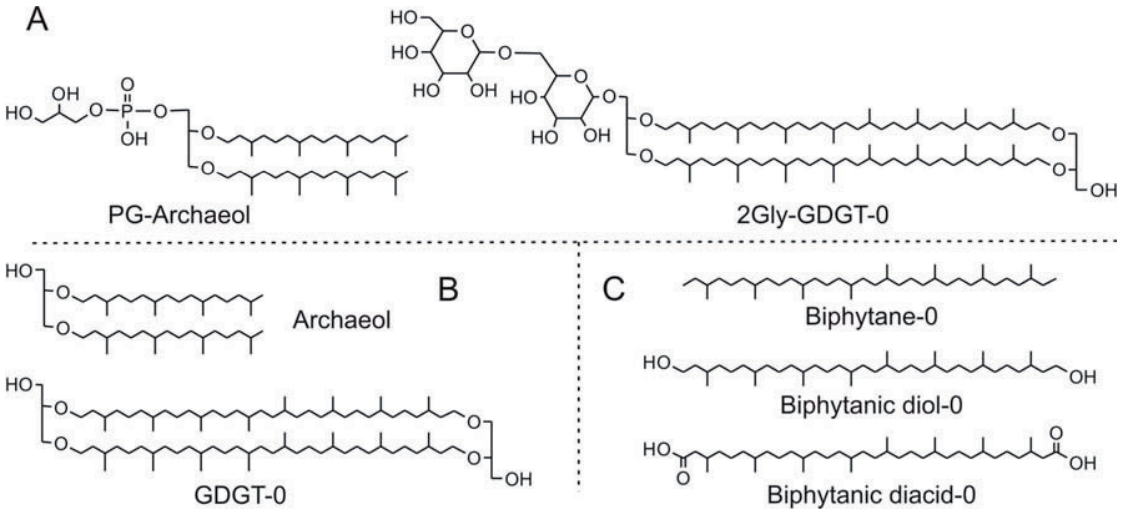

Figure 1-5. Three categories of archaeal lipids occurred in sediments, A) IPLs: PG-Archaeol (Phosphatidylglycerol archaeol), 2Gly-GDGT-0 (2glycosyl GDGT with 0 ring); B) Archaeol, GDGT-0; C) biphytane-0, biphytanic diol-0, biphytanic diacid-0 (number refer to number of rings).

Likewise, branched GDGTs, when originated from membrane lipids, should also exist in different forms ranging from IPLs to degraded fossil derivatives. While core lipids have been identified in both soils and marine sediment (e.g. Sinninghe Damsté et al., 2000; Schouten et al., 2000) intact forms of branched GDGT have been never reported. Chapter 2 of this thesis presents the first observation of intact polar branched GDGT found in soil samples. This newly found IPL provides an opportunity for tracing the source organism of the so-called 'orphan lipids' that exist in various sediments.

5. Methods

The original methods for detecting and identifying these high molecular weight glycerol ether lipids mainly relied on either thin layer chromatography (TLC) and the subsequent infrared or nuclear magnetic resonance spectrometric analysis of the isolated compounds (e.g. Langworthy, 1977), or chemical cleavage followed by gas chromatography-mass spectrometric analysis of the released derivatives (e.g. DeRosa et al., 1977). It is not until the beginning of this century that the liquid chromatography-mass spectrometric (LC-MS) methods were established for direct analysis of these big involatible compounds (Hopmans et al., 2000; Sturt et al., 2004). According to the published analytical protocols, IPLs and core lipids can be analyzed with different types of ionization, electrospray ionization (ESI) for IPLs and atmospheric pressure chemical ionization (APCI) for core lipids. In this research, the general working procedures, including sample preparation and analysis, were shown in Fig. 1-6.

To avoid degradation of lipids, especially IPLs, sediments were immediately frozen after recovery and stored at either -20 or -80°C until extraction and analysis. Depending on the expected lipid concentration and TOC content of each sample, 5-100 g of wet and frozen sediments were first freeze-dried or directly extracted in ultrasonic bath using a modified Bligh and Dyer protocol as described by Sturt et al. (2004). In order to reduce the ions suppression of complex compounds, TLE (total lipids extraction) was separated into apolar and polar fractions with preparative HPLC according to the published method of Biddle et al. (2006) or with silica gel column chromatography (modified from Oba et al., 2006). The apolar fraction containing core lipids was analyzed

with HPLC-APCI-MS and polar fraction containing IPLs with HPLC-ESI-MS. Further determination of the core lipid composition of IPLs was achieved by acid hydrolysis on the polar fraction to release the core lipids from IPLs. Detectors were chosen according to the analytical purpose. Selected ion monitoring (SIM) mode of single quadrupole mass spectrometer can provide relatively easier quantification with higher sensitivity, while ion trap and accurate-mass quadrupole time-of-flight (QTOF) mass spectrometers are ideal for structural identification by providing MS^2 spectrum or other aspects of molecular information.

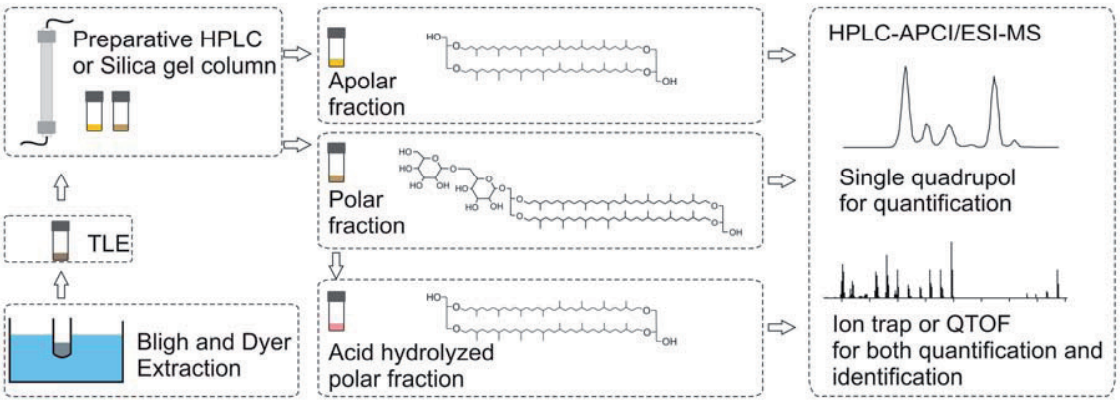

Figure 1-6. A flow chart showing the procedure of sample preparation and analysis.

6. Objectives and thesis outline

The main objective of this thesis is to get a better understanding of the sources and distribution of tetraether lipids in marine subsurface sediments. To obtain a general distribution pattern of lipids we collected a set of globally distributed marine sediments (Fig. 1-7) representing various deposition environments and geological time scales. The only exception is the peat bog samples collected from Bullenmoor in Northern Germany for the work presented in Chapter 2. These samples contained high contents of branched

GDGTs and stood for better opportunity to detect the IPLs of commonly occurred branched GDGTs in soils and marine sediment.

The identification of IPLs of branched GDGTs in Chapter 2 provided not only a closer linkage to their source organism but also good target compounds for tracing their metabolism.

In Chapter 3, the goal is to demonstrate the impact of in situ produced GDGTs by benthic archaea on the total archaeal lipids composition in sediment. We used the modified method of silica gel column chromatography to separate the GDGT core lipids from intact polar GDGTs in sediments and then compared the distribution of TEX_{86} related GDGTs in these two different fractions. A general pattern of lipids distribution in two pools was observed in the global sample set. Possible mechanisms, such as recycling of fossil lipids by benthic archaea (cf. Takano et al., 2010), were discussed.

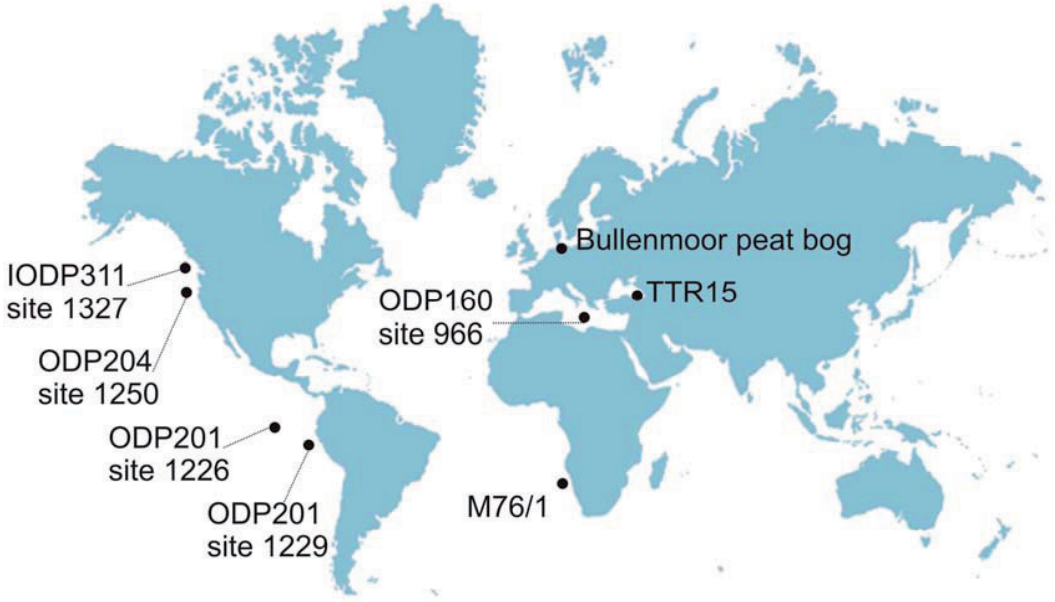

Figure 1-7. Map of major sampling sites.

In Chapter 4, a series of novel archaeal lipids, named as glycerol dibiphytanol diethers (GDDs), were first identified. GDDs with different number of rings coexist with

the corresponding GDGTs in all analyzed sediments. The origin of these lipids was discussed together with the biosynthesis and degradation mechanism of GDGTs.

In Chapter 5, several types of hydroxylated isoprenoid compounds with the hydrocarbon skeleton of biphytane were identified by means of NMR (nuclear magnetic resonance) and/or mass spectrometry. These include hydroxylated GDGTs, hydroxylated GDDs, and glycosidic hydroxyl GDGTs.

In Chapter 6, we summarized the distribution of all detected glycerol ether lipids in analyzed marine sediments. Several groups of novel compounds were also tentatively identified based on mass spectra interpretation. General fragmentation patterns of compounds that make them recognizable were also shown in MS^2 spectra. The high diversity of ubiquitously distributed ether lipids in marine sediments implied potential development of novel molecular proxies in our future works.

7. Contributions to publications

This thesis includes two published papers (Chapter 2 and 3) and three complete versions of manuscripts for submission (Chapter 4, 5 and 6).

Chapter 2-published paper

Identification of polar lipid precursors of the ubiquitous branched GDGT orphan lipids in a peat bog in Northern Germany

Xiao-Lei Liu, Arne Leider, Aimee Gillespie, Jens Gröger, Gerard J.M. Versteegh, Kai-Uwe Hinrichs

Published in Organic Geochemistry, 41, 653–660. (2010)

Xiao-Lei Liu identified lipids and provided data analysis. Arne Leider prepared samples and provided data analysis. Aimee Gillespie extracted sediments and provided part of data analysis. Jens Gröger helped with field works and sample collection. Xiao-Lei Liu, Arne Leider, Gerard J.M. Versteegh and Kai-Uwe Hinrichs wrote the paper jointly with editorial input from all coauthors.

Chapter 3-published paper

Distribution of intact and core GDGTs in marine sediments

Xiao-Lei Liu, Julius S. Lipp, Kai-Uwe Hinrichs

Published in Organic Geochemistry, doi:10.1016/j.orggeochem.2011.02.003 (2011)

Xiao-Lei Liu performed lab work and data analysis. Julius S. Lipp provided part of the samples and helped with instrument settings. Xiao-Lei Liu, Julius Sebastian Lipp and Kai-Uwe Hinrichs wrote the paper jointly with editorial input from all coauthors.

Chapter 4-full manuscript

Isoprenoid glycerol dibiphytanol diethers: a series of novel archaeal lipids in marine sediments

Xiao-Lei Liu, Julius S. Lipp, Schröder Jan, Roger E. Summons, Kai-Uwe Hinrichs

Xiao-Lei Liu performed lab work and identified lipids. Julius Sebastian Lipp helped with instrument settings. Schröder Jan extracted part of sediments. Roger E. Summons and Kai-Uwe Hinrichs provided lab facilities and supervision of works. Xiao-Lei Liu and Kai-Uwe Hinrichs wrote the paper jointly with editorial input from all coauthors.

Chapter 5-full manuscript

Novel mono- and dihydroxy-glycerol dibiphytanyl glycerol tetraethers and their intact polar analogues in marine sediments

Xiao-Lei Liu, Julius S. Lipp, Jeffrey H. Simpson, Yu-shih Lin, Roger E. Summons, Kai-Uwe Hinrichs

Xiao-Lei Liu performed lab work and identified lipids. Julius Sebastian Lipp and Yu-shih Lin helped with instrument settings and method development. Jeffrey H. Simpson provided NMR analysis. Roger E. Summons helped with lipids identification and provided lab facilities and supervision of work. Xiao-Lei Liu wrote the paper with editorial input from all coauthors.

Chapter 6-full manuscript

Distribution of glycerol ether lipids in marine sediment: structural diversity and biogeological implications

Xiao-Lei Liu, Roger E. Summons, Kai-Uwe Hinrichs

Xiao-Lei Liu performed lab work and data analysis. Roger E. Summons and Kai-Uwe Hinrichs provided lab facilities and supervision of work. Xiao-Lei Liu wrote the paper with editorial input from all coauthors.

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Chapter 2

Identification of polar lipid precursors of the ubiquitous branched GDGT orphan lipids in a peat bog in Northern Germany

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ABSTRACT

Two types of intact branched glycerol dialkyl glycerol tetraethers (GDGTs) were detected in peat bog samples from Bullenmoor, Northern Germany. Glucuronosyl and glucosyl branched GDGTs comprise on average ca. 4% of the microbial intact polar lipids in the anoxic, acidic peat layer ca. 20 cm below the surface of the bog, suggesting an important ecological role for the source microorganisms. No corresponding phospholipids were detected. Notably, glycosidic branched GDGTs are 5 to 10 times less abundant than their intact isoprenoidal counterparts derived from Archaea, while branched GDGT core lipids exceed their isoprenoid analogues by about an order of magnitude. These contrasting relationships may reflect lower standing stocks of the biomass of producers of branched GDGTs, combined with higher population growth rates relative to soil Archaea. Search strategies for the microbial producers of these conspicuous orphan lipids will benefit from the discovery of their intact polar precursors.

1. Introduction

Branched glycerol dialkyl glycerol tetraethers (GDGTs) with octacosane alkyl units with either 13,16-dimethyl- or 5,13,16-trimethyl substitution were identified almost 10 years ago (Sinninghe Damsté et al., 2000; Schouten et al., 2000). They are particularly abundant and widespread in soil (Weijers et al., 2007a), but their biological sources have not been identified. Unlike isoprenoidal GDGTs, which possess a 2,3-di-O-alkyl-sn-glycerol configuration that is considered to be diagnostic of Archaea (Kates, 1978), branched GDGTs possess a 1,2-di-O-alkyl-sn-glycerol configuration and are therefore considered to be of bacterial rather than archaeal origin (Weijers et al., 2006a). Branched GDGTs core lipids (CLs) are abundant in the anoxic horizons of acidic peat bog and soil samples (Pancost and Sinninghe Damsté, 2003; Hopmans et al., 2004; Weijers et al., 2006b), where their concentration tends to strongly exceed that of their isoprenoid counterparts (Weijers et al., 2009). This apparent dominance of branched GDGT CLs led to the suggestion that members of the acidobacteria, which substantially contribute to the pool of 16S rRNA gene sequences, are the producers of branched GDGTs (Weijers et al., 2009). The latter are not only highly unusual natural products but also serve as proxies for the reconstruction of various palaeoenvironmental properties such as soil input to marine sediments (Hopmans et al., 2004), mean annual air temperature and soil pH (Weijers et al., 2007b). Even though the branched GDGT CLs have been observed widely in the environment, their polar precursors, i.e., the intact building blocks of the membranes of their producer, have been elusive.

Isoprenoidal GDGT CLs are relatively stable and are able to survive in sediments over tens of millions of years (e.g., Kuypers et al., 2001). In contrast, intact polar lipids (IPLs) degrade rapidly upon death of the source organism (White et al., 1979; Harvey et al., 1986; Lipp and Hinrichs, 2009). The IPL precursors of branched GDGTs therefore provide a potentially more informative link to the producing organisms because the polar head groups may provide additional taxonomic information and the environmental distribution should reflect the ecology of the producing organisms rather than accumulation over time. We focussed our analysis on the zone of permanent water saturation (catotelm) in the Bullenmoor peat bog, Northern Germany. The catotelm has

been demonstrated to harbour abundant branched GDGT CLs (Weijers et al., 2006a; 2009).

2. Material and methods

2.1. Sample collection and preparation

The Bullenmoor is a small ombrotrophic peat bog in the Stader Geest in Lower Saxony, Northern Germany (53°14'40''N, 9°12'19''E). The terrain consists of fine sands overlain by a 1-2 m layer of bog that increases to 3 m at the nearby lake (Schneekloth and Tüxen, 1978). The vegetation consists of mixed forest with Betula and Pinus, as well as vegetation cover typical for ombrotrophic peat bogs, including Sphagnum, Eriophorum, Erica and Polytrichum. To the west of the lake, the vegetation is dominated by a quaking bog with Sphagnum, Vaccinium oxycoccus, Eriophorum vaginatum, Drosera rotundofolia, Erica tetralix and Andromeda polifolia (Schneekloth and Tüxen, 1978).

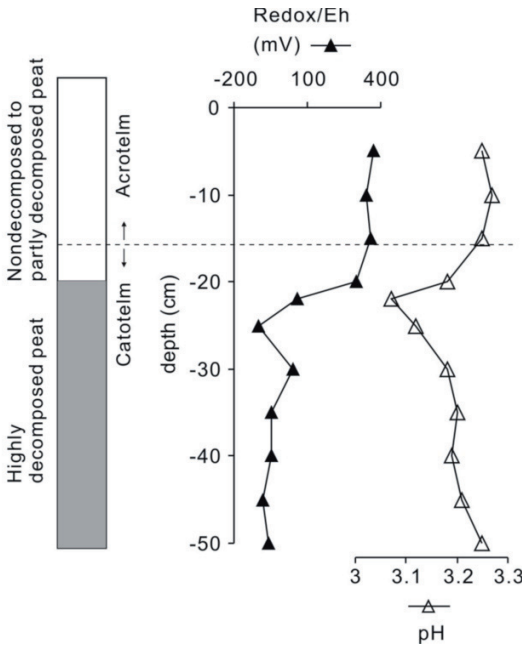

Figure 2-1. Depth profiles of redox potential and pH combined with the schematic description of the peat profile, consisting of non-decomposed to partly decomposed peat (unfilled) above 20 cm below surface, followed by the highly decomposed peat (gray). The acrotelm is the zone above the permanent water table, around 16 cm below surface; the catotelm below is continuously saturated with water.

The average depth of the water table is 16 cm below the surface. The top 20 cm of the peat core (see below) consisted of partly decomposed and non-compacted peat moss and plant roots, declared as the acrotelm layer (Fig. 2-1). Samples were collected from the underlying 30 cm from within the catotelm layer, consisting of highly decomposed peat. A 50 cm long core was obtained using an 'Eijkelkamp peat sampler'. Values of pH and Eh were measured in the field with Hamilton double pore probes for Eh and GAT ionode IJ 44 double junction for pH. Calibration of the pH probe was done with pH 1, 4 and 7 buffers. Eh values were corrected using the standard potential of the reference system (Ag/AgCl in 3 mol/l KCl solution), respectively. Then, the condensed and mostly decomposed part, 20 to 42 cm below the surface, of another fresh sediment core taken from the same site was wrapped using combusted Al foil, carried back in a cool box to the laboratory and stored at -20 °C. Sub-samples were collected from the frozen core in 2 cm intervals, the freeze-dried sediments being ground and weighed for extraction.

2.2. Analysis and purification of intact polar lipids

Total lipid extracts (TLEs) were obtained using a modified Bligh and Dyer protocol as described by Sturt et al. (2004). An aliquot of the TLE of each sample was dissolved in a mixture of MeOH and CH_2Cl_2 (1:5, v/v) for IPL analysis using a ThermoFinnigan Surveyor HPLC system coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with electrospray ionization source (ESI) under conditions described previously (Sturt et al., 2004). An aliquot (10 µl) of TLE solution was injected to a LiChrosphere Diol-100 column (150 x 2.1 mm, 5 µm, Alltech, Germany) equipped with a guard column of the same packing material.

Due to the lack of available reference standards for each class of IPLs, including the novel intact branched GDGTs, the determination of relative concentrations remains semi-quantitative. Several authentic standards, including archaeal and bacterial phospho- and glycolipids, are routinely analyzed to determine response factors and potential variations over time. During the time frame of this study, the relative response factors of authentic standards relative to the internal standard PAF (1-O-hexadecyl-2-acetoyl-sn-glycero-3-phosphocholine, platelet activation factor) ranged from 0.8 to 1.4 (Lipp et al., unpublished data). IPL standards added into sample are 1,2-dipalmitoyl-glycero-3-

phosphocholine (1,2-diC16:0 PC) and PAF, but quantification of IPLs is only based on PAF. GDGT IPLs with different polar head groups were identified according to characteristic fragments in MS^n spectra.

For identification of glycosidic polar head groups, we applied preparative HPLC in order to purify sufficient quantities of the two main analytes of interest. Fractions were obtained using a preparative LiChrosphere Diol column (250 x 10 mm, 5 μm, Alltech, Germany) with a fraction collector following established parameters (Biddle et al., 2006). The purified fractions were subsequently checked by LC-MS; no potentially interfering compounds were detected.

2.3. Analysis of GDGT core lipids

TLEs of samples with an internal standard (C_{46} GDGT, see Fig. 2-5; labelled as IS) were dissolved in *n*-hexane/isopropanol [99:1 v/v] and analyzed following a HPLC procedure modified after Hopmans et al. (2000). Both isoprenoid and branched GDGTs were separated using an Econosphere NH_2 column (250 x 4.6 mm, Alltech, Germany) operated at 30 °C in a ThermoFinnigan Surveyor HPLC system. Using a flow rate of 1 ml/min, the gradient for the mobile phase was from 99:1 *n*-hexane/isopropanol, (v/v) to 98:2 in 15 min, then held isocratically for 3 min, increased to 90:10 *n*-hexane/isopropanol, (v/v) at 20 min, held isocratically for 4 min, followed by equilibrating with 99:1 *n*-hexane/isopropanol (v/v) for 5 min before the next injection. For analysis, the HPLC-MS system was equipped with an atmospheric pressure chemical ionization (APCI) interface. APCI settings were identical to those described by Lipp and Hinrichs (2009).

2.4. Analysis of glycosidic head groups

We used a modified method of acid hydrolysis to cleave the glycosidic headgroups, followed by derivatization using aldononitrile acetate (ANA) for gas chromatographic (GC) analysis of sugar derivatives (Lin, 2009). After purification and drying, branched GDGT IPLs were hydrolyzed with 50% trifluoroacetic acid (TFA) at 70 °C for 8 h and then neutralized by evaporation. For identifying the glycosidic head groups, both hydrolyzed samples and sugar standards were treated separately with

derivatization reagents (hydroxylamine hydrochloride and 4-(dimethylamino)pyridine) at 75 °C for 30 min to convert the monosaccharides to aldononitrile derivatives; the OH groups of the aldononitrile derivatives were then acetylated using acetic anhydride at 75 °C for 20 min. For glucuronic acid, we used bis(trimethylsilyl)-trifluoroacetamide (BSTFA) instead of acetic anhydride for the final derivatization step. After reaction, the derivatives were extracted with *n*-hexane and ethyl acetate, 1:1 (v/v), for further analysis. The resulting derivatives were analyzed with a ThermoFinnigan Trace GC 2000 coupled to a ThermoFinnigan DSQ mass spectrometer. The injector temperature was 310 °C. Separation was achieved using a Rxi-5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Restek GmbH, Germany) programmed from 70 to 180°C at 4 °C min^{-1} and at 10 °C min^{-1} to 280 °C (held 2.5 min).

3. Results

3.1. Identification of glucosyl- and glucuronosyl-branched GDGTs

Based on mass spectral properties during IPL analysis using ESI-MS^n, we found two distinct series of branched GDGT IPLs differing in mass by 14 Da (blue filled circles in Fig. 2-2). These two groups were present in all analyzed samples. The molecular ions obtained from MS^1 experiments of the less polar, first cluster (Fig. 2-2b) showed dominant *m/z* values of 1229, 1215 and 1201, the corresponding fragment ions from MS^2 experiments involving loss of the polar head group were at *m/z* 1050, 1036 and 1022, respectively, i.e., the characteristic ions of branched GDGT CLs (Schouten et al., 2000). Furthermore, the ions obtained during MS^3 experiments are consistent with the structures of branched GDGTs. For example, the dominant MS^3 ions of intact branched GDGT-1022 are 948 and 511, which represent the fragments of core lipid after loss of a glycerol moiety, $C_3H_6O_2$) and after loss of glycerol linked to a 13,16-dimethyl-octacosane moiety and one oxygen, $C_{32}H_{62}O_4$), respectively. According to the neutral loss of a fragment of 179 Da, i.e., corresponding to the ammonium adduct of the hexose moiety, the first cluster of branched GDGT IPLs was assigned as consisting of monoglycosidic (Gly-) branched GDGTs (Fig. 2-3a). The second cluster (Fig. 2-2c) showed a molecular mass of 14 Da higher than the Gly-branched GDGT; dominant ions in MS^1 experiments were at *m/z* 1243, 1229 and 1215, but the fragment ions resulting from MS^2 experiments were at

m/z 1050, 1036 and 1022, identical to those obtained from the Gly-branched GDGT. Accordingly, the mass of the polar headgroup of compounds from the second cluster is 14 Da higher than the glycosidic polar head group. Based on the neutral loss of a fragment of 193 Da, corresponding to the ammonium adduct of the headgroup moiety, the second cluster of branched GDGT-IPLs was assigned as glucuronosyl-branched GDGTs (GlcA-branched GDGTs, Fig. 2-3b).

GC-MS analysis of derivatized sugars released from purified fractions of Gly-branched GDGTs and a mixture of hexose standards led to the identification of the main glycosidic head group in the first suite of branched GDGT-IPLs as glucose (Fig. 2-4a, b). The assignment is based on comparison with standards and co-injection with glucose (Fig. 2-4c). Authentic glucuronic acid (Fig. 2-4e) and the polar head moiety (Fig. 2-4d) released from the second cluster of branched GDGT IPLs resulted in identical retention times and mass spectra. The additional compounds detected during analysis of the sample (Fig. 2-4d; unlabelled peaks) were neither uronic acids nor compounds matching the calculated mass of the released head group, but undefined byproducts of the reaction or contaminants. Therefore, the compounds forming the second cluster of branched GDGT-IPLs were confirmed as GlcA branched GDGTs.

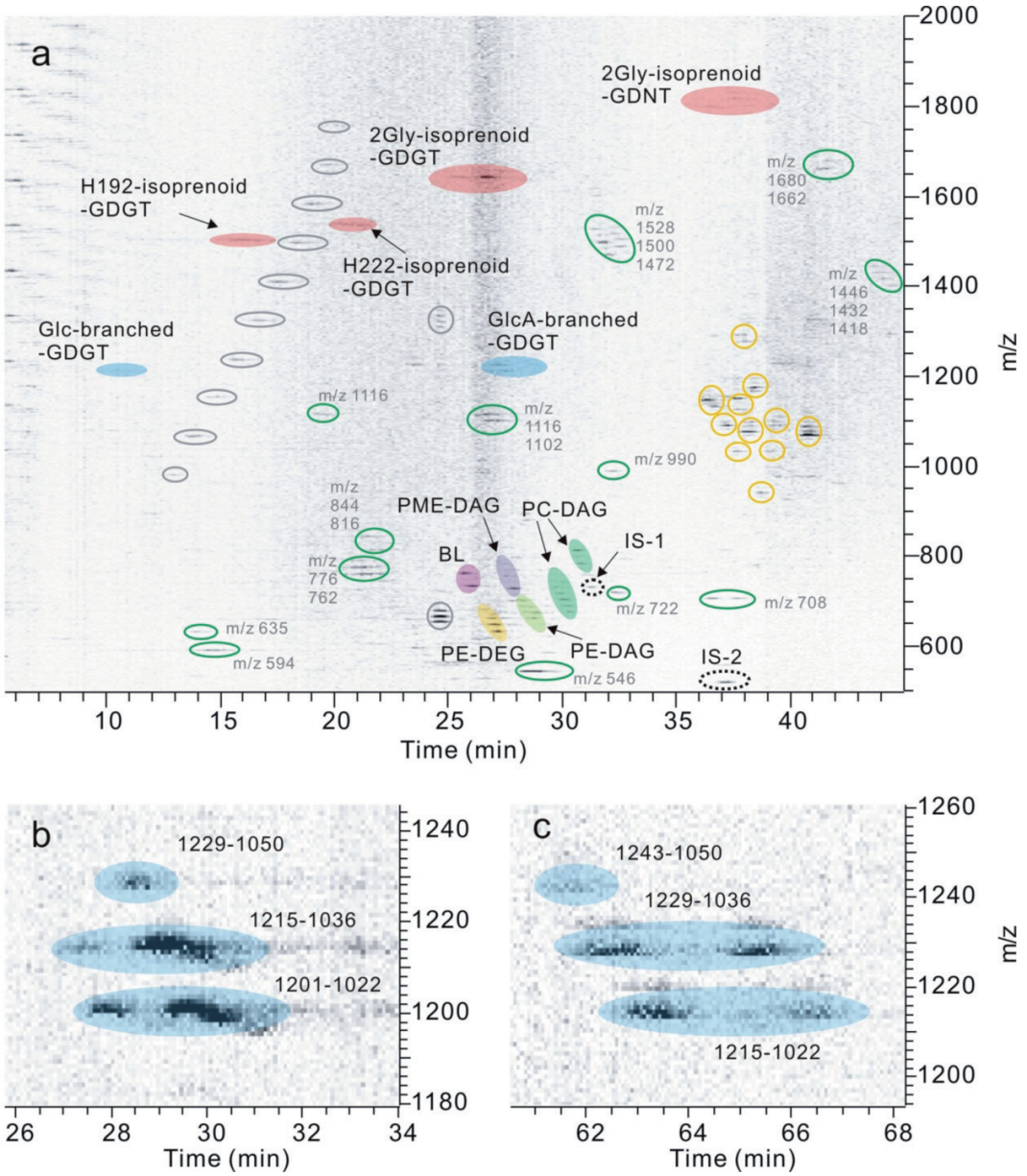

Figure 2-2. HPLC-ESI-MS density map constructed from base peak chromatograms of intact polar lipids from a representative peat sample from 40-42 cm below surface at Bullenmoor peat bog, (a) density map constructed showing all the compounds detected in the peat sample and color coded, color filled circles are identified lipids with name labeled, open green circles are unknown but presumed to be lipids (see text) with molecular mass labeled, open yellow circles are compounds that were not recognized as lipids, open grey circles are contaminants and the open black circles with dashed line are internal standards; IS-1 = PC, IS-2= PAF; detailed view of (b) Glc-branched GDGTs, and (c) GlcA-branched GDGTs prepared by preparative HPLC and then analyzed with an extended gradient program, numbers indicating mass-to-charge ratio (m/z) of molecular ion and corresponding core lipids.

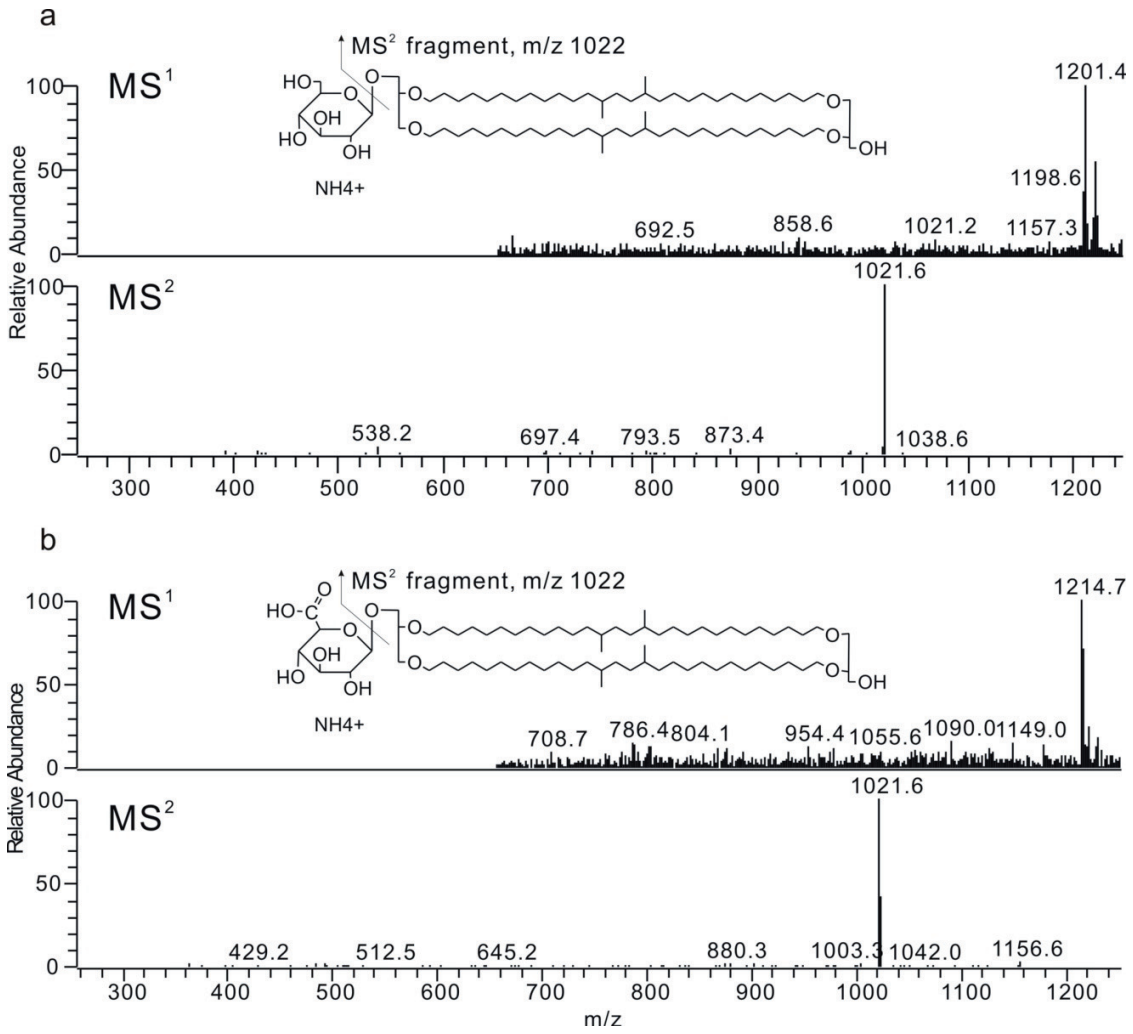

Figure 2-3. HPLC-ESI-MS mass spectrum of (a) Glc-branched GDGT corresponding to the core lipid of 1022 Da, (MS^1) molecular ion with ammonium adduct, (MS^2) fragment ion of tetraether core, and (b) GlcA-branched GDGT-1022, (MS^1) ammonium adduct of molecular ion, (MS^2) fragment ion corresponding to tetraether core.

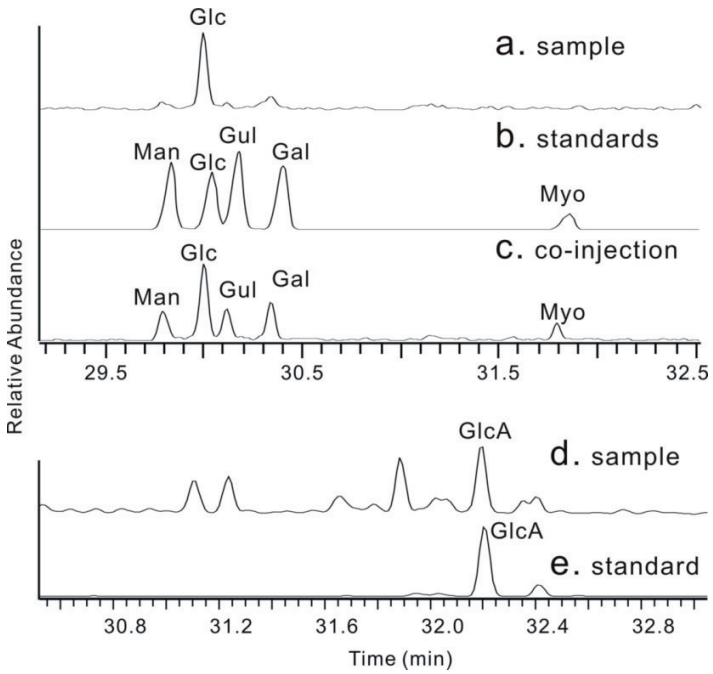

Figure 2-4. GC-MS total ion current chromatograms of (a) sugar headgroup from purified branched GDGT-IPLs from cluster 1 (Fig. 2-2b) in Bullenmoor peat bog sample, (b) a standard mixture containing galactose, glucose, gulose, mannose and myo-inositol, (c) co-injection of peat bog sample and standard mixture, (d) uronic acid headgroup (labeled peak) from branched GDGT-IPL from cluster 2 (Fig. 2-2c), (e) glucuronic acid standard. Key to abbreviations: derivatives of (Man) mannose, (Glc) glucose, (Gul) gulose, (Gal) galactose, (Myo) myo-inositol, (GlcA) glucuronic acid.

3.2. Distribution of archaeal and bacterial lipids

The Glc- and GlcA-branched GDGTs in the peat profile were accompanied by other IPLs whose distribution and concentration profiles provide complementary information on microbial community members and their potential relationships with branched GDGT producers. Four types of intact isoprenoid GDGTs of archaeal origin were detected in all samples (red filled circles in Fig. 2-2a). In the positive ionization mode, the first two (H192 isoprenoidal GDGT and H222 isoprenoidal GDGT) with dominant ions at *m/z* 1504 and 1538 are isoprenoid GDG -IPLs with unknown polar head groups attached to the isoprenoid GDGT CLs of MW 1294 and 1298. Analysis in the negative ionization mode supported the results obtained in the positive mode. Therefore, the calculated mass of these two unknown polar head groups are 192 and 222 Da. The third cluster of isoprenoid GDGT IPLs consisted of diglycosidic (2Gly-) isoprenoid GDGTs with the dominant ion at *m/z* 1644. The fourth cluster, with dominant molecular ion at *m/z* 1799 and corresponding MS^2 fragment at *m/z* 1458 was tentatively assigned to be isoprenoid 2Gly-Glyceroldialkynonitoltetraether (GDNT).

Additionally, we identified and quantified the following IPLs of presumed bacterial origin: betaine lipids (BLs, *m/z* 764 and 736, pink filled circles in Fig. 2-2a; cf. Ertefai et al., 2008; Schubotz et al., 2009), phosphatidylethanolamine dietherglycerol (PE DEG, *m/z* 650 and 637, yellow filled circles in Fig. 2-2a), phosphatidylethanolamine diesterglycerol (PE DAG, *m/z* 706, 692, 678 and 664, laurel-green filled circles in Fig. 2-2a), phosphatidyl-(N)-methylethanolamine diesterglycerol (PME DAG, *m/z* 786, 758 and 730, purple filled circles in Fig. 2-2a) and phosphatidylcholine diesterglycerol (PC DAG, *m/z* 678, 692, 706, 720, 734, 760, 774, 786, 800 and 814, green filled circles in Fig. 2-2a). In addition to these lipids, numerous unknown lipids or contaminants remain to be identified (open circles in Fig. 2-2a). Here, we only quantified the major groups of unknowns, i.e., five clusters of compounds (*m/z* 762, 776; *m/z* 1102, 1116; *m/z* 1418, 1432, 1446; *m/z* 1472, 1500, 1528; *m/z* 1662, 1680) are tentatively assigned as polar lipids on the basis of mass spectral properties consistent with variations in acyl and/or alkyl chains by way of methylene units and hydroxyl groups.

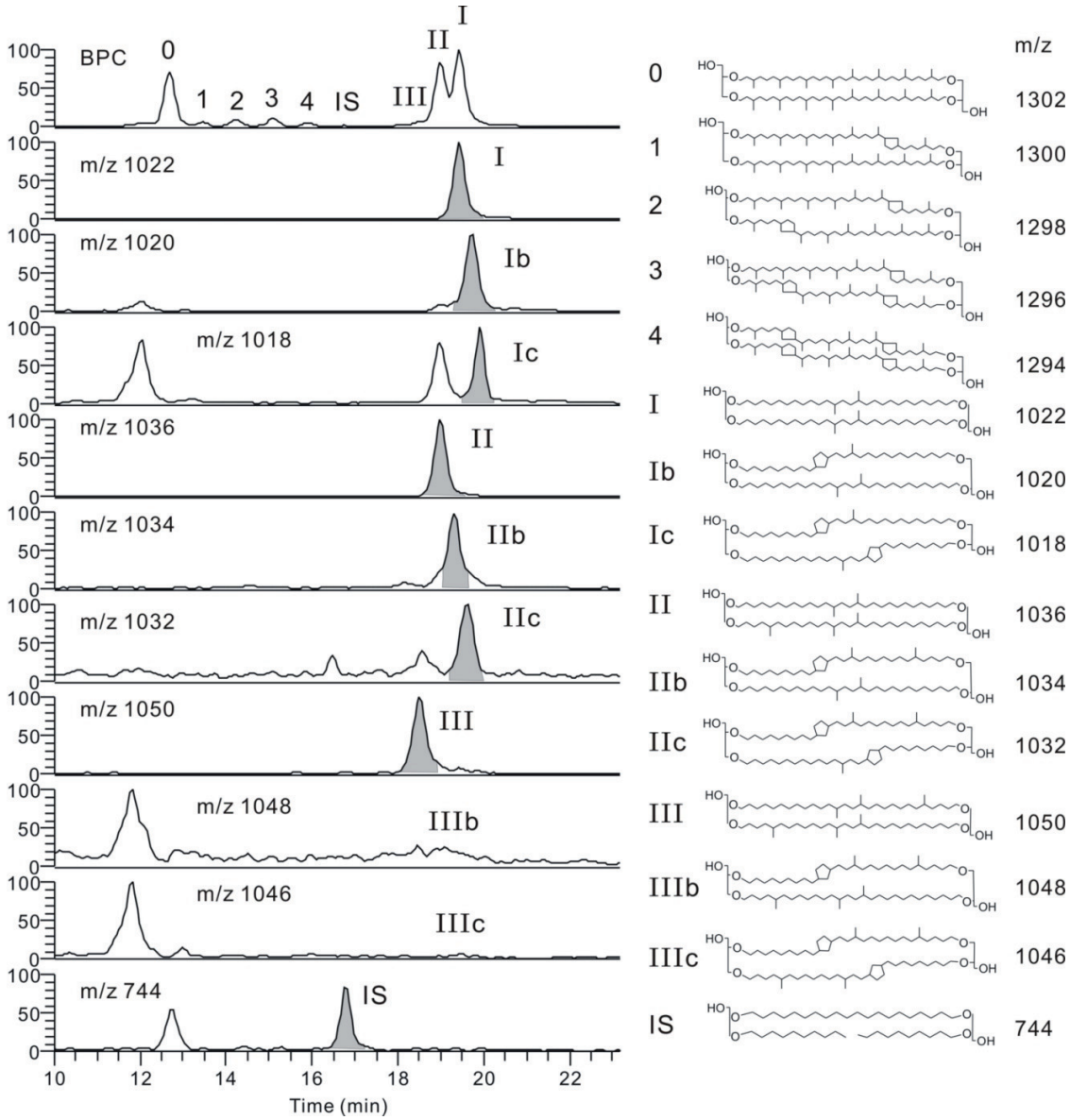

Figure 2-5. HPLC-APCI-MS data of sample at 28-30 cm below surface showing the base peak chromatogram and mass chromatograms. 0 to 4 represent the isoprenoidal GDGTs with 0 to 4 rings. IS is the internal standard, C_{46} GDGT. I, Ib, Ic; II, IIb, IIc and III, IIIb, IIIc are branched GDGTs with different numbers of methyl groups and rings. The concentration of IIIb and IIIc were too low for detection in all analyzed samples. The right panel shows schematic structures of GDGTs.

3.3. Depth profile of intact polar lipids and core lipids

Both Glc-branched GDGTs and GlcA-branched GDGTs are abundant from 22 to 34 cm below surface and their depth profiles resemble those of archaeal IPLs and the putative bacterial ether lipid IPL, PE DEG, with the exception of 2Gly-isoprenoid GDNT that increased with depth (Fig. 2-6). The isoprenoid GDGT IPLs are more abundant than their branched counterparts (Fig. 2-6). Other IPLs (betaine lipids, PE-DEG, PE-DAG, PC-DAG) of presumed bacterial origin are abundant between 20 and 30 cm and decrease strongly in deeper samples. The depth profile of total IPLs, i.e., the sum of quantified archaeal, bacterial and unidentified lipids in each sample, generally decreases below 30cm, from about 70 μg/g dry peat to less than 20 μg/g dry peat. However, the relative amounts of both branched GDGT IPLs and isoprenoid GDGT IPLs increase remarkably with depth, whereas the relative amounts of other lipids tend to decrease (Fig. 2-6).

In contrast to the IPLs, the absolute concentrations of both branched and isoprenoid GDGT CLs increased with depth. Both branched GDGT CLs and isoprenoid GDGT CLs increased in the deeper samples, with the branched type being more abundant (140-370 μg/g dry peat) than its isoprenoid counterpart (2-17 μg/g dry peat).

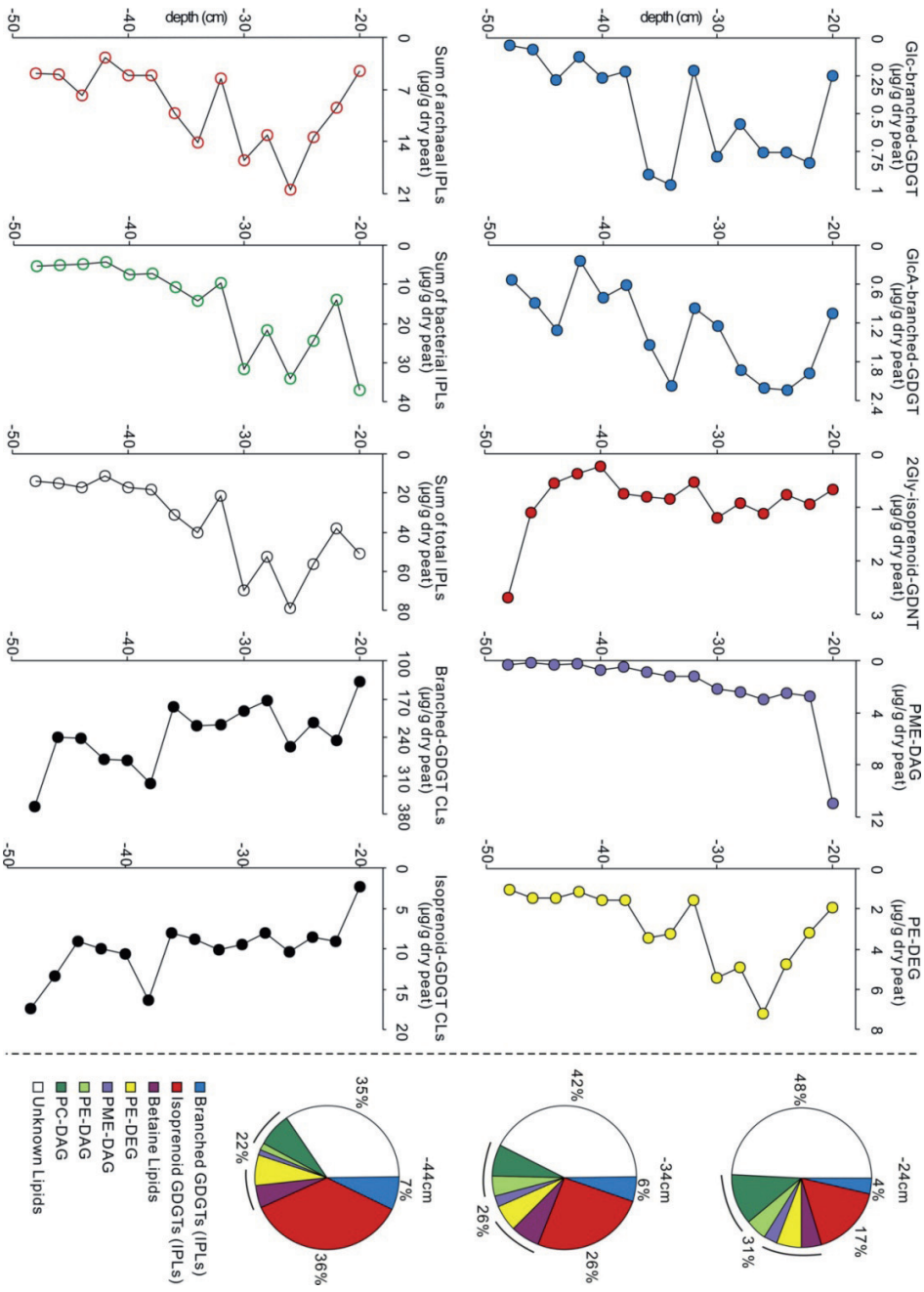

Figure 2-6. Depth profiles of concentrations of Glc-branched-GDGT, GlcA-branched-GDGT, 2Gly-isoprenoid-GDNT, PME-DAG, PE-DEG, sum of archaeal IPLs (all intact isoprenoid GDGTs), sum of bacterial IPLs (Betaine lipids, PE-DEG, PME-DAG, PE-DAG and PC-DAG), sum of total IPLs, core lipids of branched and isoprenoidal GDGTs in Bullenmoor peat bog (since PE-DAG, PC-DAG and PE-DEG have the similar depth profile, only the depth profiles of PE-DEG and sum of bacterial IPLs are shown). Right: exemplary pie charts showing the relative abundance of all IPLs at depths of -24 cm, -34 cm, and 44 cm.

3.4. Application of MBT and CBT proxies

The distributions of branched GDGT CLs (Fig. 2-5) enabled computation of proxies typically used for reconstruction of palaeoenvironmental conditions, i.e., degree of methylation (MBT) and cyclization (CBT) of branched tetraethers, respectively (Weijers et al., 2007a). The Bullenmoor peat bog samples gave MBT values of 0.49~0.58, and CBT values of 1.03~1.13 (Table 2-1). Conversion of the MBT to mean annual air temperature according to Weijers et al. (2007a) yielded estimates of 7.9-12.4 °C; the average value of 10.7 ± 1.2 °C is approximately the same as the recorded mean annual air temperature (ca. 9 °C) obtained from surrounding weather stations for the periods 1961-1990 and 2001-2009 (http://www.dwd.de/). The CBT-inferred pH at ca. 5.7-6.0 (Table 2-1) is significantly higher than the measured pH of 3.0-3.3 (Fig. 2-1).

Table 2-1. Calculated methylation index (MBT) and cyclisation ratio (CBT) of branched GDGTs and estimated mean annual air temperature (MAAT) and pH values from MBT and CBT indices according to the equations provided by Weijers et al. (2007a).

Depth (cm)	MBT	MAAT (°C)	CBT	pH
-20	0.58	12.4	1.13	5.8
-22	0.56	11.6	1.11	5.8
-24	0.53	9.6	1.18	5.7
-26	0.53	10.1	1.09	5.9
-28	0.54	10.3	1.14	5.8
-30	0.52	9.7	1.06	6.0
-32	0.55	11.3	1.09	5.9
-34	0.57	11.9	1.13	5.8
-36	0.49	7.9	1.14	5.8
-38	0.52	9.6	1.12	5.8
-40	0.54	10.8	1.10	5.9
-42	0.58	11.9	1.15	5.7
-44	0.54	9.8	1.17	5.7
-46	0.56	12.1	1.03	6.0
-48	0.56	11.4	1.10	5.9

4. Discussion

4.1. Glucosyl- and Glucuronosyl-branched GDGTs and their taxonomic significance

Glucose, as the polar head group of branched GDGTs, is taxonomically not sufficiently specific to further constrain the identity of the source organisms. Glucosyl-lipids are synthesized by members of the Archaea, Bacteria and Eukarya, and are widely distributed among the Bacteria (Hölzl and Dörmann, 2007), i.e., the prime candidates for the biosynthesis of branched GDGTs on the basis of the glycerol stereochemistry (Weijers et al., 2006a). Glucuronosyl glycerolipids are less widely distributed in the Bacteria domain; they are most common among the Alphaproteobacteria and Actinobacteria (Hölzl and Dörmann, 2007), both belonging to the most abundant bacterial phyla in peat bogs (e.g., Dedysh et al., 2006) and soils (e.g., Hugenholtz et al., 1998; Janssen et al., 2002).

4.2. Branched GDGTs in relation to other microbial lipids

The depth profiles of branched GDGT IPLs generally co-vary with those of other major microbial IPLs (Fig. 2-6). This suggests that the producing organisms in the peat profile are limited by similar factors. Striking are the opposing relationships in concentration of isoprenoid vs. branched GDGT-IPLs on one hand and the corresponding CLs on the other hand (cf. Fig. 2-6). The concentration of branched GDGT CLs is almost one order of magnitude higher than the isoprenoid GDGT CLs, while the branched GDGT IPLs are 5 to 10 times less abundant than the isoprenoid GDGT IPLs. Given the structural similarities of these two related classes of compounds, we do not anticipate that largely differing MS response factors have caused this relationship. However, we cannot completely rule out that the live cells of soil archaea and the prokaryotes producing the branched GDGTs are being extracted with different efficiencies. We note, however, that Huguet et al. (2010) arrived at similar conclusions regarding the quantitative relationships based on circumstantial evidence even though these authors utilized different extraction and analysis protocols. Huguet et al. (2008) reported the preferential preservation of branched GDGTs compared to crenarchaeol in organic rich turbidites upon long term oxygen exposure. However, this does not seem to fit with our peat samples which were taken from the permanent water saturated layer. We therefore suggest that the reverse

pattern may be caused by a higher activity and regeneration rate of microbes producing the branched GDGTs compared to the producers of isoprenoid GDGTs. Such effects of cellular regeneration rates on the lipid inventories of Archaea vs. Bacteria have been discussed for marine subsurface environments (Lipp et al., 2008) and are consistent with archaeal adaptation to minimum metabolic energy (cf. Valentine, 2007), resulting in extremely low rates of cell division. As demonstrated by the dissimilar trends for IPLs vs. their CL counterparts, the use of CLs as tracers for the biomass of their producers may result in deviating conclusions. The profiles of both isoprenoid and branched GDGT CLs show increasing concentrations with depth. When viewed in concert with profiles of their precursor GDGT IPLs, that rather tend to decrease with depth (Fig. 2-6), the pattern is suggestive of accumulation of these recalcitrant lipids in the peat over time. Hence, while CL data would imply that the branched GDGT producers must be sought among the dominant bacterial phyla (cf. Weijers et al., 2009), the IPL data suggest that the producers are an important but not dominant component of the peat microbial community. Knowledge of the IPL derivatives of branched GDGTs will enhance the chances of further constraining their biological sources, since their records can be combined with other quantitative molecular indicators of soil microbial ecology.

5. Conclusions

We have identified two prominent glycosidic IPLs that are the precursors of the ubiquitous branched GDGT “orphan lipids”. The two compounds, glucosyl- and glucuronosyl- branched GDGT, account for 4-7% of the total IPLs, including unknown lipids, and for 8-10% of all known IPLs, suggesting that their producers represent a sizeable but not dominant component of the microbial community. Combined concentration profiles of both GDGT polar and core lipids suggest that the producers have relatively high regeneration rates compared to peat archaea. Among cultured bacterial phyla that are prominent in soils, glucuronosyl lipids have been found in the Actinobacteria and the Alphaproteobacteria.

Acknowledgements

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Chapter 3

Distribution of intact and core GDGTs in marine sediments

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ABSTRACT

We conducted a survey of archaeal GDGT (glycerol dibiphytanyl glycerol tetraether) distributions in marine sediments deposited in a wide range of depositional settings. The focus was comparison of two pools presumed to have distinct geobiological significance, i.e. intact polar GDGTs (IP GDGTs) and core GDGTs (C GDGTs). The former pool has been suggested to be related to live communities of benthic archaea inhabiting marine sediments, while the latter is commonly interpreted to consist of molecular fossils from past planktonic archaeal communities that inhabited the surface ocean. Understanding the link between these two pools is important for the assessment of the validity of current molecular proxies for sedimentary archaeal biomass and past sea surface temperatures. The relative distributions of GDGTs in the two pools in a core at a methane-rich site in the Black Sea provide strong evidence for in situ production of glycosidic IP GDGTs and their subsequent degradation to corresponding C GDGTs on timescales that are short in geological terms. In addition, we monitored the relationship of the IP GDGT and C GDGT pools in a sample set from various ocean basins with subseafloor depths from a few cm to 320 m and 0 to 4 Myr in age. Notable differences between the two pools can be summarized as follows: the GDGT with acyclic biphytanes, GDGT-0, and its analogues with two and three cyclopentane moieties (GDGT-2 and -3) are generally more abundant in the pool of IP GDGTs, while crenarchaeol tends to be

more abundant in the C GDGT pool. Consequently, the ring index is generally higher for the C GDGTs while the TEX_{86}, a molecular proxy ratio not considering the two major GDGTs, tends to be higher in the IP GDGT pool. These differences in the proportion of individual GDGTs in the two pools are probably due to in situ production of IP GDGTs with distributions differing from those of C GDGTs. Despite these differences, we observed significant correlations of these two ratios between the two pools. Specifically, in both pools TEX_{86} is high in sediments from warm oceanic regimes and low in cold regimes. We discuss these relationships and suggest that recycling of core GDGTs by benthic archaea is an important mechanism linking both molecular pools.

1. Introduction

Archaeal lipids are common in sediments and soil, with glycerol dibiphytanyl glycerol tetraethers (GDGTs) being most widespread. GDGTs can be utilized as biomarkers for recent and ancient activity of Archaea. However, knowledge of the biological sources and fate of GDGTs is still in its infancy. Even though GDGT-producing Archaea inhabit the entire oceanic water column (Karner et al., 2001) and probably major portions of marine habitable sediment (Lipp et al., 2008), GDGTs in marine surface sediment are generally thought to be mainly derived from the photic zone (Wuchter et al., 2005; Menzel et al., 2006) as a result of efficient export involving algal grazing and repackaging into larger particles (Wakeham et al., 2003; Huguet et al., 2006b). Additionally, within some anoxic basins, a contribution from Archaea thriving in deeper water has been inferred (Huguet et al., 2007). Recent studies also discuss the different contributions of two major marine archaeal groups, Crenarchaeota and Euryarchaeota, which occur in the ocean at different water depths and nutrient concentrations (Turich et al., 2007; Schouten et al., 2008; Turich et al., 2008). In addition to planktonic sources, the contribution of GDGTs from benthic sedimentary archaea needs to be considered, as suggested by suspiciously ^{14}C depleted GDGTs in Santa Monica Basin (Shah et al., 2008), and the discovery of intact polar GDGTs presumed to

be produced in sediments in situ (Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009).

The deep biosphere revealed by way of deep scientific drilling into marine sediment has been estimated to constitute 10-30% of the total biomass on Earth (Parkes et al., 1994; Whitman et al., 1998) and analysis of 16S-rRNA, -rDNA, and intact membrane lipids provided evidence of active archaeal populations in deep sub-surface sediments (Biddle et al., 2006, 2008; Lipp et al., 2008). As benthic sedimentary archaea appear to produce core lipids indistinguishable from their planktonic counterparts, potential contributions from benthic archaea to the fossil record of archaeal lipids need to be constrained, since they may be relevant for applications of GDGT-based palaeoenvironmental proxies such as TEX_{86} (Schouten et al., 2002) and the BIT index (Hopmans et al., 2004). One means of constraining such impacts includes diagenetic modelling (Lipp and Hinrichs, 2009; Schouten et al., 2010) but its usefulness remains limited as long as robust information on the kinetics of the key step in the degradation of intact polar GDGT (IP GDGT) to core GDGT (C GDGT) remains unavailable. A more direct means, although at this point merely qualitative, lies in the systematic structural comparison of the distribution of IP GDGTs and C GDGTs in a set of diverse representative samples. Preliminary data generated for the Peru Margin (Lipp and Hinrichs, 2009) suggest that distributional differences between both pools can be significant.

Intact polar lipids (IPLs) of Archaea are usually composed of phospho- and/or glycolipids, with the latter being the dominant type in marine sub-surface sediments (Biddle et al., 2006; Lipp et al., 2008) and in sediments hosting methanotrophic ANME-1 archaea (Rossel et al., 2008). Phospho-IPLs have been shown to degrade rapidly upon death of the source organism (White et al., 1979; Harvey et al., 1986) while the kinetics of the degradation of the more stable glyco-IPLs remains to be constrained. Experimental data on the degradation of glycolipids and phospholipids (Harvey et al., 1986) is not directly comparable in terms of the stability of glycosidic vs. phospho head groups because the experiment monitored degradation of a glycosidic diether and phospho diacyl lipid. Nevertheless, multiple lines of circumstantial evidence (cf. discussions in Lipp et al. (2008) and Lipp and Hinrichs (2009)) suggest that glyco-

GDGTs turn over on much shorter time scales than C GDGTs and are therefore a meaningful proxy for sedimentary biomass.

We compare here the core lipid distributions of C GDGTs and IP GDGTs in a diverse range of sub-surface samples in order to further constrain the processes and organisms that contributed to the two respective pools and discuss the implications for palaeoenvironmental applications of GDGTs.

2. Material and methods

2.1. Sample collection and extraction

Sediment samples were collected from a variety of depositional environments (Table 3-1). All were immediately frozen after recovery and stored frozen at either -20 or -80°C until extraction and analysis. According to the different lipid concentration of each sample, 5-100 g of wet and frozen sediments were extracted using a modified Bligh and Dyer protocol as described by Sturt et al. (2004).

2.2. Separation of IP GDGTs and C GDGTs

IP and C GDGTs were separated using liquid chromatography with silica gel (modified from Oba et al., 2006). In brief, a glass column (1.2 cm i.d.) was packed with 1.5 g silica gel (Silica Gel 60, 60 – 200 μm; Roth, Germany) and preconditioned with EtAc; the first fraction, containing apolar neutral lipids and C GDGTs, was eluted with 8 ml EtAc and the second, containing IP GDGTs with 10 ml MeOH. The separation efficiency was evaluated by screening C GDGT fractions for IP GDGTs and IP GDGT fractions for C GDGTs; for all analyzed samples complete separation was verified. Both fractions were evaporated to dryness under N_2 before further analysis.

Table. 3-1 Samples analyzed.

Cruise	Location/Sites	Position	Water Depth (m)	Sediment Sampling Depth (m)	Total Organic Carbon (wt%)	Age (Ma)	Number of Samples
SO147	Peru Margin, station 2MC	11°35.0'S 77°33.1'W	86	0.05~0.26	2.5~4.6	<0.1	2
TTR15	Black Sea, station BS369M	41°57.5'N 41°17.6'E	850	0~0.3	1.2~1.8	<0.1	5
M76/1	Namibia Margin, site GeoB12802	25°30.0'S 13°27.0'E	790	4.3	7.2	<0.5	1
	Namibia Margin, site GeoB12806	25°0.1'S 14°23.4'E	132	0.1~2.6	7.5~8.6	<0.5	2
	Namibia Margin, site GeoB12807	25°20.7'S 13°46.5'E	300	3.2	7.4	<0.5	1
	Namibia Margin, site GeoB12815	27°14.2'S 9°60.0'E	5000	0.05	1.5	<0.5	1
Leg201	Eastern Equatorial Pacific, site 1226	3°5.7'S 90°49.1'W	3297	8~320	0.2~1.1	0.1~12	5
	Peru Margin, site 1229	10°58.6'S 77°57.5'W	152	30~190	0.4~4.6	0.1~1.8	3
Leg204	Hydrate Ridge, site 1250	44°34.1'N 125°9.0'W	796	24~130	1.2~1.3	0.5~1.4	3
Exp311	Cascadia Margin, site 1326	48°37.6'N 127°3.0'W	1828	130	0.5	~0.4	1
	Cascadia Margin, site 1327	48°41.9'N 126°51.9'W	1304	79.8~150	0.5~0.6	0.2~1	3
	Cascadia Margin, site 1328	48°40.1'N 126°51.0'W	1267	140~287	0.4~0.6	0.9~1.5	2
Leg160	Mediterranean, site 966	33°47.8'N 32°42.1'W	925	20~65	5.6~7.3	1.8~4	3

Sampling data are according to the literatures (Kudrass et al., 2000 (SO147); Akhmetzhanov et al., 2007 (TTR15); Goldhammer, 2009 (M76/1); D'Hondt et al., 2003 (Leg201); Tréhu et al., 2003 (Leg204); Akiba et al., 2009 (Exp311); Robertson et al., 1998 (Leg160). The sample ages were estimated on the basis of sediment depth, general stratigraphic position and information provided in the original references. Concentrations of total organic carbon are from this work.

2.3. IP GDGT analysis

The fraction containing IP GDGTs was dissolved in a mixture of MeOH and CH_2Cl_2 (1:5, v/v) for analysis with a ThermoFinnigan Surveyor HPLC (high performance liquid chromatography) system coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer via electrospray ionization source (ESI). Sample solution (10 µl) was injected onto a LiChrosphere Diol-100 column (150 x 2.1 mm, 5 µm particle size; Alltech, Germany) equipped with a guard column of the same packing material.

Separation of lipids was achieved with the solvent gradient used by Sturt et al. (2004). With a flow rate of 0.2 mL/min, from 100% A (79:20:0.12:0.04 of hexane / 2-propanol / formic acid / 14.8 M NH_{3aq}) to 35% A and 65% B (88:10:0.12:0.04 of 2-propanol / water / formic acid / 14.8 M NH_{3aq}) over 45 min, hold for 15 min, then 100% A for 1 h to re-equilibrate the column for next run, Mass spectrometric analysis was performed in data-dependent ion mode with fragmentation of the base peak ion up to MS^3 under conditions described by Sturt et al. (2004). IP GDGTs with different polar head groups were assigned according to retention time and characteristic fragmentation in MS^2 spectra (Sturt et al., 2004; Lipp and Hinrichs, 2009).

2.4. Preparation and analysis of C GDGTs

The fractions containing C GDGTs were dissolved in 200 µl *n*-hexane/isopropanol (99:1 v/v) for direct injection and analysis without further treatment. In order to analyze the core lipid distribution of IP GDGTs, the polar head groups were removed by way of hydrolysis of the glycosidic bond by heating an aliquot of the IP GDGT fraction in 1 ml of 6 M HCl / MeOH / CH_2Cl_2 (1:9:1, v/v) at 70°C for 24 h (cf. Lipp and Hinrichs, 2009). The hydrolyzed IP GDGT fractions were dried under a N_2 and dissolved in 200 µl *n*-hexane/isopropanol (99:1, v/v) before analysis. Analysis of C GDGTs followed a method modified from Hopmans et al. (2000). Separation of GDGTs was achieved with an Econosphere NH_2 column (250 x 4.6 mm; Alltech, Germany) operated at 30°C in a ThermoFinnigan Surveyor HPLC system using a flow rate of 1 ml/min and the following gradient: 99:1 *n*-hexane/isopropanol (v/v) ramp up to 98:2 *n*-hexane/isopropanol (v/v) at 15 min, held isocratically for 3 min, and then increasing to 90:10 *n*-hexane/isopropanol (v/v) at 20 min and holding isocratically for 4 min to remove polar constituents from the column, finally equilibrating with 99:1 n-hexane/isopropanol (v/v) for 5 min before the next injection.

Mass spectrometric identification and quantification were performed with a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer coupled to the HPLC instrument by an atmospheric pressure chemical ionization (APCI) interface. APCI settings were: capillary temperature 200°C, source heater temperature 400°C, sheath gas flow 30 arbitrary units, source current 5 µA, ms1 range *m/z* 1280-1340, while other parameters were optimized by manual tuning during infusion of a hydrolyzed commercially available intact GDGT standard compound (1Gly-GDGT-PG; Matreya, USA).

2.5. Quantification and calculation

Calculation of relative ring distributions was carried out via peak area of the $[M+H]^+$ ions of GDGTs containing 0, 1, 2, 3, and 5 rings. The contribution of tetracyclic GDGT was not quantified due to interference of the $[M+H]^+$ peak with the $[M+H+2]^+$ isotope peak of pentacyclic GDGT and lack of compounds to allow a correction

following the procedure of Zhang et al. (2006). The analytical error was calculated from duplicate analysis and was generally <10%. With added external standard, C_{46} GDGT (cf. Huguet et al., 2006a), the proportion of IP GDGT in total GDGT was estimated from directly measured C GDGT and hydrolyzed IP GDGT fraction according to (IP GDGT) / (IP GDGT + C GDGT) x 100%.

2.6. Analysis of total organic carbon (TOC) content

50 mg of freeze dried sediment of each sample was first decalcified with 3 N HCl, and then loaded on a Leco CS200 analyzer for TOC content analysis.

3. Results and discussion

3.1. Transfer of core lipids from IP GDGT to C GDGT pool: a case example

The samples from TTR-15 station BS369M in the Black Sea (Table 3-1) were collected from the Batumi seep area off the coast of Georgia. Active methane emission into the water column was observed in the area, and authigenic carbonate precipitation and gas hydrates occurred in the sediment (Klaucke et al., 2006). The depletion of porewater SO_4^{2-} in the first 15 cm below the seafloor is consistent with a high rate of anaerobic oxidation of methane (AOM). Archaeal IPLs at this site have been analyzed as part of a large survey (Lipp et al., 2008, supporting online material, where the station was incorrectly labelled BS269M) and were found to be strongly dominated by diglycoside derivatives, which appear to be major lipids in both benthic archaea (Biddle et al., 2006) and methanotrophic ANME-1 archaea (Rossel et al., 2008).

For this site, we compared the GDGT distributions in the C GDGT fraction and IP GDGT fraction in order to examine how the activity of this sedimentary process, i.e. AOM, impacts on these two pools. Methane-metabolizing archaea involved in AOM are known to produce relatively large proportion of GDGT-1 and -2 (Pancost et al., 2001; Zhang et al., 2003; Wakeham et al., 2004; Rossel et al., 2008). GDGT profiles reflect the contribution of methanotrophic archaea to the GDGT pool. C GDGTs in the top three samples (0.5, 6.5 and 13 cm; Fig. 3-1) are highly similar, with a distribution typical for a predominant origin of planktonic archaea (cf. Schouten et al., 2002). By contrast, the C

GDGTs at 17 cm, the depth of SO_4^{2-} depletion, show a substantial increase in GDGT-1 and GDGT-2; this effect is even more pronounced at 30 cm (Fig. 3-1).

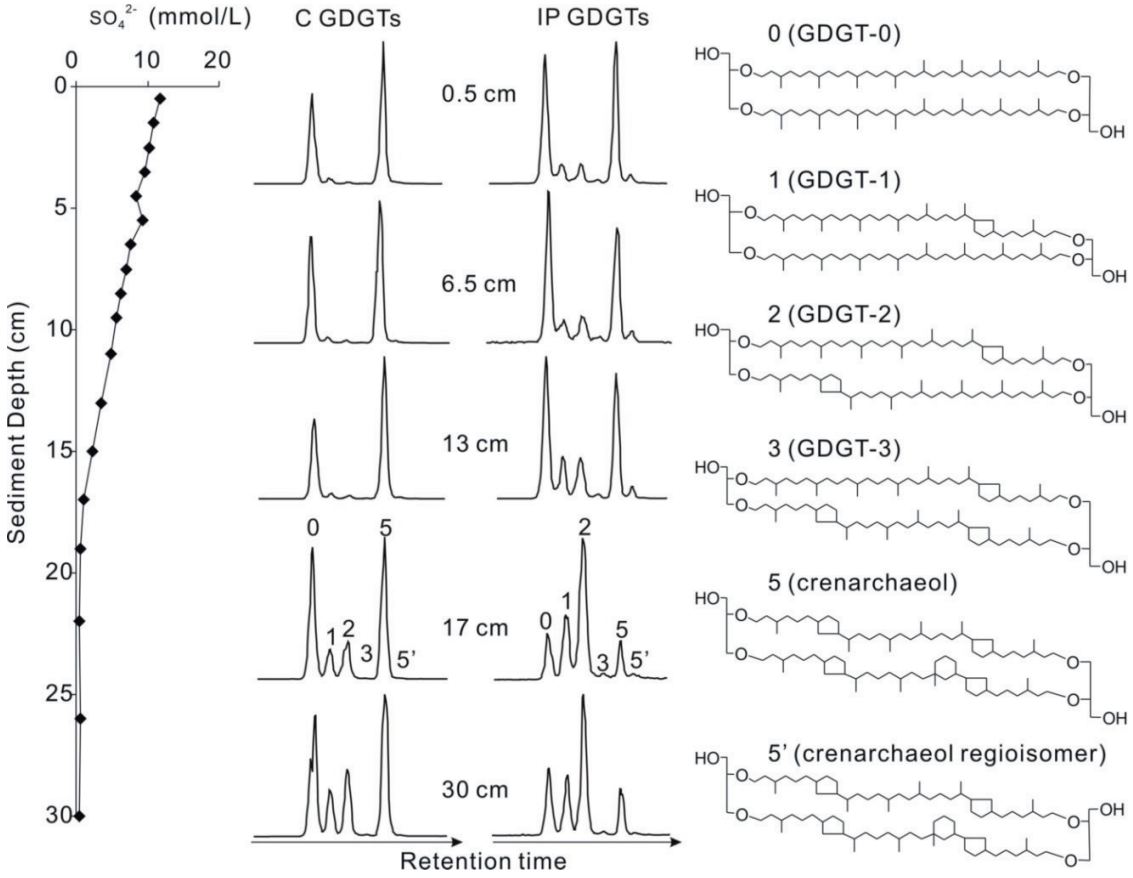

Figure 3-1. Downcore profile in sediments from Black Sea core BS369M, expedition TTR15, of sulfate (left panel, sulfate data: M. Haeckel, unpublished; depth scale is uncorrected depth and refers to recovered sediment core), and HPLC-APCI-MS base peak chromatograms showing distributions of C GDGTs (middle), and IP GDGTs (right). The IP GDGTs were analyzed as C GDGTs after hydrolysis of the polar fraction containing glycoside GDGTs. Note the change in C GDGT distribution at 17 and 30 cm, which we interpret to result from hydrolysis of the IP GDGT precursors.

The IP GDGT distribution is distinct from the corresponding C GDGT distribution and exhibits stronger changes downcore. In the surface sample, the IP

GDGT distribution is relatively similar to that of the C GDGTs; in both pools crenarchaeol (GDGT-5) is more dominant than GDGT-0 but compared to the C GDGT pool, GDGT-1 and -2 are more abundant and probably reflect the contribution of sedimentary archaea to this pool. In samples from 6.5 and 13 cm, the most obvious change vs. the surface sample is the increasing relative abundance of GDGT-0, a pattern previously linked to the presence of benthic sedimentary archaea (Lipp and Hinrichs, 2009). The IP GDGT distribution at 17 cm is dramatically changed; GDGT-1 and -2 are more abundant than GDGT-0 and -5, suggesting that the archaeal biomass is strongly impacted by AOM archaea. The sample from 30 cm is similar in distribution to the 17 cm sample. A linkage between both pools is evident: in situ production of a distinct population of IP GDGTs in the zone influenced by AOM. The increased proportion in GDGT-1 and-2 is consistent with hydrolytic cleavage of the glycoside head groups and subsequent preservation of the resulting fossil C GDGTs. Although we cannot quantify the flux of GDGTs from the IPL to the core lipid pool, we can state qualitatively that, at a site with relatively active archaeal methanotrophs, the "normal" planktonic and benthic archaeal lipid fingerprint in the "fossil" C GDGT fraction is strongly altered by in situ production of fresh IP GDGTs and subsequent loss of glycoside head groups. Given the late Holocene age of the sediments, this transfer of GDGTs from the IPL to the core lipid pool takes place on millennial or shorter timescales. Other data from natural settings are consistent with our finding. For example, in recent microbial mats of ANME-1 archaea in the anoxic water column of the Black Sea, the major lipid type (2Gly-GDGT; cf. Rossel et al., 2008) is identical to that found in sub-surface sediments. Analysis of the C GDGT and IP GDGT distributions in the mats suggests substantial loss of the diglycosidic polar headgroup on the timescale of the mat's life cycle (Thiel et al., 2007).

3.2. Survey of core lipid distributions in IP GDGT and C GDGT fractions

The process demonstrated above, i.e. a flux of GDGTs from the IPL pool to the core lipid pool, is presumed to take place wherever new archaeal biomass is formed. Its magnitude and impact on the sedimentary record of C GDGTs depends on the microbial growth rate and the enzymatic activity, as well as the efficiency at which C GDGTs produced in situ are preserved on geological timescales. Since marine sediments are a

habitat of benthic archaea (Biddle et al., 2006; 2008; Lipp et al., 2008; Lipp and Hinrichs, 2009; Parkes et al., 2005; Teske and Sørensen, 2008), a certain flux of GDGTs from newly formed archaeal biomass into the record of preserved C GDGTs is conceivable.

We examined the distribution of GDGTs in both the IP GDGT and C GDGT pool in a diverse set of sediments in order to characterize these two pools and constrain the factors that influence their distribution. Prior to C GDGT analysis of hydrolysis products of IP GDGTs, we analyzed the latter group in a selected set of samples. In line with a previous report (Lipp and Hinrichs, 2009), 2Gly-GDGT and an unidentified GDGT lipid (H341-GDGT) were the most abundant compounds, with proportions of >70%, followed by 1Gly-GDGT and 3Gly-GDGT (Table 3-2, relative concentration not shown). In the respective set of 12 samples, the proportion of IP GDGTs from total GDGTs ranged from 4% to 11% (Table 3-2), similar to the range reported by Lipp and Hinrichs (2009).

Table 3-2. Composition of IP GDGTs and ratio of IP GDGTs to total GDGTs ([IP GDGT]/ [IP GDGT + C GDGT]*100%) in 12 selected samples further concentrated for IP GDGT analysis with HPLC-ESI-MS.

Samples		Sediment Depth (m)	Identified IP GDGTs	Ratio
M76/1	GeoB 12806-2	0.1	1Gly, 2Gly-GDGT	8.8%
(Nami. Mar.)	GeoB 12807-2	3.1	1Gly, 2Gly, H341-GDGT	3.9%
ODP201	1226B 10H3	83.8	2Gly, 3Gly, H341-GDGT	3.1%
(Equ. Pacific)	1226E 20H3	320	1Gly, 2Gly, H341-GDGT	9.7%
ODP201	1229D 4H4	30.7	2Gly, H341-GDGT	10.9%
(Peru Mar.)	1229A 22H1	185.9	2Gly-GDGT	6.8%
ODP204	1250D 6H5	43.5	2Gly, H341-GDGT	7.1%
(Hydr. Rid.)	1250D 12H5	100.3	2Gly, 3Gly, H341-GDGT	6.9%
IODP311	1327C 10H5	79.8	2Gly, 3Gly, H341-GDGT	7.9%
(Cas. Mar.)	1327C 13X6	109.8	2Gly, H341-GDGT	5.5%
ODP160	966C 5H02	40	2Gly, 3Gly, H341-GDGT	8.4%
(Med. Sap.)	966C 7H04	65	2Gly-GDGT	11.2%

Several features stand out in the data set. GDGT-0 is generally more abundant in the IP GDGT fraction, while crenarchaeol (GDGT-5) tends to be less abundant (Fig. 3-2A, E). In the group of less abundant GDGTs, i.e. GDGT-1, -2, -3 and 5' – the compounds used for calculation of TEX_{86} (Schouten et al., 2002) - the two fractions differ as well. No clear trend to lower or higher abundance is recognizable for GDGT-1, although the data clearly do not scatter along the 1:1 line (Fig. 3-2B). GDGT-2 and -3

have higher relative abundance in the IP fraction for the majority of samples (Fig. 3-2C and D). Only the relative amount of crenarchaeol regioisomer (GDGT-5') in both pools is similar (Fig. 3-2F). The GDGT distribution from the AOM-influenced Black Sea seep site is shown for comparison and plots close to the 1:1 line (Fig. 3-2A-F), indicating the strong connection of the two GDGT pools at this site by in situ production IP GDGT and their subsequent degradation to C GDGTs.

The overall difference in the distribution of both pools can be summarized by way of the ring index (Eq. (1), cf. Uda et al., 2001; Pearson et al., 2004), which reflects variation of all detected C GDGTs (Fig. 3-2G).

$$Ring\ Index = [(\%GDGT-1)+2(\%GDGT-2)+3(\%GDGT-3)+5(\%Cren.+Cren\,Iso.)]/100 \quad (1)$$

The ring index in the IP GDGT pool is typically 0.5 to 1 units lower than in the corresponding C GDGT pool and indicates the lower relative abundance of cyclic GDGT derivatives in the former pool. Notably, the ring index of the IP GDGTs and C GDGTs is linearly correlated (R^2 0.67, $P<0.001$) suggesting some relationship between the two lipids pools.

TEX_{86} (Eq. (2)) is a molecular ratio based on the typically least abundant C GDGTs, which is positively correlated with modern sea surface temperatures (SSTs), when determined in surface sediments (Schouten et al., 2002).

$$TEX_{86} = \frac{(\%GDGT-2)+(\%GDGT-3)+(\%Cren\,Iso.)}{(\%GDGT-1)+(\%GDGT-2)+(\%GDGT-3)+(\%Cren\,Iso.)} \quad (2)$$

The validity of the proxy is based on the assumption that the signal is formed by planktonic archaea in the photic zone. No clear relationship was revealed between TEX_{86} and sedimentary in situ temperature for IP GDGTs (Fig. 3-2H), suggesting that the temperature dependence observed for core lipids from planktonic archaea is not valid for benthic archaea. We note, however, that verification of this statement will require further testing, e.g. at a single site with a substantial temperature gradient and a phylogenetically relatively homogeneous benthic archaeal community to avoid effects resulting from changing community composition.

For most of the samples, the IP GDGTs tend to give higher TEX_{86} values than the corresponding C GDGTs (Fig. 3-2I). The difference in values (ΔTEX_{86}) ranged from 0.02

to 0.17, so ΔSST values were from <1°C to 10°C (calculation of SST from TEX_{86} according to Kim et al., 2008). Interestingly, TEX_{86} values for the IP GDGT pool are strongly correlated with those determined for the C GDGT pool (Fig. 3-2I; R^2 0.68, P 0.002). Most notably, even though there is considerable variation in sample sets from each location, palaeoenvironments with relatively warm SST display higher (warmer) TEX_{86} values for both GDGT pools than samples from the cooler Northeast Pacific (Hydrate Ridge and Cascadia Margin). For example, samples from the Cascadia margin with low TEX_{86} values in both pools were deposited at SST of ca. 5-14°C (Herbert et al., 2001; alkenone-based reconstruction of SST off Northern California), while samples from warmer areas at the Equatorial Pacific and the Peru margin (22-28°C; Etourneau et al., 2010; Lawrence et al., 2006; Wara et al., 2005) have higher TEX_{86} values in both pools. Settings with intermediate paleo SST such as the Namibia Margin (12-18°C; Marlow et al., 2000) are characterized by intermediate TEX_{86} values in both pools. The general similarity of TEX_{86} values for both pools suggests, by analogy with the ring index, that both pools are related.

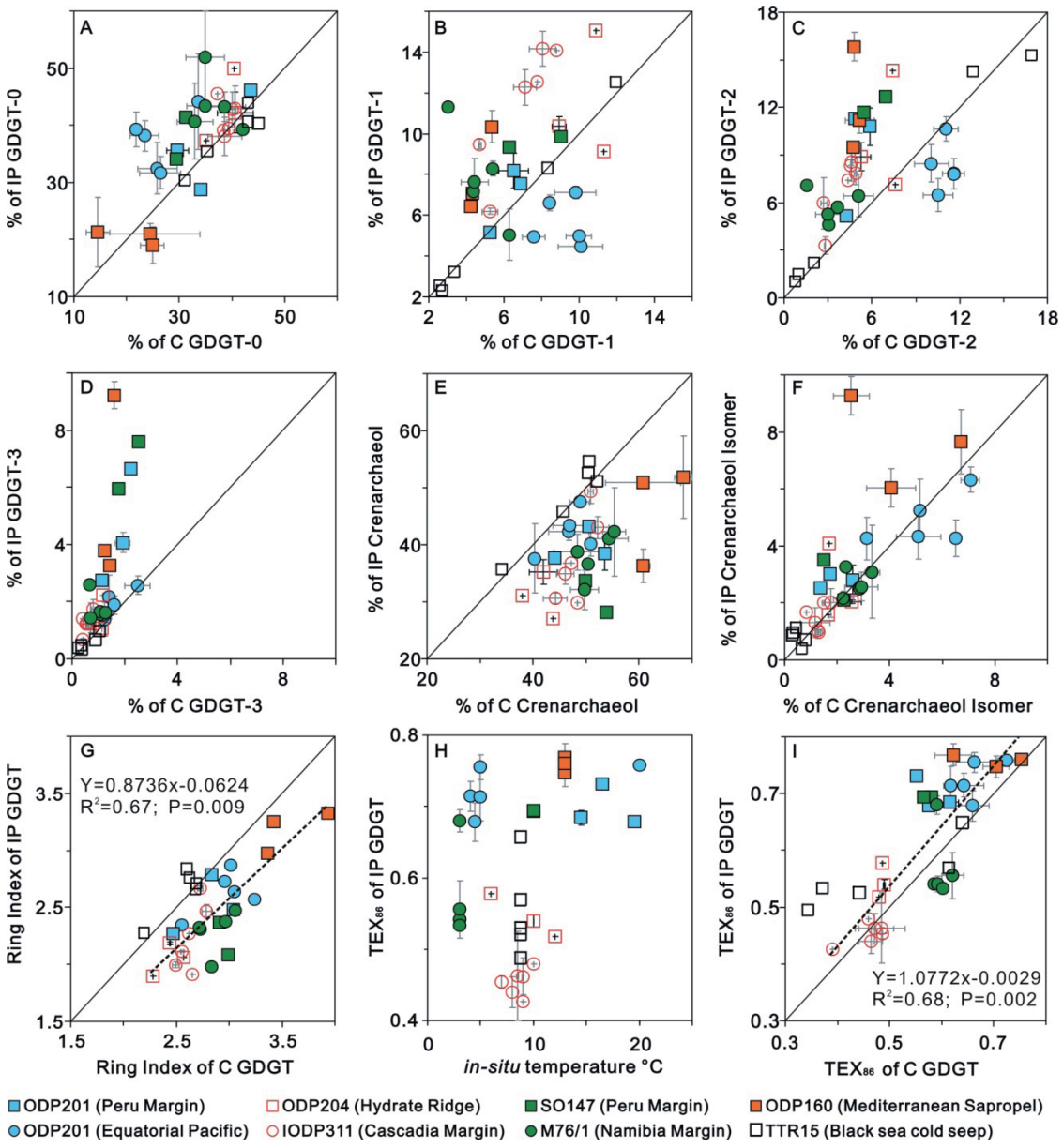

Figure 3-2. X-Y diagrams showing the relative abundance of individual GDGTs in pools of C GDGTs and IP GDGTs in a diverse sample set of marine subseafloor sediments (A-F), Ring Index for C vs. IP GDGTs (G), TEX_{86} values from IP GDGTs in relation to estimates of in situ temperature (H) and TEX_{86} for C vs. IP GDGTs. Data for 27 non-seep samples were plotted with error bars. For comparison, five samples from the Black Sea seep site (Fig. 3-1) are included (no error bars because of single measurements). The linear regression of Ring Index and TEX_{86} were calculated without the samples from the Black Sea seep site. The estimation of in situ temperature is based on the IODP online database (http://iodp.tamu.edu/janusweb/general/dbtable.cgi) and the basis of bottom water temperatures, sediment depth and the geothermal gradient. See legend for assignment of symbols to samples.

Three principle explanations could account for compositional connection of the two GDGT pools: (1) The pool of C GDGTs is strongly influenced by sedimentary in situ production and subsequent hydrolysis of IP GDGTs, to the effect that both pools resemble each other (cf. Fig. 3-1). (2) The pool of IP GDGTs largely represents molecular fossils derived from planktonic archaea (cf. discussion in Lipp and Hinrichs (2009) and Schouten et al. (2010)). (3) The partial recycling of fossil, exogenous C GDGTs, derived from planktonic archaea, by benthic archaea (Takano et al., 2010). We discuss these three alternatives as follows.

(1) New production of IP GDGTs by benthic archaea (cf. Lipp et al., 2008) coupled with subsequent hydrolysis of the (predominantly glycosidic) bond between polar head group and GDGT could explain the structural similarity in the two pools if rates of new production and preservation of the resulting C GDGTs were high enough to overwhelm the C GDGT pool originating from the planktonic archaeal community (Fig. 3-1). Although the distributional differences for individual compounds between the two GDGT pools are likely the result of synthesis of new lipids by benthic archaea and their contribution to the total lipid pool is not ignorable, this scenario fails to explain the general resemblance of molecular ratios sensitive to SST for both pools.

(2) This alternative – that a substantial fraction of IP GDGTs is fossil in nature and derives from planktonic archaeal communities – is in principle attractive because it could account for most observed relationships. As one consequence, previous estimates of sub-surface archaeal biomass (Lipp et al., 2008) would have been too high since only a fraction of the predominant glycosidic IPLs would be associated with live cells.

The degradation kinetics of IP GDGTs, especially glycoside types, has been discussed controversially, with modelling results not providing an unequivocal picture (cf. Lipp and Hinrichs, 2009; Schouten et al., 2010). For example, Schouten et al. (2010) noted the similarity between modelled IPL concentration profiles and decay of total sedimentary organic carbon (TOC) in sub-surface sediments and interpreted this as support for degradation (rather than in situ production) of IPLs being the major factor controlling their vertical distribution in the sub-surface; this is in contrast to suggestions by Lipp and Hinrichs (2009), who interpreted the profiles to be largely being reflective of their in situ production. We note that the relationship with TOC described by Schouten et

al. (2010) is not necessarily support for degradation as principal control. Instead, such a relationship would also be expected when IPL concentration is scaled with biomass of a heterotrophic archaeal community that is involved in TOC degradation (cf. Biddle et al., 2006; Lipp et al., 2008).

Based on the above discussion, no final conclusion can be drawn without further experimental tests that address to which degree the IP GDGTs represent fossil vs. live archaeal biomass. Previous short term experiments on the degradation of glycosidic archaeal lipids (Harvey et al., 1986) do not enable extrapolation to geological timescales considered relevant for cell turnover in the deep biosphere (cf. Biddle et al., 2006; Parkes et al., 2000). However, numerous lines of circumstantial evidence arguing against a predominant planktonic origin of IP GDGTs can be cited here: (i) concentration patterns of archaeal IP GDGTs in shallow sediments are inconsistent with their origin from a planktonic community, i.e., concentrations of IP GDGT are close to the detection limit in the uppermost sediment layer and increase with depth (Lipp et al., 2008; Schubotz et al., 2009); (ii) the diagenetic behaviour of 2Gly-GDGTs in natural settings (Fig. 3-1 and related discussion) argues against a predominant origin from surface water communities; (iii) last but not least, there is now overwhelming evidence for an important role of subseafloor archaeal communities based on molecular-genetic techniques (e.g. Biddle et al., 2006; 2008; Lipp et al., 2008; Teske and Sørensen, 2008); the archaeal communities detected by these techniques are in situ producers of IPLs. On the other hand, we cannot exclude the possibility that a fraction of the IPLs is of extracellular (fossil) origin (cf. Dell'Anno and Danovaro, 2005, who discuss the corresponding phenomenon for DNA), as a result of slowed degradation of benthic archaeal IP GDGTs in low activity sediments. In conclusion, planktonic archaeal communities as a primary source of sedimentary IP GDGTs appear unlikely; hence this scenario cannot satisfactorily explain the observed relationship with SST (Fig. 3-2I).

(3) Alternative 3, reutilization of exogenous C GDGTs by the benthic archaeal community (Takano et al., 2010; cf. Lin, 2009, for supporting evidence), combines elements of the two other options (1 and 2), in that IP GDGTs are representative of benthic archaeal communities but the lipophilic molecular moieties are to a substantial extent fossil remnants from planktonic archaeal C GDGTs that are incorporated via yet

undefined mechanisms and intermediates. The principle mechanistic requirements for utilization of exogenous lipids by microbes are established. Numerous pathways of ether lipid scission involving a range of ether substrates are developed in prokaryotes (White et al., 1996) but poorly investigated. The incorporation of exogenous lipids such as cholesterol and phospholipids into the membrane of several of the cell wall-free Mollicutes has been demonstrated (e.g. Razin et al., 1980). Moreover, the uptake and utilization of free fatty acids for the production of phospholipids has been demonstrated for fatty-acid auxotrophic *E. coli* (e.g., Silbert et al., 1968).

Two recent studies using stable isotopic probing to target archaeal communities in marine sediments showed strong, preferential label uptake into the glycoside headgroup (Lin, 2009) and the glycerol backbone (Takano et al., 2010) of archaeal GDGTs, while the biphytanyl moieties remained largely unlabelled. The results have provided evidence for benthic archaea utilizing an anabolic shortcut to save metabolic energy while taking advantage of the abundant fossil C GDGTs in the sedimentary habitat. Such a mechanism could account for the observations in this study. It can explain the observed relationships of TEX_{86} and ring index between pools of C GDGTs and IP GDGTs, without invoking the unlikely predominant origin of sedimentary archaeal IP GDGTs from fossil planktonic communities. We note that the importance of this mechanism is probably highest in energy-starved subseafloor archaeal communities (cf. Biddle et al., 2006; Lipp et al., 2008) but may not apply to ANME archaea which synthesize lipids de novo as suggested by GDGT distributions (Fig. 3-1) and incorporation of ^{13}C label into biphytane moieties when amended with ^{13}C-labelled substrates (e.g., Wegener et al., 2008). Accordingly, the compositional differences between both pools, especially those affecting the less abundant C GDGTs, could result from new synthesis of selected GDGTs by benthic archaea (cf. Shah et al., 2008), and distinct diagenetic pathways for the GDGTs and/or their related intermediates. As a corollary, the impact of benthic archaeal communities on palaeoceanographic proxies based on C GDGT distribution may be limited as long as such recycling of GDGTs is not associated with selective loss of some GDGTs. More research is required to shed light into these geobiological processes and their impact on palaeoenvironmental reconstruction and to further examine the importance of the three alternative scenarios discussed above.

4. Conclusions

In sub-surface sediments at a site with high methane flux in the Black Sea, the in situ production of polar glycosidic GDGTs by methanotrophic archaea goes along with their relatively rapid hydrolysis and production of the corresponding C GDGTs, suggesting that under these conditions IP GDGT production will have an impact on the sedimentary record of fossil C GDGTs.

A systematic comparison of two fractions of archaeal tetraether core lipids, C GDGTs and IP GDGTs, in a wide range of marine sub-surface sediments not affected by methane seepage revealed a number of important similarities and differences: The acyclic GDGT-0 is generally more abundant in the IP GDGT pool, while crenarchaeol is more abundant in the C GDGT pool. On the other hand, GDGT 2 and GDGT 3 are relatively more abundant as intact lipid, which results in mostly somewhat higher TEX_{86} values for the IP GDGT pool. Despite these differences, a relationship between low abundance GDGTs (GDGT-1, -2, -3, -5') in both pools, as expressed by way of TEX_{86}, is apparent; notably warmer oceanic regions are associated with higher TEX_{86} values for both pools and colder regions with lower values for both pools. A number of alternative explanations could account for these relationships. Based on the cumulative lines of evidence, we suggest that recycling of fossil GDGTs from planktonic archaea by the benthic archaeal community is an important process. At selected sites, in situ production of new GDGTs by benthic archaea and preservation of water column-derived IP GDGTs, however, may also contribute to the apparently related molecular ratio found in the two molecular pools.

Interesting questions arise from this study and remain to be addressed. For example, does the proposed recycling of lipids have an impact on the fossil record of C GDGTs and related molecular proxies? To what degree do glycoside GDGTs, i.e. the major polar GDGT type in sub-surface sediments, represent cellular vs. extracellular (fossil) components?

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Chapter 4

Isoprenoid glycerol dibiphytanol diethers: a series of novel archaeal lipids in marine sediments

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ABSTRACT

We report a new series of archaeal lipids, widespread in marine sediments and tentatively assigned as isoprenoid glycerol dialkanol diethers (GDDs). These lipids are structural analogues of isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) with one glycerol unit missing and with each biphytanyl moiety possessing a terminal hydroxyl. The structural identification is based on molecular formulae determined from accurate mass measurement and interpretation of mass spectral fragmentation patterns. Acetylation of GDD-0 confirmed the presence of three hydroxyl groups, and ether cleavage and reduction of the products afforded two biphytanyl chains. Tests of different protocols for both extraction and acid hydrolysis indicate that GDDs are not formed during sample preparation. The co-existence of GDDs and GDGTs in 12 selected marine sediment samples of varying origin showed that the ring distribution in these two ether lipid pools is related and implies that the two compound classes share a common biological source. The presence of isoprenoid GDDs is likely linked to the occurrence of biphytane diols in marine sediments, favoring the recently proposed fossil lipid recycling by benthic archaea. Intact polar lipids (IPLs) containing GDDs as core lipids were not detected in the samples analyzed.

1. Introduction

During the last decade, isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) have been recognized as widespread archaeal lipids in marine and terrestrial environments (Schouten et al., 2000, 2002; Lipp et al., 2008), while the other archaeal lipid type, glycerol dialkyl diethers, is usually abundant in hydrocarbon seeps at the ocean's seafloor (e.g. Hinrichs et al., 1999; Pancost et al., 2001). These ether lipids have attracted increasing interest from different fields of geobiological research since they can be used to trace the activity of Archaea in modern and ancient environments. The development of liquid chromatography mass spectrometry (LC-MS) has facilitated identification and quantification of archaeal lipids (e.g. Hopmans et al., 2000; Sturt et al., 2004) and opportunities for exploring novel types of archaeal lipids have become available as a result.

Previous studies of archaeal cultures, such methanogens (Tornabene and Langworthy, 1979; Koga et al., 1993), have found both diether- and tetraether-based lipids as major membrane constituents. In general, three categories of archaeal lipids are found in environmental samples: IPLs, glycerol ether core lipids (e.g. GDGT core lipids) and degraded fossil products. Archaeal lipids in their intact form, mainly glyco- and phospholipids, contain one or two labile polar head groups attached to the glycerol ether core. The polar head groups of IPLs, especially phospholipids, can be rapidly hydrolyzed after cell decay (e.g. White et al., 1979) and can therefore be used as proxies for living archaea. They can be detected with LC-MS in lipid extracts of biomass from cultures, marine particulate organic matter (OM) and sediments (e.g. Sturt et al., 2004; Schouten et al., 2008; Lipp et al., 2008; Rossel et al., 2010). GDGT core lipids are thought to represent a fossil signal and are ubiquitous in various depositional environments where they occur with high structural diversity (Schouten et al., 2000). They are relatively stable and are able to survive in sediments over tens of millions of years (e.g. Kuypers et al., 2001), so that they can serve as biomarkers of past archaeal activity and components of molecular proxy ratios, such as the paleo sea surface temperature indicator TEX_{86} (e.g. Schouten et al., 2002). Smaller fossil derivatives of archaeal lipids amenable to analysis by gas chromatography (GC), such as biphytanediols (Schouten et al., 1998; Saito and

Suzuki, 2010) and biphytanic diacids (Meunier-Christmann, 1988; Birgel et al., 2008) have been found in both recent sediments and ancient rock samples. However, it is not clear whether these biphytane derivatives are products of isoprenoid GDGT degradation or intermediates in lipid biosynthesis. In addition, a series of C_{80}, C_{81} and C_{82} isoprenoid biphytanyl tetraacids were identified in crude oil (Lutnaes, et al., 2006, 2007) and the authors assumed that they were of recent archaeal origin.

Our knowledge of the biosynthesis of archaeal lipids is limited although a pathway, based on both IPL analysis and genetic studies, has been proposed (Koga and Morii, 2007). Expanding knowledge of the distribution of archaeal lipid derivatives in nature should help better understanding of the activity of Archaea, the origin and fate of archaeal lipids in different geologic settings and the limitations of related molecular proxies. Here we report the identification of a novel series of lipids, i.e., so-called isoprenoid glycerol dialkanol diethers (GDDs). We discuss their distribution in marine sediments as well as the evidence pertaining to their source and potential biosynthetic significance.

2. Experiments

2.1. Materials and sample preparation

Twelve globally distributed marine sediments from various geological settings were collected (Table 4-1). More detailed information on sampling sites is given by Liu et al. (2011). Samples were freeze-dried and extracted using the modified Bligh and Dyer protocol as described by Sturt et al. (2004). In addition, one archaeal culture, *Methanothermococcus thermolithotrophicus* strain (DSM 2095), grown at 85 °C in enamel-protected fermentors with stirring (400 rpm) and continuous gassing (H_2/CO_2, 80:20) was provided by M. Baumgartner and K. Stetter (University of Regensburg, Germany). Using an ultrasonic probe (HD 2200, Bandelin electronic GmbH & Co. KG, Germany), 0.5 g freeze-dried biomass of *Methanothermococcus thermolithotrophicus* was extracted (4 x) with dichloromethane (DCM):MeOH [20 ml;1:1, v/v] for 15 min. An aliquot (1%) of the total lipid extract (TLE) of *Methanothermococcus*

thermolithotrophicus was spiked with 200 ng of a C_{46} GDGT standard (Huguet et al., 2006) before splitting it into two aliquots for direct lipid analysis and for treatment with 6 M HCl/MeOH/DCM [1 ml;1:9:1, v/v] at 70 °C for 3 h to hydrolyze the IPLs to core lipids.

Table 4-1. The ratio of total GDD vs. the sum of total GDGT and GDD in twelve marine sediment samples[a] and other samples

Sample		GDD/(GDGT+GDD) (%)
M 76/1	GeoB 12806-2	4.5
Namibia Margin	GeoB 12807-2	9.3
ODP Leg 201	1229D 4H4	17.0
Peru Margin	1229A 22H1	11.1
ODP Leg 201	1226B 10H3	8.6
Equatorial Pacific	1226E 20H3	8.8
ODP Leg 204	1250D 6H5	11.3
Hydrate Ridge	1250D 12H5	9.9
IODP Exp. 311	1327C 10H5	10.9
Cascadia Margin	1327C 13X6	10.7
ODP Leg 160	966C 5H02	3.5
Mediterranean	966C 7H04	7.6
Methanothermococcus	TLE	8.6
thermolithotrophicus	hydrolyzed TLE	1.1
GDGT-0 + extracted sediment + B&D[b]		n.d.[c]
GDGT-0 + acid hydrolysis at 70 °C for 24 h		n.d.[c]
GDGT-0 + acid hydrolysis at 70 °C for 72 h		0.2

[a] Detailed sampling data of marine sediment samples is given by Liu et al. (2011); [b]B&D, Bligh and Dyer extraction; [c] n.d., GDD not detected .

2.2. Analysis of GDGTs and GDDs

One aliquot of each sample was dissolved in 200 µl hexane/isopropanol (99:1, v/v) for HPLC-MS. Separation was performed with a Prevail Cyano column (2.1×150 mm, 3 µm; Grace, Germany) maintained at 35 °C in an Agilent 1200 series HPLC instrument. Using a flow rate of 0.25 ml min^{-1}, the gradient of the mobile phase was first held for 5 min with 100% eluent A [*n*-hexane/isopropanol, 99:1 (v/v)], followed by a linear gradient to 90% A and 10% B [*n*-hexane/isopropanol, 90:10 (v/v)] in 20 min, followed by a linear gradient to 100% B at 35 min, and finally holding at 100% B for 5 min. After analysis the column was equilibrated with 100% A at 0.6 ml min^{-1} for 5 min. Detection was achieved with an Agilent 6130 MSD single quadrupole mass spectrometer, coupled to the Agilent 1200 series HPLC instrument via a multimode ion source in atmospheric pressure chemical ionization (APCI) mode. APCI settings were: nebulizer pressure 60 psi, vaporizer temperature 250 °C, drying gas (N_2) flow 6 L min^{-1} and drying gas temperature 200 °C, capillary voltage 2 kV, and corona current 5 µA. With Chemstation software the detector was set for selective ion monitoring (SIM) of $[M+H]^+$ ions (*m/z* 1302, 1300, 1298, 1296, 1292, 1246, 1244, 1242, 1240 and 1236, fragmentor voltage 190 V).

In addition, small amounts of GDD-0 (acyclic GDD) and GDD-cren (GDD corresponding to crenarchaeol) were isolated from the combined extracts of the twelve sediments by collecting fractions manually through a flow splitter installed between the HPLC instrument and detector. The LC method used for isolating small amounts of GDD was as described above for GDD analysis. In order to record MS^2 spectra and accurate molecular masses, isolated GDDs were injected again into an Agilent 1200 series HPLC system coupled to an Agilent 6520 quadrupole time-of-flight (qTOF) mass spectrometer through an APCI interface. The APCI source temperature was 350 °C with a gas (N_2) flow of 4 l min^{-1}. The qTOF parameters for auto MS/MS scanning mode with MS^1 range *m/z* 500-2000 and MS^2 *m/z* 100-2000 were: capillary voltage 1 kV, corona current 5 µA, fragmentor voltage 150 V, skimmer voltage 65 V and octapole voltage 750 V. The qTOF system was tuned and calibrated with Agilent commercial tuning mix to reach a mass accuracy better than 2 ppm.

Relative ring distributions of GDGTs and GDDs were determined using peak areas of the $[M+H]^+$ ions of compounds containing 0, 1, 2, 3, and 5 rings. Because proper standards were unavailable, we assumed an identical response factor for GDDs and GDGTs when we calculated absolute concentrations, and the ratio of GDD vs. GDGT was estimated from the peak area of isoprenoid GDGTs and GDDs according to (GDD) / (GDGT + GDD) x 100%.

2.3. Analysis of acetylated GDD

For determination of the number of OH groups in GDD, an aliquot of purified GDD-0 was transferred into a 2 ml vial and dried with N_2; 1 ml acetic anhydride and pyridine (1:1,v:v) was added, and the mixture was kept at 50 °C for 1 h. Acetylated GDD-0 was analyzed with APCI-MS by infusing the sample directly into the ion source with a syringe pump set at 0.2 m min^{-1}.

2.4. Analysis of alkyl moieties released by ether cleavage

One aliquot of isolated GDD-cren was transferred to a 2-mL vial and dried with N_2, then 0.5 ml BBr_3 (1M in DCM; Aldrich) was added. The reaction was performed at 60 °C for 2 h. After evaporating the solvent and residual BBr_3 with N_2, 0.5 ml superhydride ($LiEt_3BH$, in THF, Aldrich) were added to reduce the bromides to hydrocarbons at 60 °C for 2 h. Adding a few drops of water quenched the reaction. The hydrocarbons were extracted (3 x) with *n*-hexane and the extracts combined for GC-MS. In order to analyze potential hydroxylated compounds, one aliquot of the ether cleavage/reduction products was transferred into a 2 ml vial, dried with a flow of N_2 and mixed with 100 µl BSTFA (N, O-bis(trimethylsilyl)trifluoroacetamide) and 100 µ pyridine at 70 °C for 1 h. GC-MS was performed with an Agilent 5975C inert XL MSD system equipped with an Agilent DB-5HT column (30 m x 250 µm x 0.25 µm). Separation was achieved at an oven temperature program of 60 °C (1 min) to 150 °C at 10 °C min^{-1} and then to 320 °C (held 36 min) at 5 °C min^{-1}.

2.5. Tests for production of GDDs from GDGTs

To test potential production of GDD via degradation of GDGT during sample extraction and preparation, three experiments were conducted. In the first, 1 µg of purified GDGT-0 was subjected to acid hydrolysis as described above for the *M. thermolithotrophicus* culture (experiment A). For the second, pre-extracted sediment was spiked with another aliquot of 1 µg of purified GDGT-0 before extraction with the Bligh and Dyer method (experiment B). The third experiment evaluated the influence of three different extraction protocols on the yield of GDGT and GDD (experiment C). Freeze-dried and homogenized sediment (M76/1 GeoB 12806-2) was separated into six aliquots of 1.5 g dry wt. Homogenization was achieved using a freezer mill (CryoMill, Retsch GmbH, Haan, Germany) cooled with liquid N_2 (cf. Lipp et al., 2008). Each aliquot was spiked with 1 µg C_{46} GDGT standard (Huguet et al., 2006). The first two aliquots were extracted with the Bligh and Dyer protocol in four steps, twice with phosphate buffer and twice with trichloroacetic acid (TCA) buffer (cf. Sturt et al., 2004); the second two aliquots were extracted with only phosphate buffer f(4 x), and the last two aliquots were extracted using an accelerated solvent extractor (ASE 200, DIONEX) with a mixture of DCM:MeOH (9:1, v/v) and three cycles of 5 min each at 100 °C and 7.6×10^6 Pa (cf. Leider et al., 2010).

3. Results and discussion

3.1. Identification of isoprenoid GDDs

A novel series of compounds structurally related to the known isoprenoid GDGTs with 56 Da lower molecular mass was recognized in extracts of marine sediment samples (Table 4-1). The components eluted several min after GDGT during normal phase HPLC (Fig. 4-1). For instance, the analogues of GDGT-0 ($[M+H]^+$, *m/z* 1302) and crenarchaeol ($[M+H]^+$, m/z 1292) show molecular ions $[M+H]^+$ at *m/z* 1246 and 1236, respectively. Their MS^2 spectra gave dominant fragment ions at *m/z* 669 and 663, respectively,

representing the fragments of a glycerol moiety plus one ether-bound biphytanediol (with one free OH; no ring for *m/z* 669 and three rings for *m/z* 663). Consequently, the neutral losses of 577 Da and 573 Da correspond to biphytanediols with 0 and 2 rings (Fig. 4-2).

Accurate mass determination of the two compounds via time-of-flight mass spectrometry with less than 2 ppm of mass uncertainty showed a $[M+H]^+$ at *m/z* 1246.2950 and 1236.2166, respectively, affording calculated formulae $C_{83}H_{168}O_5$ and $C_{83}H_{158}O_5$. Based on this, their elution pattern relative to GDGTs and their MS^2 spectra, these lipids were tentatively identified as isoprenoid GDDs.

Three additional observations support this structural assignment. Firstly, the acetylated GDD-0 gave a molecular ion at *m/z* 1372, which can be interpreted as acetylation of three OH groups with an added mass of 126 Da. Secondly, identical compounds were released by ether cleavage/reduction from GDD-cren and crenarchaeol, i.e. mixtures of biphytanes with two and three rings. Theoretically, biphytane mono-ols might be released by ether cleavage of GDD, but no alcohols were detected in the BSTFA-derivatized sample. This confirms that C-O bonds of hydroxyl functions are susceptible to attack by BBr_3 with subsequent reduction of the bromides to biphytane hydrocarbons by superhydride. Thirdly, the degradation tests on GDGT-0 showed that after 72 h acid hydrolysis at 70 °C only small amounts of GDD-0 had been generated from GDGT-0 (Table 4-1). This detection of GDD-0 as a degradation product of GDGT-0 provides further evidence for the proposed structure of GDD.

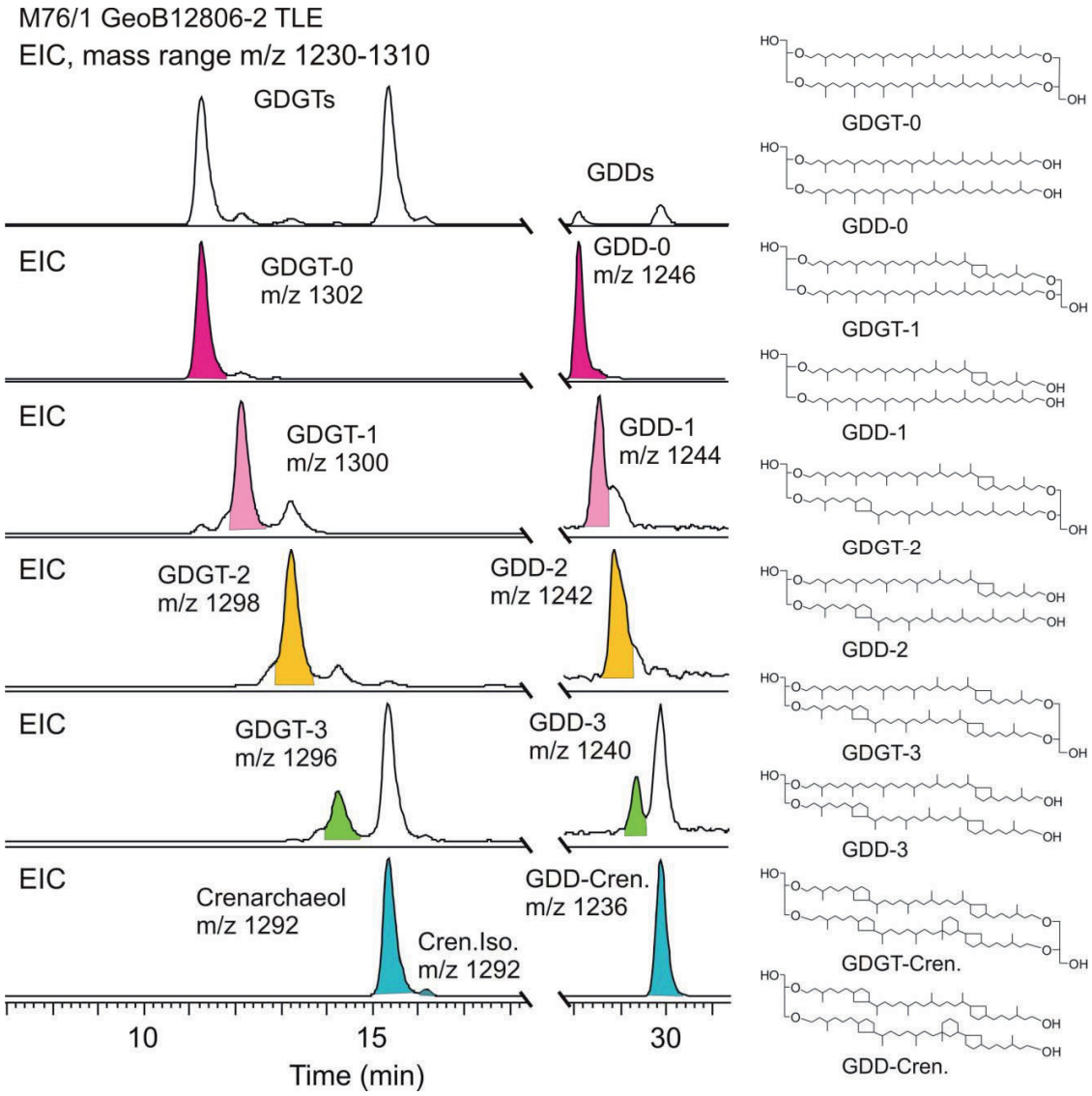

Figure 4-1. HPLC-APCI-MS chromatogram (SIM mode), showing GDDs and corresponding GDGTs in the TLE of marine sediment sample M76/1 GeoB 12806-2. EIC represents extracted ion chromatogram, respectively. GDGT and its corresponding GDD are labeled in the same color.

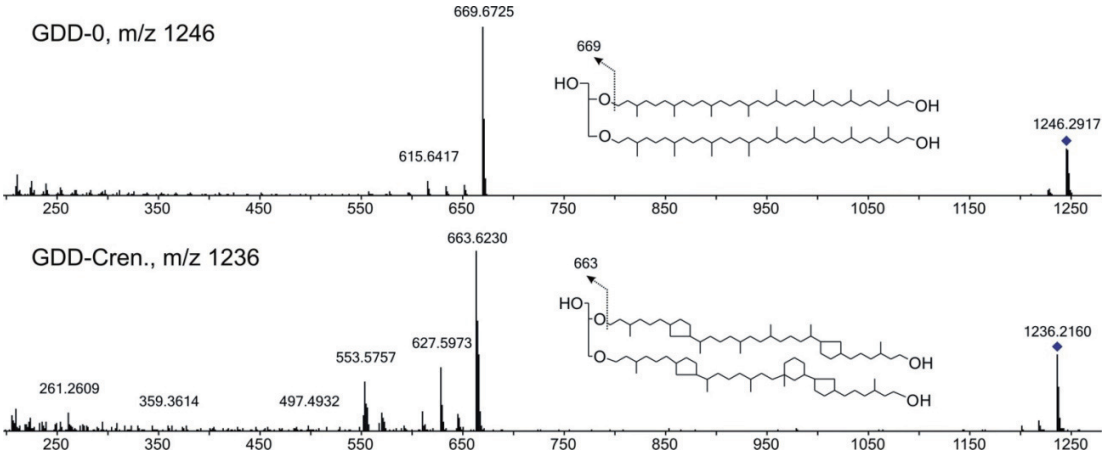

Figure 4-2. MS^2 (qTOF) mass spectra showing dominant fragment ions for GDD-0 and GDD-cren.

3.2. Occurrence of GDDs and GDGTs

Twelve marine sediment samples from six sites (Table 4-1) were analyzed for the occurrence of isoprenoid GDDs and GDGTs. In all of them GDDs coexisted with the corresponding GDGTs. The ratio of signal responses of total GDD vs. sum of total GDGT and GDD ranged from 3.5% to 17% (Table 4-1), suggesting a substantial contribution of GDDs to the overall pool of sedimentary archaeal lipids. The ring distribution of GDDs and GDGTs with identical numbers of rings is illustrated as a cross plot in Fig. 4-3 in order to expose potential relationships of the two compound classes. Since GDDs contain only one glycerol, no cis vs. trans regioisomers (cf. Sinninghe Damsté et al., 2002) were observed for GDD-cren (Fig. 4-3e).

In general, the data plots of compounds with one, two and three rings scatter within a deviation of a few % along the 1:1 line (Fig. 4-3b-d), but GDD-0 shows a trend to lower relative abundance than GDGT-0 (Fig. 4-3a) while GDD-cren is relatively more abundant than the corresponding GDGT (Fig. 4-3e). This phenomenon is analogous to the relationship between biphytanediols and GDGTs (Schouten et al., 1998), where, in a selection of marine sediments, the tricyclic biphytane diol was present in higher relative abundance than the tricyclic biphytanes released by ether cleavage. This observation is

consistent with isoprenoid GDDs sharing common sources and/or formation pathways with biphytanediols.

Methanothermococcus thermolithotrophicus contained both the core lipids of GDD-0 and GDGT-0 in abundance of 22 ng GDD-0 and 240 ng GDGT-0 g^{-1} biomass, respectively. Afteracid hydrolysis of the TLE their abundance increased to 140 ng GDD-0 and 12000 ng GDGT-0 $^{g-1}$ biomass. Although no IPLs of GDD were found in either the marine sediments or the archaeal culture, the increase in GDD abundance in acid-hydrolyzed TLE may reflect the presence of GDD IPLs.

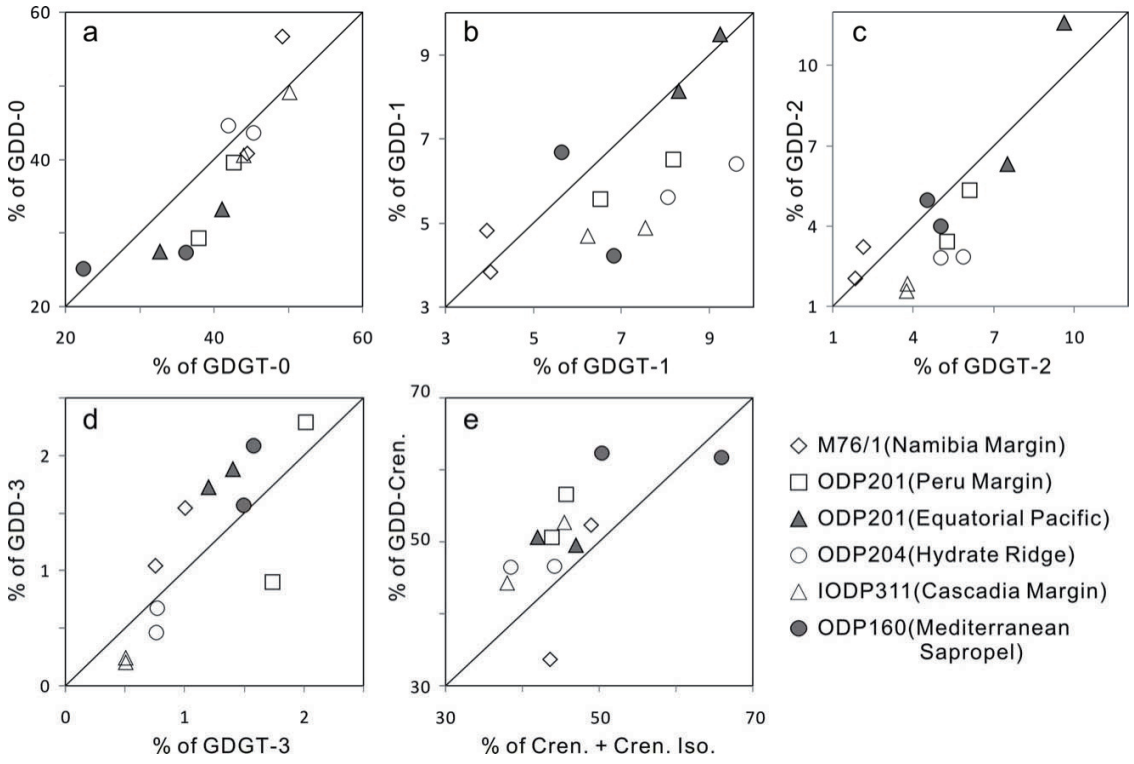

Figure 4-3. GDGT vs. GDD plots showing ring distribution of GDGTs and GDDs in twelve marine sediments.

3.3. Source of GDDs

By analogy with previously identified biphytanediols (Schouten et al., 1998), the coexistence of GDD and GDGT with similar ring distribution in the biphytanyl chains suggests that these compounds have closely related if not identical biological sources. However, for interpreting the environmental occurrence of GDDs, knowledge of their formation pathway is crucial. Three principal scenarios need to be considered and are briefly discussed: (i) production during sample preparation; (ii) diagenetic production in sediments; (iii) production as intermediates in GDGT biosynthesis.

Firstly, GDD production during sample preparation cannot be entirely excluded but the experimental evidence argues against this as major mechanism. Different extraction protocols (experiment C) provided different yields of GDDs and GDGTs (Fig. 4-4) but the effect of TCA or elevated temperature and pressure during ASE extraction did not result in an increased relative yield of GDD within the uncertainty of quantification. Likewise, in the experiment in which purified GDGT-0 was added to extracted sediment, no production of GDD-0 occurred during Bligh and Dyer extraction (experiment B). Finally, no GDD was detected within 24 h of subjecting GDGT-0 to acid hydrolysis (experiment A). The production of 0.2% GDD after 72 h of acid hydrolysis is low compared to the relative abundance of GDD in sediments (Table 4-1). These experiments strongly suggest that GDDs were already present in the sediments and not formed during sample preparation.

Secondly, the formation of small quantities of GDD during prolonged acid hydrolysis shows that, in principle, diagenetic production of these compounds in sediments is a conceivable mechanism. A diagenetic link of GDGTs and GDDs is consistent with the general relationship of their ring distributions (Fig. 4-3). Diagenetic processes could also involve biotic mechanisms such as the recently proposed recycling of exogenous GDGTs by benthic archaea (Takano et al., 2010; cf. Liu et al., 2011). The conclusions drawn by Takano et al. (2010) were based on selective uptake of stable isotope label in the glycerol moiety of GDGTs during incubation. The mechanism would require an intermediate to which newly synthesized is added; GDDs fulfill the criteria as intermediate in such a scenario. Nevertheless, the detection of GDD in the TLE of

Methanothermococcus thermolithotrophicus suggests that other mechanisms need to be considered.

The last scenario that needs to be considered is whether GDDs are normal functional lipids or intermediates in GDGT synthesis. The presence of GDD-0 as a free core lipid in *Methanothermococcus thermolithotrophicus* argues for a biological origin. However, no GDD IPLs were found in the samples. Assuming that GDDs represent components of archaeal membranes or biosynthetic intermediates, their existence may provide new insights into the biosynthetic pathway leading to ether lipids. According to the proposed pathway, the biphytane skeleton should be formed via head-to-head condensation of two archaeol units (Nemoto et al., 2003). This pathway seems inconsistent with a biological function of GDDs in archaeal membranes.

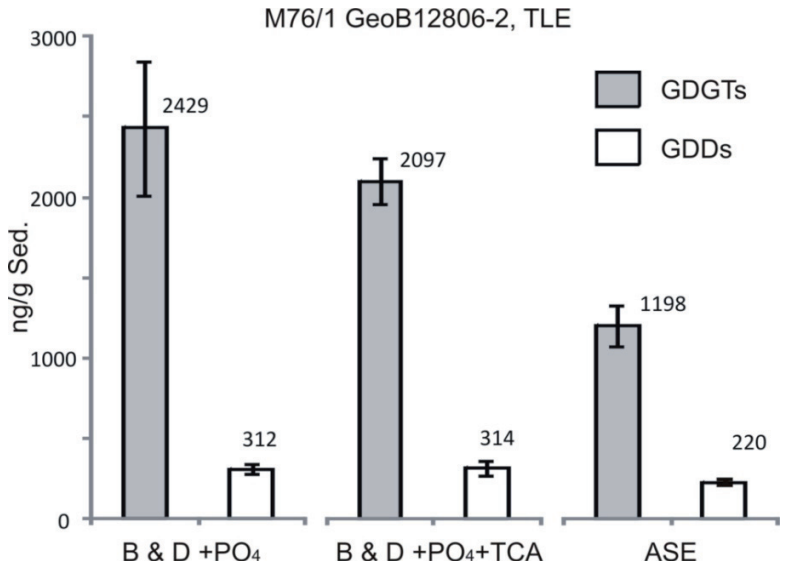

Figure 4-4. Yield of GDGTs and GDDs with three different extraction protocols. Error bars were calculated from two replicate samples for each extraction protocol. Concentrations of GDGTs and GDDs (ng/g dry sediment) in the TLE of each sample are based on duplicated analysis. 'B&D +PO_4' and 'B&D +PO_4 +TCA' represent the Bligh and Dyer extraction with only phosphate buffer and with both phosphate and TCA buffers, respectively. 'ASE' stands for extraction performed on accelerate solvent extractor.

4. Conclusion

A series of novel archaeal lipids was tentatively assigned as glycerol dibiphytanol diethers, in which a primary OH group was located on the terminal (ω) carbon. Future nuclear magnetic resonance (NMR) analysis of isolated compounds should aid in the determination of the exact structure of GDD. The coexistence of GDDs with corresponding GDGTs, their widespread distribution and remarkable abundance in marine sediments qualify these compounds as important archaeal lipid components in sediments and important targets in future studies. Diagenesis of GDGTs, especially biodegradation by benthic archaea is a possible source of GDD in sediments. Free GDD core lipid wasdetected in one archaeal culture, *Methanothermococcus thermolithotrophicus* indicated, however, a biological origin as another possible source of GDDs in sediments. So far no IPLs with GDD as the core lipid have been detected.

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Chapter 5

Novel mono- and dihydroxy-glycerol dibiphytanyl glycerol tetraethers and their intact polar analogues in marine sediments

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ABSTRACT

Hydroxy-GDGTs were detected in marine sediments deposited under contrasting conditions. Using two-dimensional (2D) ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy one major compound has been tentatively identified as monohydroxy-GDGT with acyclic biphytanyl moieties (OH-GDGT-0). Three other related compounds were assigned as acyclic dihydroxy-GDGT (2OH-GDGT-0) and monohydroxy-GDGT with one (OH-GDGT-1) and two cyclopentane rings (OH-GDGT-2). Based on the identification of hydroxy-GDGT core lipids, a group of previously reported unknown intact polar lipids (IPLs), including the ubiquitously distributed H341-GDGT (Lipp and Hinrichs, 2009) and its analogues were tentatively identified as glycosidic hydroxy-GDGTs. These hydroxy-GDGTs contain only up to two rings, implying a distinctive biological source from that of non-hydroxylated GDGTs. We detected hydroxy-GDGTs also in *Methanothermococcus thermolithotrophicus*. Given the previous finding of the putative polar precursor H341 in the planktonic marine crenarchaeon *Nitrosopumilus maritimus*, these compounds are synthesized by representatives of both cren- and euryarchaeota. The ubiquitous distribution and substantial abundance (4%-16% of total GDGTs) of hydroxy-GDGTs in marine sediments point to their potential application as molecular biomarker and geological proxies. The products of dehydrated hydroxy-GDGTs can possibly interfere with molecular proxy applications of non-hydroxylated

GDGTs since two major dehydrated products of OH-GDGT-0 gave the exact same molecular masses and nearly identical retention times as GDGT-1 and H-shaped GDGT-0, respectively.

1. Introduction

As one of the three domains of life, Archaea differ from the other two domains by not only its genetic affiliation but also through its unique membrane lipids of isoprenoid glycerol ethers. Since the first report of glycerol diether lipids in a halophilic archaeon, *Halobacterium salinarum* (Kates et al., 1963), various archaeal ether lipids have been discovered in different cultivated species of extremophiles (Langworthy, 1977), methanogens (Tornabene and Langworthy, 1979; Koga et al., 1993) and also in the uncultivated mesophilic marine crenarchaeota (DeLong et al., 1998). Among these diverse structures of archaeal lipids, such as glycerol dibiphytanyl glycerol tetraethers (GDGT) with different number of cyclopentane rings (e.g., De Rosa et al., 1980), unsaturated phytanyl diether lipids (archaeol) with double bonds (Gonthier et al., 2001) and the macrocyclic diether (Comita and Gagosian, 1983), there are also hydroxylated diethers recognized as distinctive group of archaeal lipids. Ferrante et al. (1988a) first reported the identification of hydroxy-archaeol isolated from *Methanothrix concilii*. Nuclear magnetic resonance (NMR) analysis indicated a tertiary hydroxyl group on C-3 of the phytanyl chain at the *sn*-3 position of glycerol. In the same species, the intact polar lipid (IPL) of hydroxy-archaeol, a diglycosidic hydroxy-archaeol, was also identified (Ferrante et al., 1988b). According to the inhibited synthetic pathway of GDGT in cultivated archaea, the biphytane skeleton was proposed to be formed via head-to-head condensation of two archaeol units (Nemoto et al., 2003). Therefore, given the presence of hydroxy-archaeol, synthesis of hydroxy-GDGT was also expected. Summons et al. (2002) reported their detection of hydroxylated biphytanes in *Methanothermococcus thermolithotrophicus* after ether cleavage of intact lipids, and Lipp and Hinrichs (2009) observed one unknown IPLs producing very weak MS^2 signal of putative hydroxy-GDGT, but to date on identification of hydroxy-GDGT has been confirmed.

Based on former studies of lipids in cultures, archaeal ether lipids have been intensively studied in different fields of geological research as well. For instance, the existence of methane-consuming archaea in marine sediment was revealed by the detection of ^{13}C depleted archaeol and hydroxy-archaeol (Hinrichs et al., 1999). And, the ^{13}C content of archaeal fossil lipids in a Mid-Cretaceous black-shale were reported to indicate the massive expansion of chemoautotrophic archaea in Mid-Cretaceous ocean anoxic event (Kuypers et al., 2001). On the other hand, archaeal IPLs were analyzed to reflect the in-situ activity of living archaea in specific environments, such as the anoxic water column of the Black Sea (Schubotz et al., 2009), marine methane seep sites (Rossel et al., 2008) and subseafloor sediments (Biddle et al., 2006). In several recent IPL studies one unknown compound, H341-GDGT named by Lipp and Hinrichs (2009), was detected as major putative archaeal IPL in a wide range of depositional settings (e.g. Sturt et al., 2004; Lipp and Hinrichs, 2009; Schubotz et al., 2009; Pitcher et al., 2011). Knowing the structure of this unknown but widely occurring compound H341-GDGT and its biological source will help us better understand both the composition of related archaeal communities and their activity in these environments.

With the development of liquid chromatography mass spectrometry (LC-MS), identification and quantification of big and polar archaeal lipids have been facilitated, such as the analysis of GDGT core lipids with HPLC-atmospheric pressure chemical ionization (APCI)-MS (Hopmans et al., 2000) and the analysis of archaeal IPLs with HPLC-electrospray ionization (ESI)-MS (Sturt et al., 2004). In this study, we describe the identification of novel hydroxylated GDGT core lipids and a corresponding series of glycosidic hydroxy-GDGTs.

2. Material and methods

2.1. Materials and sample preparation

Twelve globally distributed marine sediments from various geological settings were collected for lipid analysis (Table 5-1). Detailed descriptions pertaining to these samples were published previously in Liu et al. (2011). For isolating target compounds

for NMR analysis, we extracted more than 1 kg of combined dry sediment from Aarhus Bay (position: 56°07.06'N, 10°20.85'E, 15 m water depth and 6-7 m sediment depth below surface) and Baltic Sea (position: 58°56.04'N, 17°43.81'E, 52 m of water depth, surface sediment). All samples were freeze-dried and extracted using the modified Bligh and Dyer protocol as described by Sturt et al. (2004). The total lipid extracts (TLE) of the twelve marine sediments were first separated into core lipid and IPL fractions with preparative LC before analysis in order to increase sensitivity for the detection of minor compounds. Using a preparative LiChrospher Si60 column (250 x 10 mm, 5 µm, Alltech, Germany) fractions were collected with a fraction collector (GILSON, FC 204). Mobile phase gradient and instrument settings were following the method of Biddle et al. (2006).

Table. 5-1 Samples analyzed.

Samples		OH-GDGT/total Core GDGT (%)	2OH-GDGT/total Core GDGT (%)	Identified Intact Polar GDGTs
M76/1 Namibia Margin	GeoB 12806-2	11	0.2	1Gly-, 2Gly-GDGT; 1Gly-OH-GDGT
	GeoB 12807-2	13	n.d.	1Gly-, 2Gly-GDGT; 1Gly-, 2Gly-OH-GDGT
ODP201 Peru Margin	1229D 4H4	16	0.3	2Gly-GDGT; 2Gly-, 3Gly-OH-GDGT
	1229A 22H1	11	n.d.	2Gly-GDGT
ODP201 Equatorial Pacific	1226B 10H3	6	3	2Gly-, 3Gly-GDGT; 2Gly-, 3Gly-OH-GDGT; 2Gly-2OH-GDGT
	1226E 20H3	4.1	0.6	1Gly-, 2Gly-GDGT; 2Gly-OH-GDGT
ODP204 Hydrate Ridge	1250D 6H5	11.0	0.3	2Gly-GDGT; 2Gly-OH-GDGT
	1250D 12H5	11.0	0.4	2Gly-, 3Gly-GDGT; 2Gly-, 3Gly-OH-GDGT
IODP311 Cascadia. Margin	1327C 10H5	8.1	0.6	2Gly-, 3Gly-GDGT; 2Gly-, 3Gly-OH-GDGT; 3Gly-2OH-GDGT
	1327C 13X6	6.9	0.3	2Gly-GDGT; 2Gly-OH-GDGT
ODP160 Mediterranean Sapropel	966C 5H02	5.1	n.d.	2Gly-, 3Gly-GDGT; 2Gly-, 3Gly-OH-GDGT
	966C 7H04	4.2	0.1	2Gly-GDGT; 2Gly-OH-GDGT
Methanococcus Thermolithotrophicus		14.3	n.d.	1Gly-, 2Gly, 3Gly-GDGT; 2Gly-, 3Gly-OH-GDGT

(n.d. not detected)

Detailed information on the twelve marine sediment samples has been published in Liu et al., (2011). Ratio of hydroxy-GDGT vs. the total core GDGT was calculated based on the detection of hydroxy-GDGT at 3.0 eV slope collision energy.

In addition, one archaeal culture, *Methanothermococcus thermolithotrophicus* strain (DSM 2095), grown at 85 °C in enamel-protected fermentors with stirring (400 rpm) and continuous gassing (H_2/CO_2, 80:20) was provided by M. Baumgartner and K. Stetter (University of Regensburg, Germany). Using an ultrasonic probe (HD 2200, Bandelin electronic GmbH & Co. KG, Germany), 0.5 g freeze-dried biomass of *Methanothermococcus thermolithotrophicus* was extracted (4 x) with dichloromethane (DCM):MeOH (20 ml;1:1, v/v) for 15 min. One aliquot (1%) of the TLE of *Methanothermococcus thermolithotrophicus* was blown to dryness in a 2-mL vial and added 1 mL of 6 M HCl/MeOH/DCM (1:9:1, v/v/v) for reaction at 70 °C for 3 h to hydrolyze the glycosidic IPLs into core lipids.

2.2. HPLC-APCI-MS analysis of hydroxy-GDGT core lipids

One aliquot of the core lipid fractions of each sample was dissolved in 100 μL n-hexane/isopropanol (99:1, v/v) for HPLC-MS analysis. Separation of compounds was performed on a Prevail Cyano column (2.1×150 mm, 3 μm; Alltech, Grace) maintained at 35 °C in an Agilent 1200 series HPLC with the following gradient program: flow rate of 0.25 mL min^{-1}, the gradient of the mobile phase was first held at 100% of eluent A (n-hexane/isopropanol, 99:1, v/v) for 5 min, followed by a linear gradient to 90% of A and 10% B (n-hexane/isopropanol, 90:10, v/v) in 20 min, followed by a linear gradient to 100% B at 35 min, after holding 100% B for 5 min the column was re-equilibrated with 100% A at a flow rate of 0.6 mL min^{-1} for 5 min before the next injection. Detection was achieved with an Agilent 6130 MSD single quadrupole mass spectrometer, coupled to the Agilent 1200 series HPLC via a multimode ion source set in APCI mode. APCI settings are nebulizer pressure 60 psi, vaporizer temperature 250 °C, drying gas (N_2) flow 6 L min^{-1} and drying gas temperature 200 °C, capillary voltage 2 kV, and corona current 5 μA. With Chemstation software the detector was set for selective ion monitoring (SIM) of $[M+H]^+$ ions (m/z 1302, 1300, 1298, 1296, 1292, fragmentor voltage 190 V).

In order to obtain the MS^2 spectra with detailed fragmentation features, selected samples with higher abundance of targeted compounds were also analyzed with an Agilent 1200 series HPLC system coupled to an Agilent 6520 quadrupole time-of-flight

(qTOF) mass spectrometer through an APCI interface. The APCI drying gas temperature was set at 350 °C with a gas (N_2) flow of 4 L min^{-1}. The qTOF parameters were set to: capillary voltage 1 kV, corona current 5 µA, fragmentor voltage 150 V; skimmer 65 V and octopole 750 V in auto MS/MS scanning mode with MS^1 range of m/z 500-2000 and MS^2 mass range of m/z 100-2000. To achieve the ideal fragmentation of targeted compounds, the mass-dependent collision energy for each precursor was automatically set by the Agilent MassHunter control software (version B.03.01) according to the following equation: [(m/z) / 100]*slope + intercept, where the slope was 3.0 eV and the intercept was -0.5 eV for the normal analysis of GDGTs, but 1.0 eV of slope and -0.5 eV of intercept for the thermolabile hydroxy-GDGTs.

2.3. Isolation of OH-GDGT-0

The TLE of combined sediment from Aarhus Bay and Baltic Sea were dissolved with hexane/isopropanol (99:1, v/v). For each run 200 µL of sample was loaded onto a PerfectSil 100 CN-3 preparative LC column (250*10 mm, 5 µm particle size, MZ Analysentechnik, Germany) equipped with a guard column of the same packing material. With the Agilent 1200 series HPLC system compounds were separated by applying the following solvent gradient at the eluent flow rate of 3 mL min^{-1}: first 100% of eluent A (n-hexane/isopropanol, 99:1 [v/v]) for 3 min, increase to 90% of A and 10% B (n-hexane/isopropanol, 90:10 [v/v]) with a linear gradient in 12 min, then to 100% B at 20 min and hold 100% B for 10 min, before the column was equilibrated with 100% A for 15 min for the next injection. OH-GDGT-0 was collected with a fraction collector (Waters Fraction Collector III) in the time interval from 22 to 23 min.

2.4. NMR analysis of OH-GDGT-0

Around 0.3 mg of purified OH-GDGT-0 was dissolved with 40 µL deuterated benzene (benzene-D_6) and transferred into a 40-µL Zirconia nanoprobe sample tube for 2D ^{1}H-^{13}C-NMR analysis. All spectra were obtained using a Varian Unity-INOVA 500 MHz NMR spectrometer with a Varian Nanoprobe which spins the 40-µL sample

tube at ca. 2 kHz at the magic-angle (54.7°) relative to the applied field axis. The 90° ^{1}H pulse was 10 μs and, for the indirect detection experiments, the ^{13}C 90° pulse was 12.5 μs.

The 1-D 500 MHz ^{1}H NMR spectrum was obtained by collecting 64 transients. Each transient was collected for 3 s, with an additional 2 s relaxation delay prior to each acquisition. The ^{1}H spectral window spanned 4.3 kHz (8.6 ppm) and contained 12881 complex (25762 actual) points. No apodization was applied. The time domain data was zero-filled to obtain a total size of 128k complex (256k actual) data points prior to Fourier transformation. Phase correction and a small amount of baseline correction were applied. The total experiment time was 5 min and 20 s.

The 2-D 500 MHz ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) NMR spectrum was obtained using the same ^{1}H spectral window parameters, except that the digitization time of the FID was restricted to 238.5 ms and the relaxation delay was 1 s, making the total time required for a single pass through the pulse sequence 1.29 s. For each t_1 evolution time, 32 transients were averaged together. 200 complex points were collected in the t_1 time domain (the ^{13}C dimension), but linear prediction was used to extend the data set to 512 complex points (312 complex points were added to the 200 that were collected). The ^{13}C spectral window spanned 10 kHz (80 ppm) and was centered at 40 ppm. The t_2 time domain data was apodized with a Gaussian function of 0.110 s, and the t_1 time domain data was apodized with a Gaussian function of 0.024 s. Baseline correction parallel to the f_2 frequency axis was performed on the half-Fourier-transformed interferogram prior to Fourier transformation of the t_1 time domain into the f_1 frequency domain. The final data matrix was 1k x 1k complex (2k x 2k actual).

The 2-D 500 MHz ^{1}H-^{13}C gradient-selected heteronuclear multiple bond correlation (gHMBC) NMR spectrum was obtained with parameters similar to those used in obtaining the 2-D HSQC NMR spectrum, except that 400 FIDs with unique t_1 evolution times were collected and no linear prediction was used. Each FID was the result of 64 scans. The t_2 time domain data was apodized with a sine bell with a period of 0.119 s (minimum to maximum), and the t_1 time domain data was apodized with a sine bell function with a period of 0.025 s. The final size of the data matrix was 1k x 1k.

2.5. HPLC-ESI-MS analysis of glycosidic hydroxy-GDGTs

For each sample one aliquot of IPL fraction was dissolved in 100 μL of MeOH and DCM (1:5, v/v), and analysis was performed on an Agilent 1200 series HPLC system coupled to a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer via ESI interface. 10 μL of each sample was injected onto a LiChrosphere Diol-100 column (150*2.1 mm, 5 μm particle size; Alltech, Germany) equipped with a guard column of the same packing material.

Separation of lipids was achieved with modified solvent gradient established by Sturt et al. (2004). With a flow rate of 0.2 mL min^{-1}, from 100% A (79:20:0.12:0.04 of hexane / 2-propanol / formic acid / 14.8 M $NH_{3,\ aq}$) to 35% A and 65% B (88:10:0.12:0.04 of 2-propanol / water / formic acid / 14.8 M $NH_{3,\ aq}$) over 45 min, then 100% A for 15 min to re-equilibrate the column for the next analysis. MS analysis was performed in data-dependent ion mode with fragmentation of the base peak ion up to MS^2 under conditions described by Sturt et al. (2004). Intact polar GDGTs with different polar head groups were identified according to the characteristic fragmentation in MS^2 spectra (Sturt et al., 2004; Lipp and Hinrichs, 2009). In addition, different levels of collision energy (normalized collision energy 25% and 100%) for the ion trap setting were applied to optimize detection of labile ions.

2.6. Degradation test on OH-GDGT-0

One aliquot of purified OH-GDGT-0 was subjected to a degradation test. After transferred into a 2-mL vial and dried with a flow of N_2, 1 mL of 6 M HCl/MeOH/DCM (1:9:1, v/v/v) was added into the vial containing OH-GDGT-0 for reaction at 70°C for 3 h. And then, the reaction mixture was dried with N_2 and dissolved in 100 μL of n-hexane/isopropanol (99:1, v/v) for analysis.

3. Results and discussion

3.1. Detection of hydroxy-GDGT with HPLC-APCI-MS

By increasing both the flow rate and the polar gradient of the mobile phase used by the established GDGT core lipid analysis (Hopmans et al., 2000), a series of unknown compounds with nearly identical pattern of molecular and fragment ions but later retention time when compared to the known archaeal GDGTs was generally observed during normal phase HPLC-APCI-MS analysis of marine sediments (Fig. 5-1). For instance, in Fig. 5-1 the extracted ion chromatogram (EIC) of m/z 1298 shows two unknown compounds with the same mass but higher polarity than GDGT-2. By setting the qTOF mass spectrometer to a normal collision energy level (3.0 eV slope) for GDGTs, the MS^2 fragmentation patterns of these three compounds are quite similar (Fig. 5-2a), which suggests similar molecular structures. One small difference is that GDGT-2 gives the major fragment ion of m/z 741, which represents two glycerol units with one monocyclic biphytane, while the other two unknowns both yielded the fragment of m/z 743 resulting from the loss of one biphytane moiety, and thus representing a fragment comprising two glycerol units with one acyclic biphytane.

Under a lower collision energy (1.0 eV slope) the second compound (only yielding m/z 1298 under higher collision energy of 3.0 eV slope) showed a precursor ion of m/z 1316 that generated the daughter ion of m/z 1298 (Fig. 5-2b). And, a precursor ion (molecular ion) of m/z 1334, which yielded the daughter ions of m/z 1316 and 1298, was detected for the third compound (Fig. 5-2b). The observed mass differences of 18 and 36 Da could represent loss of H_2O units due to the labile feature of compounds. Therefore, these lipids do not yield significant molecular ions under collision energy settings typically used for GDGT analysis. Based on their fragmentation features, these two series of unknowns were tentatively identified as OH-GDGT-0, -1, -2 (numbers refer to the numbers of cyclopentane rings) and 2OH-GDGT-0. In addition, according to the fragment ion of m/z 743 in the MS^2 spectrum of 2OH-GDGT-0 (Fig. 5-2a), representing two glycerol units ether bonded to one acyclic biphytane, the two hydroxyl groups of 2OH-GDGT-0 were believed to be located at a single biphytane moiety. Isomers with

different positions of the hydroxyl group(s) may also exist but cannot be distinguished with the analytical protocols applied in this study. The first unknown compound detected at a retention time of about 27 min, which possesses at low collision energy the molecular ion of m/z 1318 and product ion of m/z 1300, is usually the most abundant component among these unknowns. Based on its mass spectrum this compound was identified as OH-GDGT-0 and was then isolated for further structural verification with NMR.

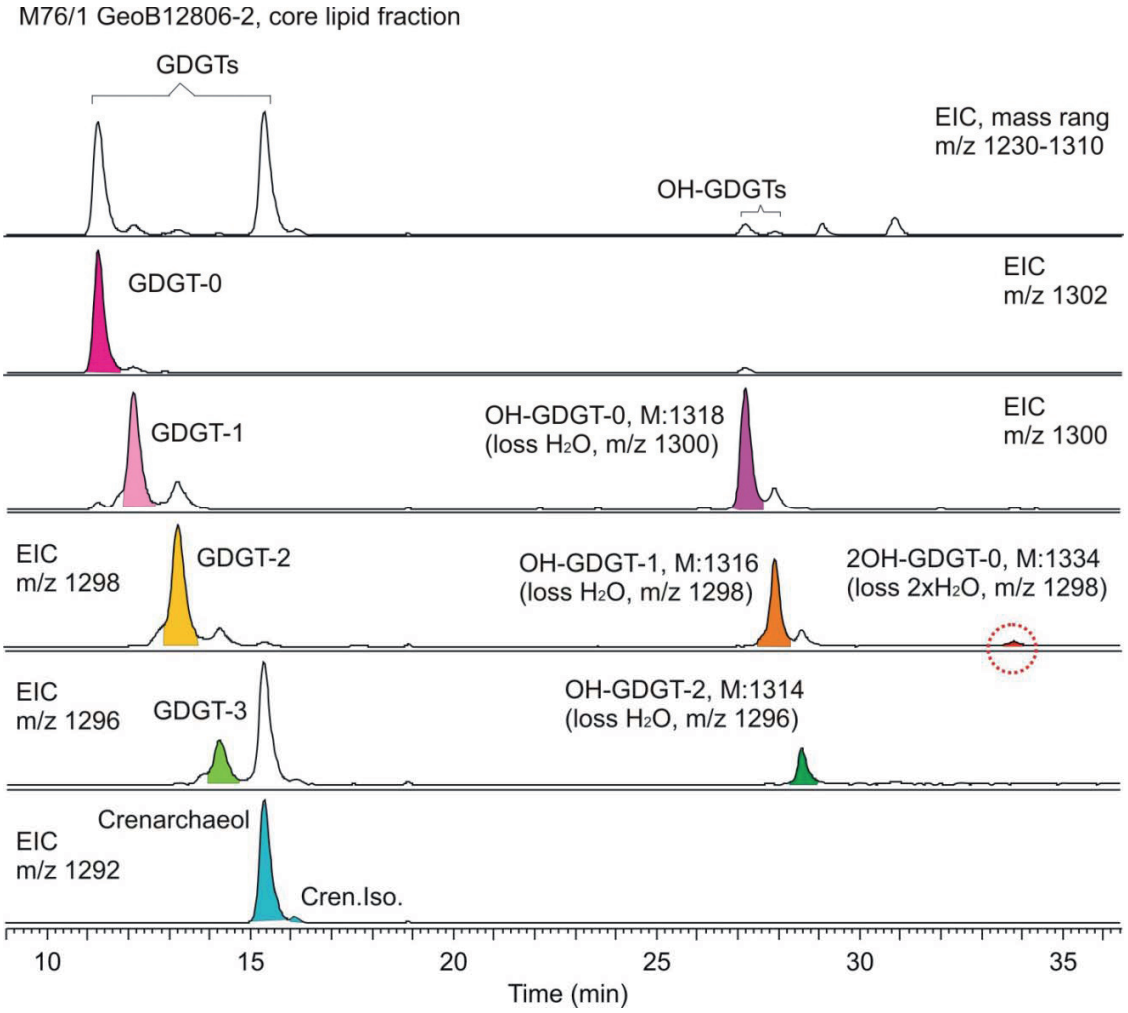

Figure 5-1. HPLC-APCI-MS chromatogram generated by the Agilent 6130 MSD single quadrupol mass spectrometer, showing GDGTs and hydroxy-GDGTs in the core lipid fraction of GeoB 12806-2. Dehydrted ions of monohydroxy-GDGTs and dihydroxy-GDGT are displayed together with GDGTs in separate mass window of EIC. The peak of 2OH-GDGT-0 is highlighted with the dashed line open circle.

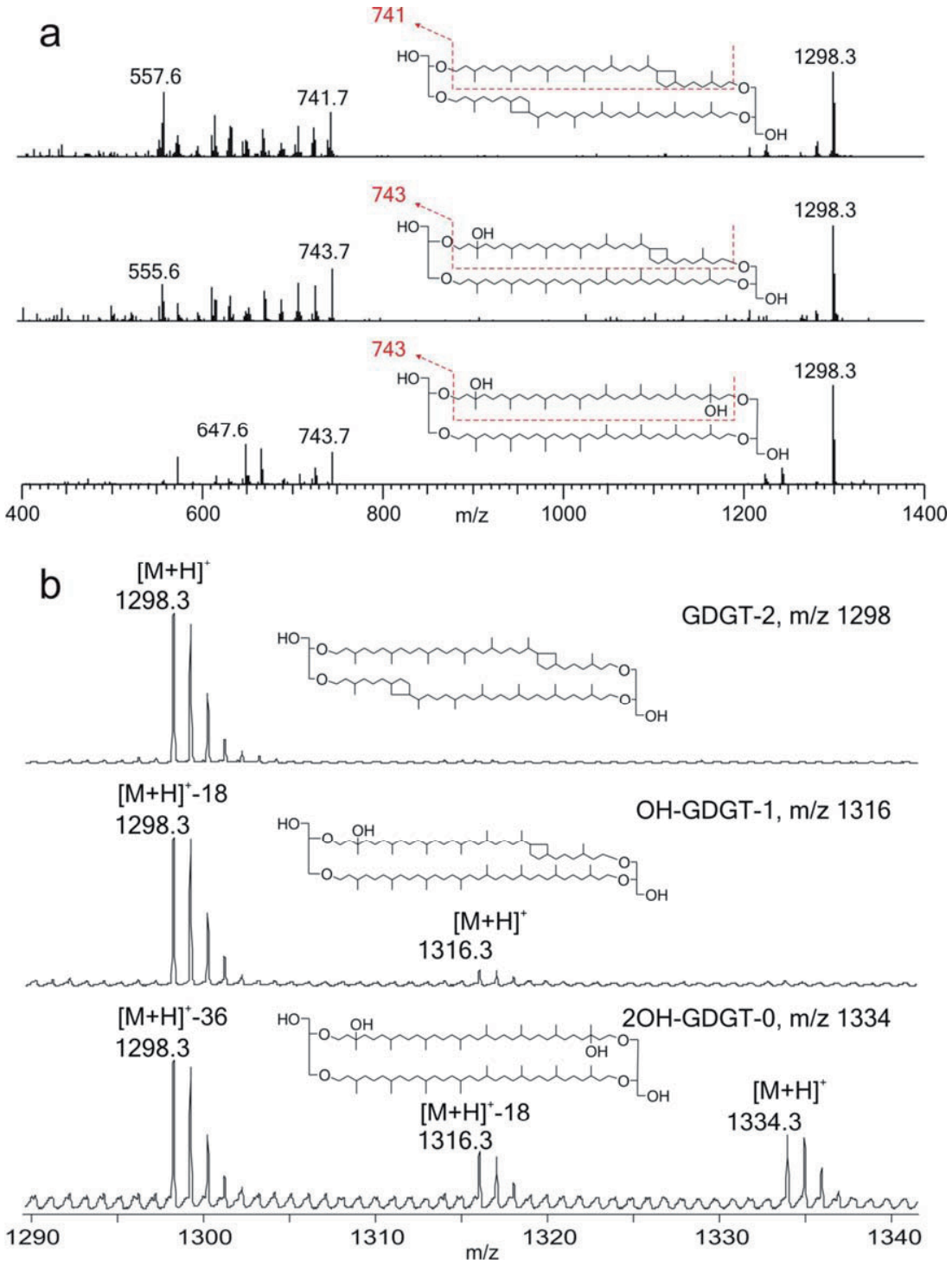

Figure 5-2. MS^2 mass spectra of GDGT-2, OH-GDGT-1 and 2OH-GDGT-0 generated by APCI-qTOF at two different collision energy levels. a) 3.0 eV slope in centroid mode, showing the major fragment ions, and b) 1.0 eV slope in profile mode, showing the dehydration of molecular ions.

3.2. NMR analysis of putative OH-GDGT-0

After two times of purification with preparative HPLC, around 0.3 mg of putative OH-GDGT-0 was isolated from more than 1 kg (dry mass) of combined sediment sample. Due to the inadequate amount, it is difficult to define all the carbon and proton shifts in this big glycerol tetraether molecule, however, the present of one extra hydroxyl group can be confirmed. Given that the carbon skeleton of OH-GDGT-0 has been previously analyzed with NMR for initial determination of acyclic isoprenoid GDGT (cf. Langworthy, 1977; Heathcock, et al., 1988; Sinninghe Damsté, et al., 2002), we here focus our discussion of NMR data on the position of the extra hydroxyl group.

The extra hydroxyl group is attached to one of the isopranyl chains at a position that was formerly a methine. The evidence for this is two-fold: (1) the ^{1}H NMR resonance arising from the methyl group near the hydroxyl group is a singlet, thus indicating it is an isolated spin system (it does not J-couple to any other ^{1}H's 3 bonds removed), and (2) the ^{1}H resonance from the methyl group couples (in the 2D gHMBC NMR spectrum) to a carbon at about 71.77 ppm. No protonated carbon resonances appear in the 2D HSQC NMR spectrum with this chemical shift, thus indicating that this carbon shift arises from a non-protonated carbon site.

Having located the hydroxyl group on a non-protonated carbon site for one of the isopranyl units, we see that the singlet methyl ^{1}H resonance (near the hydroxyl group) correlates with two ^{13}C resonances, one at 43.31 ppm and the other at 40.26 ppm. If the site of the extra hydroxyl group were well removed from the end of the chain (i.e., on the second or third methylated C-atom in the biphytanyl chain), the two adjacent carbon resonances for the methylene groups (CH_2's) adjacent to the non-protonated (OH-bearing) carbon would be nearly the same. This cannot explain the observed difference of more than 3 ppm, unless the hydroxyl group is located at C-3 in one of the biphytanyl chains. A second possibility is that the hydroxyl group resides near the middle of the chain, i.e., at a methylated C-atom adjacent to the head-to-head isopranyl linkage. If this were the case, however, the six carbon resonances arising from the ethyl end groups would show only three unique chemical shifts, instead of the six we observe. That is, the symmetry-

disrupting hydroxyl group must be near one end of the biphytanyl moiety in order for the chemical shifts of the two ethyl ends to be distinct.

Based on the evidence discussed above, we assume that the hydroxyl group is located at a tertiary C-atom closest to one of the two glycerol moieties. If the GDGT skeleton is a trans regioisomer, there will be two different C-3 positions in the molecule. Due to the size of this molecule, it is difficult to determine whether the extra hydroxyl group is located on the glycerol *sn2* or *sn3* ether-bound C-3 site.

Summons et al. (2002) reported the tentative structures of biphytan-N-ol where N= 3 or 7 or 11 or 15, however, we observed isomers of hydroxy-GDGTs with presumably different hydroxyl group positions. Acid hydrolyzed TLE of *Methanothermococcus thermolithotrophicus* yielded two compounds with identical mass spectra (compounds (3) and (4), Fig. 5-3E and F). Compound (3) is the isolated OH-GDGT-0 from marine sediment, so that the later eluting compound (4) could be an isomer of OH-GDGT-0, in which the hydroxyl group is located at another terminal tertiary C-atom in the biphytanyl chain (structures shown in Fig. 5-3). Theoretically, if the hydroxylation only occurred on the C-3 position, two isomers can be formed for the trans regioisomer OH-GDGT-0, four isomers for OH-GDGT-1, two for OH-GDGT-2 (with fixed cyclopentane ring position) and three for the 2OH-GDGT-0 (see the Appendix for the possible structures). In this work we detected only OH-GDGT-0 isomers, while the presence of other hydroxy-GDGT isomers need to be verified with further study. In addition, hydroxy-GDGTs with more than two hydroxyl groups may also exist.

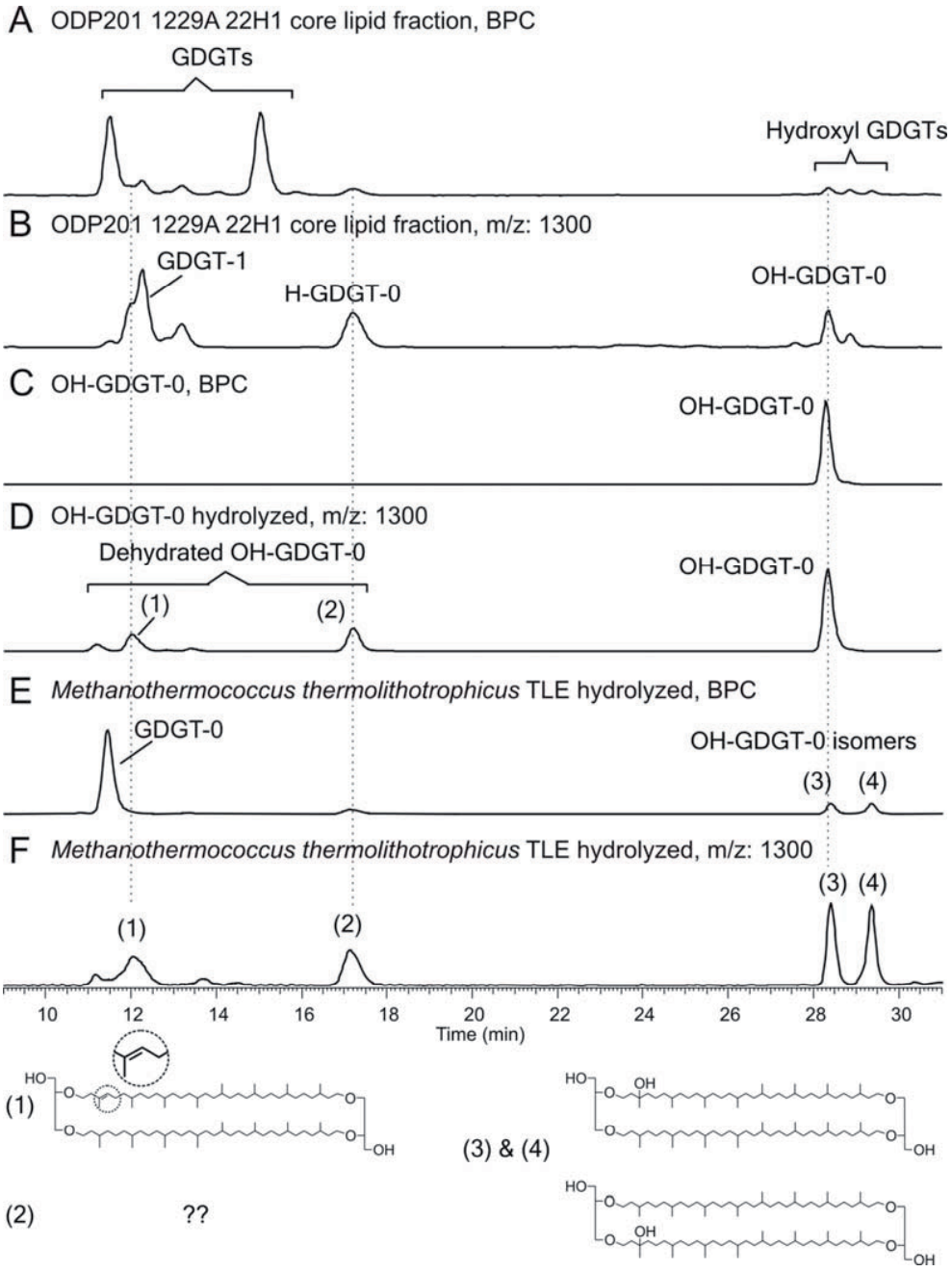

Figure 5-3. HPLC-APCI-MS chromatogram generated by MSD single quadrupole mass spectrometer in SIM mode showing the detection of compounds in ODP201 1229A 22H1 core lipid fraction: (A) base peak chromatogram (BPC), (B) EIC, m/z 1300; in purified OH-GDGT-0: (C) BPC; in acid hydrolyzed OH-GDGT-0: (D) EIC, m/z 1300; and in hydrolyzed TLE of *Methanothermococcus thermolithotrophicus*: (E) BPC and (F) EIC, m/z 1300. Compounds (1) and (2) are two major dehydration products of OH-GDGT-0, (3) and (4) are two isomers of OH-GDGT-0. Possible molecular structures of (1), (3) and (4) are provided, the structure of compound (2) remains unclear.

3.3. Identification of glycosidic hydroxy-GDGT

A number of previous IPL studies have reported in marine samples and archaeal cultures an unknown intact polar GDGT showing a neutral loss of 18 Da heavier than the dihexose moieties in the MS^2 spectrum (Sturt et al., 2004; Schouten et al., 2008b; Schubotz et al., 2009; Lipp and Hinrichs, 2009; Rossel et al., 2011; Pitcher et al., 2011). Termed as H341-GDGT by Lipp and Hinrichs (2009), this unknown lipid was a major archaeal IPL in a wide range of marine sediments (Lipp and Hinrichs, 2009; Rossel et al., 2011) and was also observed in the TLE of the Crenarchaeon *Cand. Nitrosopumilus maritimus* (Schouten et al., 2008b). In the preparative LC cleaned up IPL fractions of twelve marine sediment samples, we detected IPLs with both 18 Da and 36 Da higher molecular weight than mono-, di- and triglycosyl GDGTs (Fig. 5-4).

Fragmentation behavior of H341-GDGT was tested under 100% and 25% collision energy of the ion trap mass spectrometer (Fig. 5-5). With 100% collision energy, the $[M+NH_4]^+$ ion of H341-GDGT (m/z 1660.4) generated only one dominant fragment ion of m/z 1300 (m/z 1299.8 in Fig. 5-5a), but with the moderate 25% collision energy a more detailed fragmentation behavior, including the loss of H_2O and sugar units has been revealed (Fig. 5-5b). Together with the $[M+NH_4]^+$ ion of m/z 1660.4 there are also fragment ions of m/z 1641.8, 1479.8, 1461.5, 1318.3 and 1300.0. The 180.6 Da difference between m/z 1660.4 and m/z 1479.8 represents the loss of one hexose moiety with ammonia adduct. The fragment ion of m/z 1318.3 was then assigned to the core lipid of OH-GDGT-0, and 18 Da difference between m/z 1318 and 1300 represents the loss of H_2O. Therefore, this unknown IPL, H341-GDGT, was tentatively identified to be 2Gly-OH-GDGT. Likewise, all other 18 Da and 36 Da plus IPLs related to 1Gly-, 2Gly- and 3Gly-GDGT were tentatively identified as glycosidic hydroxy-GDGTs (see molecular structures provided in Fig. 5-4).

Composition of these glycosidic hydroxy-GDGTs varied within different samples. Different distributions of intact polar GDGTs in analyzed samples were shown in Table 5-1. 2Gly-OH-GDGT was generally the most common OH-GDGT IPL, which is consistent with the earlier observations (cf. Lipp and Hinrichs, 2009; Schubotz et al., 2009).

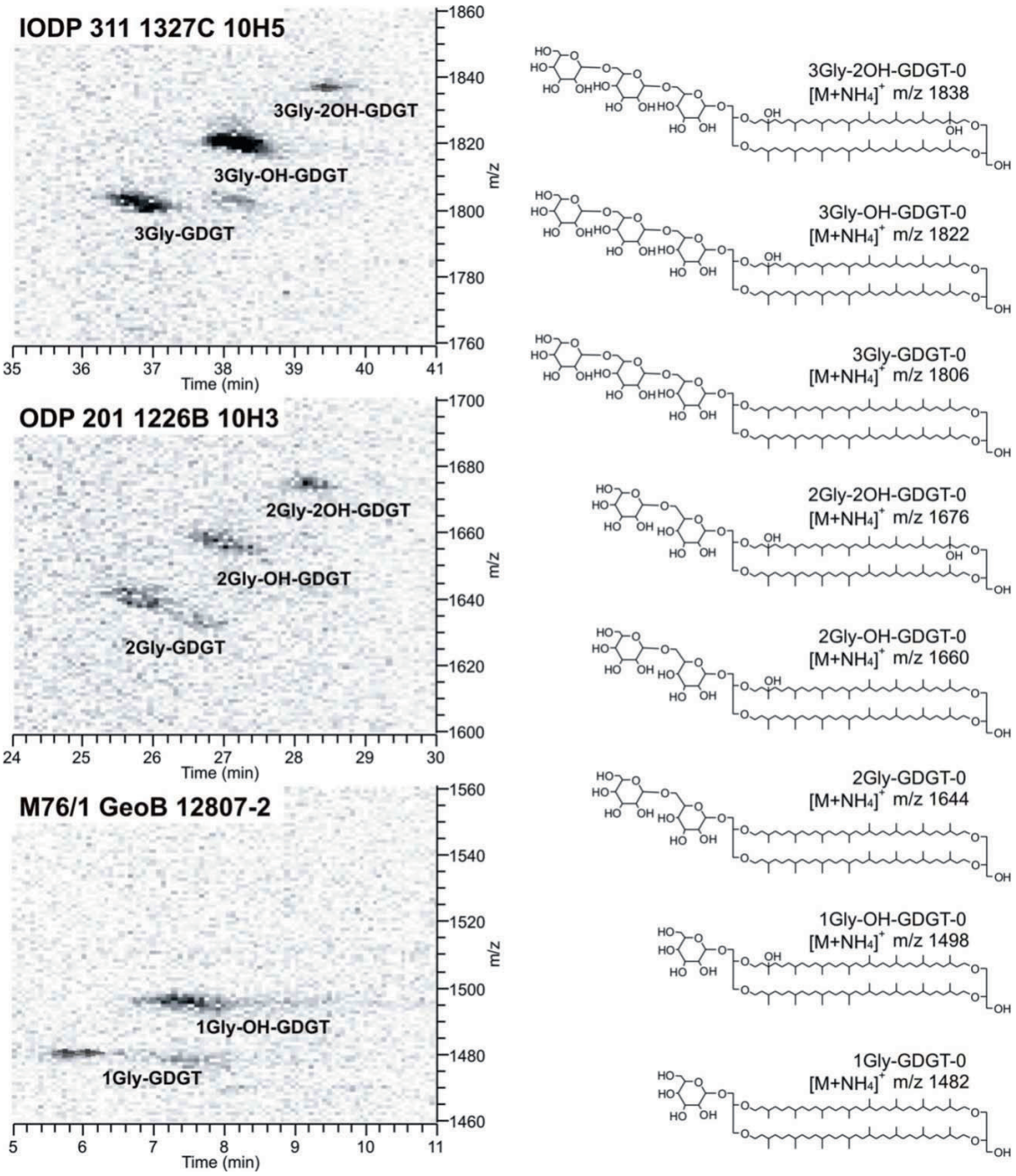

Figure 5-4. Density maps generated by ESI-ion trap mass spectrometer showing the detection of glycosidic GDGTs and hydroxy-GDGTs in the IPL fractions of samples IODP 311 1327C 10H5, ODP 201 1226B 10H3 and M76/1 GeoB 12807-2. Tentative molecular structure of compounds were also shown.

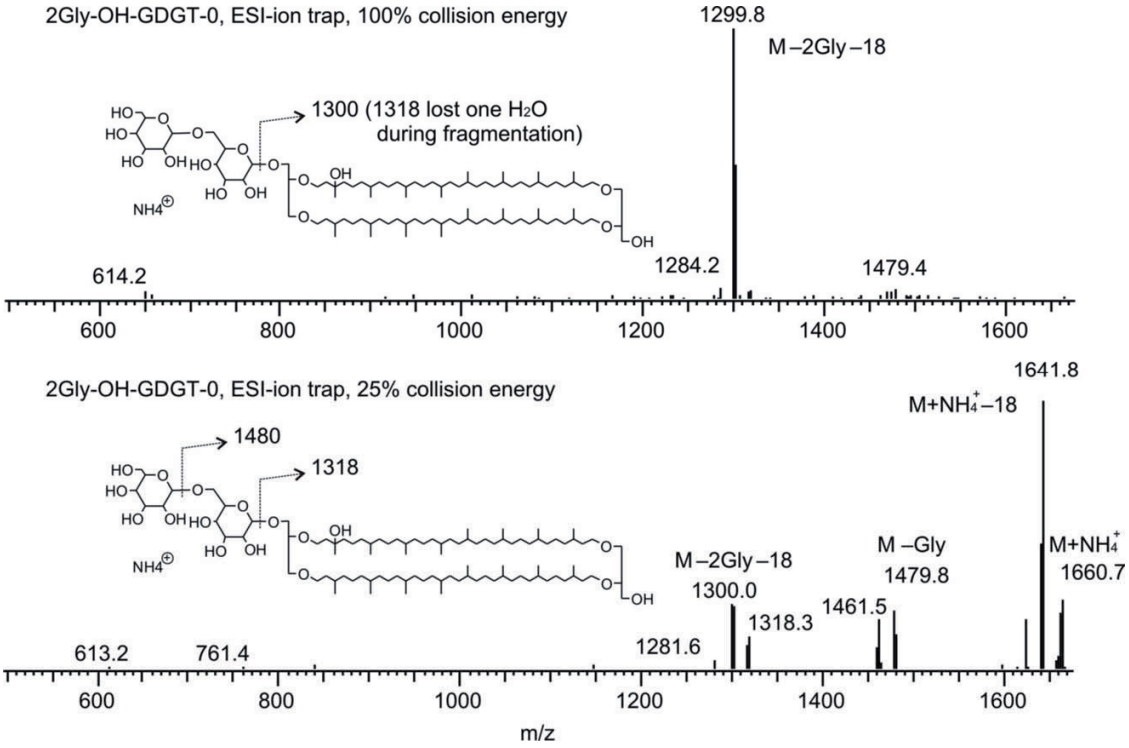

Figure 5-5. MS^2 mass spectra of 2Gly-OH-GDGT-0 in sample ODP 201 1229D 4H4 generated by ESI-ion trap mass spectrometer, showing the fragmentation features that under 100% collision energy (upper panel) the neutral loss of 2 hexose moieties together with the hydroxyl group (as H_2O) on biphytanyl moiety, and under 25% collision energy (lower panel) the successive losses of hexoses and the biphytanyl hydroxyl group.

3.4. Distribution and abundance of hydroxy-GDGT core lipids

Hydroxy-GDGT core lipids occurred in all analyzed marine sediments. With our present analytical protocol it is difficult to obtain the actual abundance because of their dehydration during ionization. However, relative abundance of hydroxy-GDGTs compared to total GDGTs was estimated with the APCI-MS detection of both GDGT and hydroxy-GDGT core lipids in the same analysis (data shown in Table 5-1). Indicated ratios are based on the peak areas of $[M+H]^+$ ions of GDGTs, $[M+H-18]^+$ ions of OH-GDGTs and $[M+H-36]^+$ ion of 2OH-GDGT-0, for example, m/z 1298 for GDGT-2, OH-GDGT-1 and 2OH-GDGT-0 (Fig. 5-1). This calculation must have underestimated the actual abundance of hydroxy-GDGTs but are useful for comparing relative distributions

between samples. In analyzed core lipids fractions of marine sediments the calculated ratio of hydroxy-GDGTs ranged from 4% to 16% of the total detected tetraether core lipids while 2OH-GDGT-0 comprises less than 1%. The widespread occurrence and remarkable abundance of hydroxy-GDGTs in the marine environment made these compounds an important target for future studies concerned with archaeal lipids.

3.5. Source of hydroxy-GDGT

In all analyzed marine sediments the ring composition of hydroxy-GDGT core lipids is quite different to that of non-hydroxylated GDGTs, which implies probably different sources of two lipid groups. As it is shown in Fig. 5-1, GDGTs consist of 0-, 1-, 2-, 3- and 5-ring structures (GDGT-4 was not taken into account because of the co-elution with crenarchaeol and the interference of $[M+H+2]^+$ signal of crenarchaeol) and with GDGT-0 and crenarchaeol as the dominant components, however, there are only 0-, 1- and 2-ring structures detected in hydroxy-GDGTs, and with the acyclic structure as the most abundant component in both mono- and dihydroxy-GDGTs.

Except for marine sediments, in the TLE of *Methanothermococcus thermolithotrophicus* GDGT-0 and OH-GDGT-0 were found in the form of free core lipids with low abundance, while as IPLs we detected 2Gly- and 3Gly-OH-GDGT (Table 5-1). Reported by Schouten et al. (2008), a 2Gly-GDGT with 18 Da elevated molecular mass was detected in *Candidatus Nitrosopumilus maritimus*, a marine group I crenarchaeon, and we suggest that this compound, as the H341-GDGT in a wide range of marine sediments, is 2Gly-OH-GDGT. The presence of hydroxy-GDGT in these two archaeal cultures showed that the capability of synthesizing hydroxy-GDGTs is not limited to either crenarchaeota or euryarchaeota (*M. thermolithotrophicus*). However, the sources of hydroxy-GDGTs in marine sediments may be diverse and remain to be constrained.

3.6. Degradation of hydroxy-GDGTs

Due to the labile property of tertiary alcohols (cf., Sprott et al., 1990), degradation of hydroxy-GDGT in sedimentary environments needs to be considered for their relevant studies. A simple degradation test was conducted by acid hydrolysis on purified OH-GDGT-0 and the TLE of *Methanothermococcus thermolithotrophicus*. Two major dehydrated products resulting from hydrolyzed OH-GDGT-0 were detected (Fig. 5-3D). The first dehydrated product (compound (1) in Fig. 5-3), which nearly co-eluted with GDGT-1, showed the same molecular mass of 1300 Da as GDGT-1 and identical fragmentation behavior in MS^2. Compound (1) was assumed to be an acyclic GDGT with one double bond (see structure showed in Fig. 5-3). During diagenesis in sediments, GDGTs with one double bond and zero to two rings can be generated by dehydration of OH-GDGT-0, 1 and 2. Presumably, the dehydrated products of OH-GDGT-1 and 2 may also elute out right before GDGT-2 and 3. This could explain the commonly observed small front shoulders of GDGT-1, 2 and 3, but none for GDGT-0, in the LC chromatograms obtained by analyzing marine sediments and cultures. These earlier-eluting compounds were usually presumed to be isomers of GDGT-1, 2 and 3 (e.g., Pitcher et al., 2009; 2011).

The second dehydration product (compound (2) in Fig. 5-3D and F) has also the molecular mass of 1300 Da and reacts similarly in MS^2 as GDGT-1, but has a higher polarity as indicated by its elevated retention time. It is questionable whether a different double bond position, as a result of dehydration from a different OH-GDGT isomer, can cause this shift in retention time. Pitcher et al. (2011) reported the detection of glycosidic hydroxy-GDGTs (shown as GDGTs with M+180 and 2x180 head groups) in marine sediment enrichments. In their core lipid fractions treated with acid hydrolysis, these authors observed both earlier and later eluting 'isomers' of GDGT-1, -2 and -3. According to the LC method used in Pitcher et al. (2011), their later eluting 'isomers' could be the second dehydrated product (compound (2)) and other analogues derived from OH-GDGT-0, -1 and -2.

Interestingly, we also noticed that compound (2) nearly co-elutes with H-shaped GDGT-0 (structure determined by MS^2 spectrum, Liu et al., unpublished data) that was

abundant in one of our marine sediments, ODP201 1229A 22H1 (Fig. 5-3A and B). The same molecular mass, m/z 1300, and co-elution of compound 2 and H-shaped GDGT-0 points to the danger of identifying H-shaped GDGT only on the basis of retention time and molecular mass (e.g., Schouten et al., 2008a).

GDGTs are stable molecular biomarkers which are abundant even in marine sediments of Cretaceous age (Kuypers et al., 2002), and planktonic archaea thriving in the surface water column are considered to be the predominant source of GDGT in marine sediments (Schouten et al., 2002; Wuchter et al., 2006). However, these less stable hydroxy-GDGTs, especially the intact polar hydroxy-GDGTs, detected in deep subsurface marine sediments up to 12 Ma of age, for instance ODP201 1226E 20H3 (see sample description in Liu et al. 2011), may strongly reflect the *in-situ* contribution of benthic archaea living in sediments. On the other hand, the two isomers of OH-GDGT-0 occurred with almost equal abundance in the extract of *Methanothermococcus thermolithotrophicus* (Fig. 5-3F), but no later isomer detected in marine sediments (e.g., Fig. 5-3B). This could be a phylogenetic signal of different species composition or a diagenetic effect involving two isomers with possibly different molecular stability. Potential proxies indicating thermal maturity of organic matter or lipid input of benthic archaea may be developed based on the analysis of hydroxy-GDGTs in the future.

4. Conclusion

We identified OH-GDGT-0 on the basis of its mass spectrometric behavior and NMR analysis. 2D-NMR analysis revealed an extra hydroxyl group located at the tertiary C-3 of one biphytanyl moieties. Tentative identification of monohydroxy-GDGTs with zero to two rings and dihydroxy-GDGT extended the knowing diversity of archaeal lipids in marine sediments. These widespread hydroxy-GDGTs are subject to dehydration during the commonly applied protocols for LC-APCI-MS analysis of GDGTs, and consequently gave the artificial signal of 18 Da less than their molecular mass. We also successfully identified the corresponding IPLs, comprising of a series of glycosidic hydroxyl-GDGTs that had been reported as unknowns in several previous studies.

Because of the occurrence of hydroxyl-GDGTs in twelve globally distributed marine sediments, we suggest that these lipids are as ubiquitous and widespread in marine sediments as their non-hydroxylated analogues. In our analyzed samples the relative abundance of OH-GDGT is substantial, 4%-16% of total isoprenoid GDGTs.

In marine sediments hydroxy-GDGTs contain up to two rings with the acyclic compound being the most abundant component. Their biological source is not clear, but we identified hydroxy-GDGT in one methanogen species, *Methanothermococcus thermolithotrophicus,* and based on previous findings of putative 2Gly-OH-GDGT in *Candidatus Nitrosopumilus maritimus,* it appears that both crenarchaeota and euryarchaeota are able to synthesize this compound. Dehydrated products of OH-GDGT, which can be produced by acidic hydrolysis, are candidates for the commonly observed fronting shoulders of peaks of GDGT-1, -2 and -3.

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Chapter 6

Distribution of glycerol ether lipids in marine sediment: structural diversity and biogeological implications

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ABSTRACT

Glycerol-based alkyl ether lipids are prominent constituents of archaeal lipid membranes but also occur in some Bacteria. Due to the relatively high stability of ether lipids and their widespread biological sources, glycerol alkyl ethers are major lipids in soils and sediments. Examination of mass spectral data obtained from analysis of extracts from twelve globally distributed marine subsurface sediments extended the known range of the structural diversity of glycerol ether lipids. The structures of more than forty glycerol di- and tetraethers were recognized by their characteristic fragment ions and fragmentation patterns. A general distribution of ubiquitously occurring ether lipids in marine sediments was summarized into eleven structural groups, in which isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) were counted to be the most dominant component (in average over 70% of total ethers), followed by 8% of hydroxyl GDGTs and 7% of glycerol dibiphytanol diethers. The percentages of archaeol and branched GDGTs were only 2% and 1%, respectively. Newly identified hybrid isoprenoid/branched GDGTs account for 2% of the total ether lipids, while one tentatively identified H-shaped GDGT occurred with a remarkable abundance of more than 6%, while the other lipids, such as acyclic glycerol trialkyl glycerol tetraether, and

the first reported hydroxyl glycerol dibiphytanol diethers, overly branched GDGTs and sparsely branched GDGTs only exist in minor amounts of less than 1% for each group.

1. Introduction

Isoprenoid glycerol ethers are characteristic membrane lipids of Archaea that are primarily observed in cultivated extremophiles (Kates et al., 1963; DeRosa et al., 1976; Langworthy, 1977) and methanogens (Makyla and Singer, 1978; Tornabene et al., 1979). Phylogenetic analysis based on ribosomal RNA and DNA revealed the widespread occurrence of non-extremophilic archaea in marine water column and sediments (DeLong et al., 1992; Fuhrman et al., 1992; Vetriani et al., 1998, 1999). Archaeal ether lipids were also detected in non-extreme environments with mesophilic crenarchaeota as the major biological source (Hoefs et al., 1997; DeLong et al., 1998; Schouten et al., 1998, 2000).

In sediments the most commonly detected isoprenoid ether lipids are glycerol diphytanyl diether, also called archaeol, and glycerol dibiphytanyl glycerol tetraethers (isoprenoid GDGTs) with 0~3 cyclopentane rings and crenarchaeol. These ether lipids had been used as the indicator for the presence of Archaea and their carbon metabolism. For example, in 1999 Hinrichs et al. reported the existence of methane consuming archaea in marine sediment based on the detection of ^{13}C depleted archaeol and hydroxy-archaeol. A sea surface temperature (SST) proxy, TEX_{86}, defined by the ratio of isoprenoid GDGTs with different number of rings, was developed by assuming planktonic archaea in surface water column as the primary source of sediment preserved isoprenoid GDGTs (Schouten et al., 2002). On the other hand, the structural diversity of ether lipids in natural settings is high (Schouten et al., 2000), including not only the isoprenoid archaeal diethers and tetraethers but also non-isoprenoid ether lipids, such as branched GDGTs (Sinninghe Damsté et al., 2000) that are likely of soil bacterial origin (Weijers et al., 2006). And, based on the dominant distribution of branched GDGTs in terrestrial environments, a molecular proxy called BIT had been developed for reconstructing soil input into marine environments (Hopmans et al., 2004). Exploring the diversity of ether lipids in marine sediments will provide us more insight into the composition of source organism and their role in relative environments.

In the early stage of lipids analysis the detection and identification of these high molecular weight ether lipids was mainly relied on gas chromatograph mass spectrometer (GC-MS) analysis of the ether cleavage reaction released derivatives. Even though the later established analytical methods with liquid chromatograph mass spectrometer (LC-MS) can analyze the intact core isoprenoid GDGTs (e.g. Hopmans et al., 2000), application of these methods only restricted to those well-known GDGTs. Using ion trap and quadrupole time-of-flight (QTOF) mass spectrometer as the detectors, we conducted a survey of the diversity of ether lipids in various marine sediments. We also described in this work the method for analyzing some specific lipids with electron spray ionization (ESI). The basic structures of known diethers, GDGTs and numerous not yet described ether lipids are recognizable according to their fragmentation patterns. Most of these newly detected ether lipids present in all analyzed sediments. Biological sources and implications of these ether lipids were discussed in each structural group.

2. Experiments

2.1. Materials and sample preparation

Twelve globally distributed marine sediments from various geological settings were collected for lipid analysis (Table 6-1). More detailed information on sampling sites has been published previously in Liu et al. 2011. All marine sediment samples were freeze-dried and extracted using the modified Bligh and Dyer protocol as described by Sturt et al. (2004). In addition, four archaeal cultures, *Methanococcus Thermolithotrophicus* strain (DSM 2095), *Methanococcus igneus* (DSM 5666) and *Methanopyrus kandleri*, (DSM 6324) grown at 85°C in enamel-protected fermentors with stirring (400 rpm) and continuous gassing (H_2/CO_2, 80:20) were kindly provided by Manuela Baumgartner (University of Regensburg, Germany); *Archaeoglobus fulgidus* was provided by Michael Thomm (University of Regensburg, Germany) and cultivated according to the method described in Rothe and Thomm (2000).

Using an ultrasonic probe (Bandelin electronic GmbH & Co. KG), 0.5 g freeze-dried biomass of each culture was extracted with 20 ml dichloromethane and methanol

[1:1, v/v] mixture for four times for 15 min. One aliquot TLE of each biomass was treated with 1 ml of 6 M HCl/MeOH/CH_2Cl_2 [1:9:1, v/v] at 70 °C for 3 h to hydrolyze the intact polar lipids into core lipids.

2.2. HPLC-APCI-MS analysis

One aliquot of each sample was dissolved in 200 µl hexane/isopropanol [99:1, v/v] for HPLC-MS analysis. Separation of compounds was performed on a Prevail Cyano column (2.1×150 mm, 3 µm; Grace) maintained at 35 °C in an Agilent 1200 series HPLC. Using a flow rate of 0.25 ml min^{-1}, the gradient of mobile phase was first hold for 5 min with 100% of eluent A (n-hexane/isopropanol, 99:1 [v/v]), followed by a linear gradient to 90% of A and 10% B (n-hexane/isopropanol, 90:10 [v/v]) in 20 min, followed by a linear gradient to 100% B at 35 min, after hold at 100% B for 5 min. After analysis the column was equilibrated with 100% A at a flow rate of 0.6 ml min^{-1} for 5 min before the next injection.

Mass spectrometric identification of lipids were performed on a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer coupled to the HPLC by an atmospheric pressure chemical ionization (APCI) interface. APCI settings were as follows: capillary temperature 200 °C, source heater temperature 400 °C, sheath gas flow 30 arbitrary units, source current 5 µA, MS^1 mass range m/z 500-1500.

In order to get more detailed fragmentation features and higher mass resolution than ion trap, selected samples with higher abundance of targeted compounds selected samples were also analyzed with an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer connected to an Agilent 1200 series HPLC system via an APCI interface. The APCI source temperature was set at 350°C with a gas (N_2) flow of 4 L min^{-1}. Q-TOF parameters of capillary voltage 1 kV, corona 5 µA, fragmentor 150 V, skimmer 65V and octopole 750V were set for auto MS/MS scanning mode with MS^1 range of m/z 500-2000 and MS^2 m/z 100-2000.

Quantification of lipids was achieved with an Agilent 6130 MSD single quadrupole mass spectrometer, coupled to the Agilent 1200 series HPLC via a multimode ion source set in atmospheric pressure chemical ionization (APCI) mode. APCI settings

are nebulizer pressure 60 psi, vaporizer temperature 250°C, drying gas (N2) flow 6 L/min and drying gas temperature 200°C, capillary voltage 2 kV, and corona current 5 μA. Selective ion monitoring (SIM) was set on $[M+H]^+$ ions of targeted compounds.

2.3. HPLC-ESI-MS analysis

One aliquot TLE of *Methanococcus Thermolithotrophicus* and *Archeaoglobus Fulgidus* was directly analyzed with ESI coupled mass spectrometer, but with the same LC condition, column and mobile phase as for the APCI-MS analysis of core lipids. A buffer mixture of H_2O/MeOH/formic acid/NH_{3aq} [1:1:0.01:0.01, v/v] was infused in 0.02 ml min^{-1} with a syringe pump via a T-piece set between column and ion source. ESI settings were according to the published parameters (Sturt et al., 2004).

3. Results and discussion

Within this survey of ether lipids distribution in marine sediments, around forty compounds, including the well-known lipids, newly identified lipids and unknowns, were found present in all analyzed marine sediments. In an artificially constructed composite chromatogram compounds were shown in the order of theoretical retention time (Fig. 6-1). The relative abundance of eleven lipid groups in twelve analyzed marine sediments and their average values were listed in Table 6-1. Calculation is based on the peak area of individual compound of single injection by ignoring the various response factors of different compounds and for those hydroxylated compounds detection is only based on the dehydrated ion. The calculation of relative abundance was therefore considered as only rough estimation. These ether lipids are recognizable by their $[M+H]^+$ ion and characteristic fragment patterns. In the following paragraph we will discuss the identification of these lipids, their origin and the resulting implications; this discussion will be structured according to distinct structural groups.

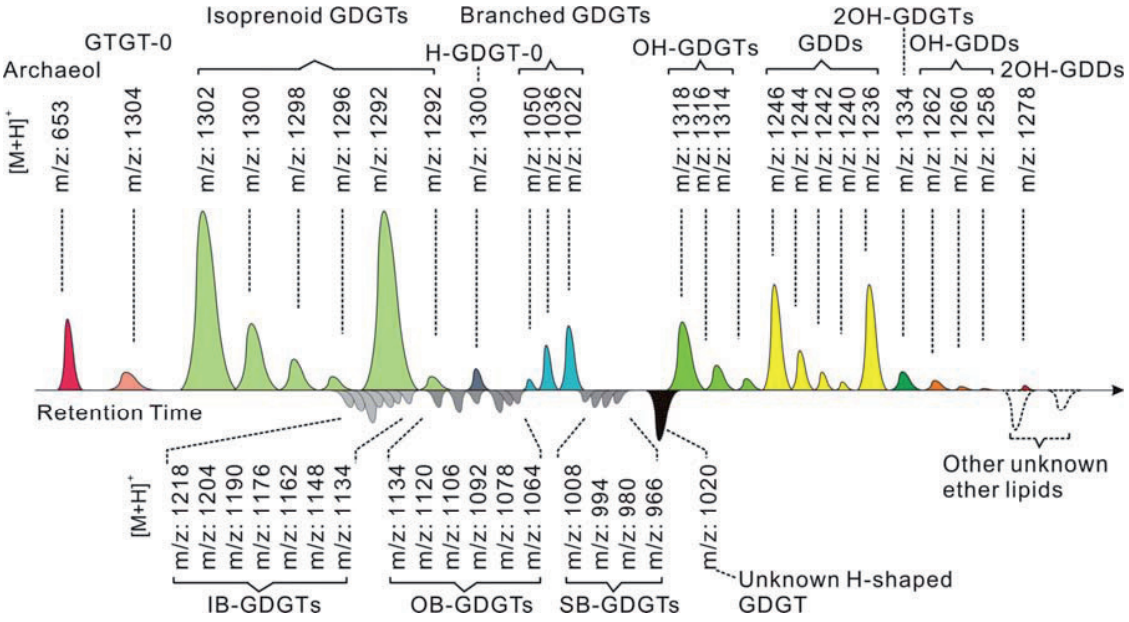

Figure 6-1. Artificially constructed composite chromatogram showing the distribution of commonly occurred ether lipids in marine sediments. Theoretical retention time of compound reflects only the order of elution on real measurement of normal phase LC performed with Prevail Cyano column (2.1×150 mm, 3 μm; Grace). Compounds having similar structure were coded with the same color. Peaks under the base line of chromatogram are compounds with undetermined structures. Abbreviations: acyclic glycerol trialkyl glycerol tetraethers (GTGT-0), acyclic H-shaped GDGT (H-GDGT-0), monohydroxy-GDGTs (OH-GDGTs), glycerol dibiphytanol diethers (GDDs), dihydroxy-GDGTs (2OH-GDGTs), monohydroxy-GDDs (OH-GDDs), dihydroxy-GDDs (2OH-GDDs), hybrid isoprenoid/branched GDGTs (IB-GDGT), overly branched GDGTs (OB-GDGTs), sparsely branched GDGTs (SB-GDGTs).

3.1. Archaeal diethers

Glycerol diphytanyl diether, also called archaeal (Ar), is the most commonly observed archaeal diether lipids in natural environment; on average, it accounts for about 2% of total recognized ether lipids in the analyzed marine sediments (Table 6-1). In normal phase liquid chromatography, Ar is the first eluting ether lipid with the characteristic $[M+H]^+$ ion of m/z 653 and fragment ion m/z 373 (Fig. 6-1). Ar is present in a wide range of marine environments (e.g. Turich et al., 2007) but usually dominant in methane seep settings and attributes to the presence of methanogenic and methanotropic archaea (Hinrichs et al., 2000; Pancost et al., 2000; 2001; Rossel et al., 2008).

The intact archaeol determined as phosphatidyl glycerophosphate was first isolated and characterized in the culture of *Halobacterium cutirubrum* (Kates et al., 1963 and 1965), and then Ar was also identified in methanogenic archaea, (Tornabene et al., 1979). Besides, there are also other kinds of archaeal diethers, such as hydroxy archaeol (OH-Ar) first identified in *Methanothrix concilii* by Ferrante et al. (1988), extended C_{20}, C_{25} Ar and C_{25}, C_{25} Ar (DeRosa et al., 1982; 1983a) in alkaliphilic halophiles, OH-C_{25}-Ar in a marine seep environment (Stadnitskaia et al., 2008), and macrocyclic Ar first identified in a methanogen isolated from hydrothermal vent (Comita et al., 1983). A detection of macrocyclic Ar and Ar in lipid extract of *Methanococcus igneus* was shown in Fig. 6-2. However, these bizarre archaeal diethers only occurred in minor abundance and restricted to specific geological settings.

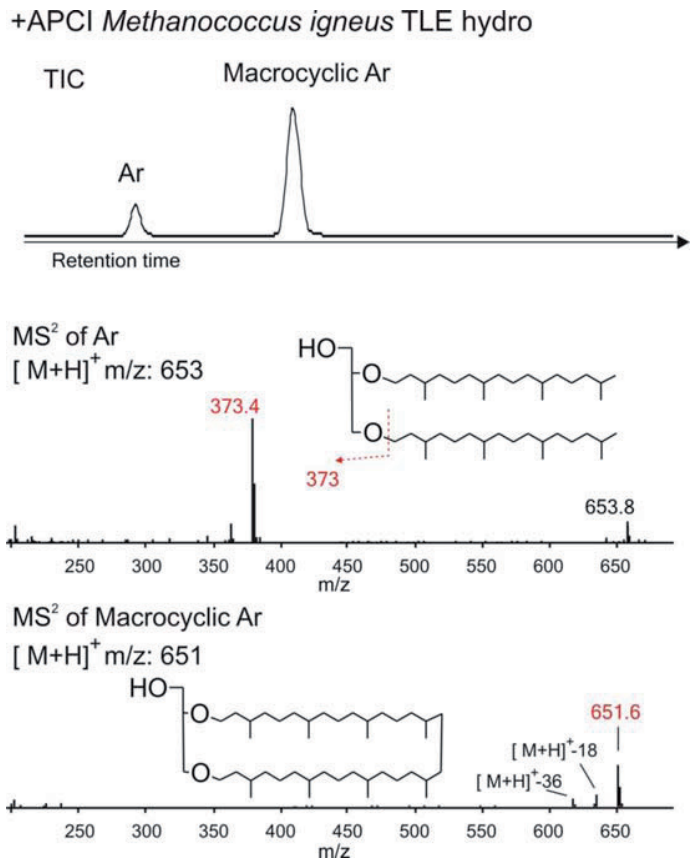

Figure 6-2. LC-APCI-MS (QTOF) chromatogram of hydrolyzed TLE of *Methanococcus igneus*, and the MS^2 spectra of Ar, $[M+H]^+$ m/z: 653, showing characteristic fragment: m/z 373, and of macrocyclic Ar, $[M+H]^+$ m/z: 651, showing fragments related to loss of water.

After the derivatization of hydroxyl groups with BSTFA, Ar and hydroxyl Ar are detectable with gas chromatography (GC) (Hinrichs et al., 1999; 2000; Pancost et al., 2000), but in this work we detected OH-Ar with LC-MS. TLE of *Methanococcus thermolithotrophicus* was injected for OH-Ar analysis. Due to the reactivity of the tertiary hydroxyl group, OH-Ar and the stronger ionization energy of APCI compared to ESI, no clear signal of hydroxy-Ar was obtained with APCI-MS, but an acceptable detection of Ar, Sn2-OH-Ar, Sn3-OH-Ar and 2OH-Ar has been achieved with ESI-MS (Fig. 6-3).

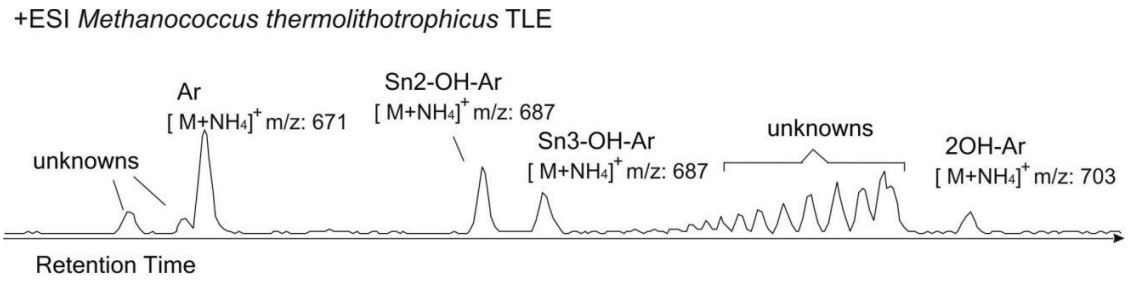

Figure 6-3. LC-ESI-MS (QTOF) chromatogram of Ar, Sn2-OH-Ar, Sn3-OH-Ar and 2OH-Ar detected in the TLE of *Methanococcus thermolithotrophicus*.

In addition, because of the acid lability of OH-Ar, it was known that with strong acid treatment OH-Ar would be converted to glycerol monophytanyl monoether (GMM) (Koga et al., 1993; Oba et al., 2006). However, in this work we identified mono-glycosidic GMM in the TLE of *Archeaoglobus Fulgidus* (Fig. 6-4), which suggested the presence of GMM as a natural lipid. (1Gly-DMM, $[M+NH_4]^+$ m/z: 552, characteristic fragment: m/z 373, neutral loss: 180 Da). Presumably, there could be GMM in natural environment as well. Further studies searching for GMM in natural environment is needed.

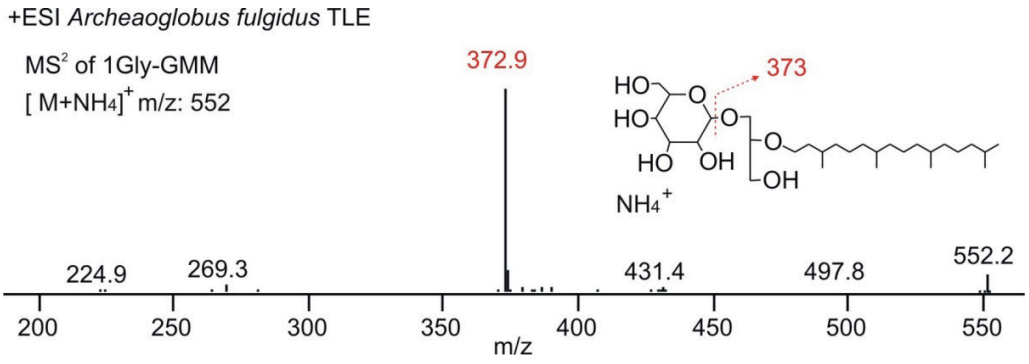

Figure 6-4. MS^2 (LC-ESI-MS, ion trap) mass spectrum of 1Gly-GMM identified in the TLE of *Archeaoglobus fulgidus*. $[M+NH_4]^+$ m/z: 552, characteristic fragment: m/z 373.

3.2. Glycerol Trialkyl Glycerol Tetraethers (GTGT)

GTGT-0 containing one biphytane and two phytanes as its alkyl chain is also a generally present archaeal tetraether in our analyzed marine sediments that elutes between Ar and GDGT-0 (Fig. 6-1). Comparing to glycerol diethers and GDGTs, GTGT gives a very distinctive fragmentation pattern in the mass spectrum from APCI-MS analysis (Knappy et al., 2009), which makes the general structure of GTGT easily recognizable, for example GTGT-0 possesses the $[M+H]^+$ ion of m/z 1304 and characteristic fragment ion m/z 373, 931, 1023 (Fig. 6-5A). Accordingly, one of the unknowns, m/z 1117, in ODP 207 4R, can be recognized to have a glycerol trialkyl glycerol tetraether structure (Fig. 6-4B).

GTGT-0 was first identified in *Sulfolobus solfataricus* (DeRosa et al., 1983b), and a recent study showed its presence in another species of Crenarchaeota (Schouten et al. 2008c). In this work we detected GTGT-0 also in a Euryarchaeota species, *Archeaoglobus fulgidus,* and all marine sediments. Even though the relative abundance of GTGT-0 in analyzed marine sediments is very low, around 0.2% (Table 6-1), this compound is a potential molecular precursor of both biphytane and phytane after degradation in natural environments. The presence of phytanes in sediments should therefore not only be attributed to Chlorophyll and Ar but also to GTGT.

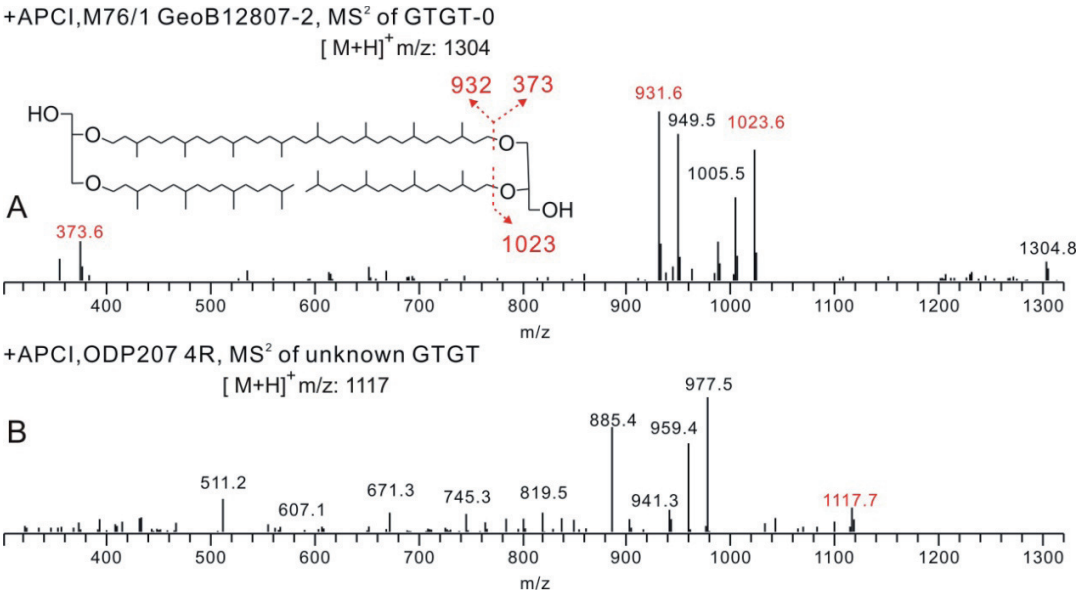

Figure 6-5. MS^2 (LC-APCI-MS, ion trap) mass spectrum of GTGT-0 (A), $[M+H]^+$ m/z: 1304, characteristic fragment: m/z 373, 931, 1023; and one unknown GTGT (B), $[M+H]^+$ m/z: 1117.

3.3. Isoprenoid GDGTs

The most commonly observed isoprenoid GDGTs in marine sediments are compounds with 0-3 cyclopentane rings, crenarchaeol and the regioisomer of crenarchaeol (Schouten et al., 2000; 2002). Isoprenoid GDGTs are also usually the most abundant archaeal ether lipids in marine sediments (Schouten et al., 2000), and in the analyzed marine sediments of this work, an average relative abundance of around 71% was detected (Table 6-1). One example of the common distribution of isoprenoid GDGTs in marine sediments and their characteristic MS^2 pattern was given in Fig. 6-5. Together with ions of $[M+H]^+$, $[M+H]^+$-18 and $[M+H]^+$-74, the most distinguishable fragments of isoprenoid GDGTs are the loss of one biphytane ions (Fig. 6-5B). With the MS^2 pattern of isoprenoid GDGTs, we observe that preferentially the more unsaturated biphytane moiety in each molecule is initially lost during fragmentation.

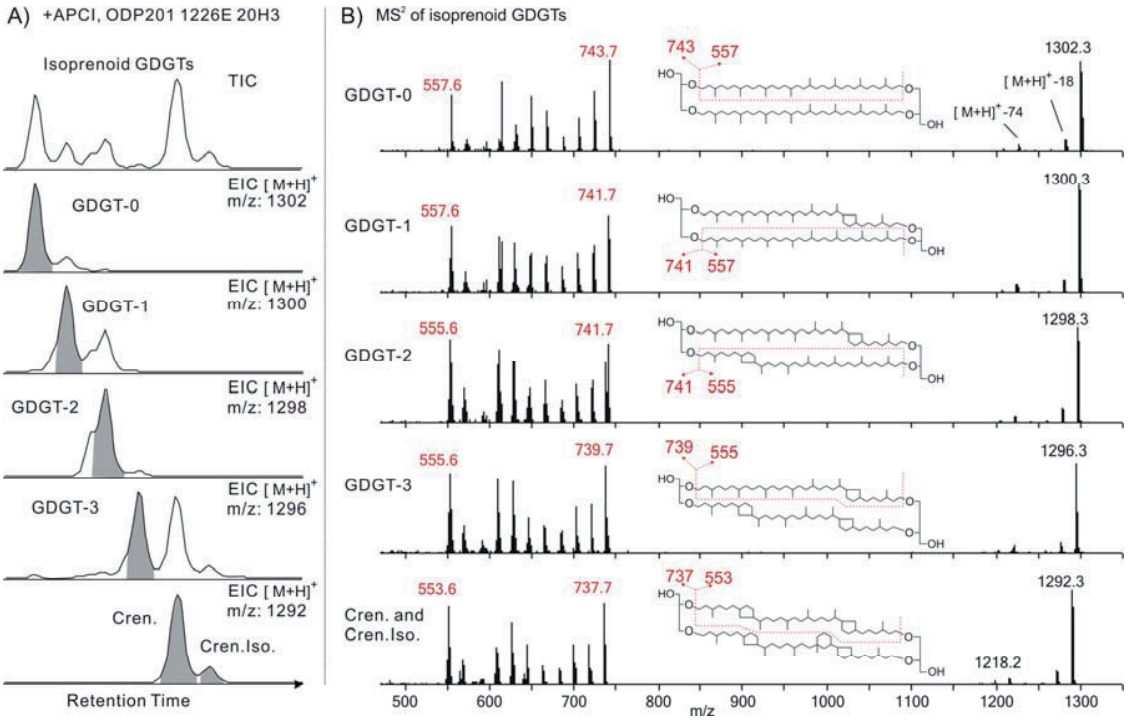

Figure 6-6. LC-APCI-MS (QTOF) chromatogram showing the distribution of isoprenoid GDGTs in the extraction of one marine sediment, ODP201 1226E 20H3 (A)), and the MS^2 spectra of isoprenoid GDGTs (B)), $[M+H]^+$ m/z: 1302, 1300, 1298, 1296 and 1292, showing the characteristic fragments of losing one biphytane.

Because of the coelution of GDGT-4 (with 4 rings) and crenarchaeol with the present methods, analysis of GDGT-4 usually is not taken into account (Zhang et al., 2006). Improved methods that are able to separate and analyze these two compounds need to be developed in the future. Isoprenoid GDGTs preserved in marine sediments was believed majorly deriving from planktonic archaea in the water column, although in situ contribution from benthic archaea had been argued in some studies (Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009). A survey of globally distributed marine subsurface sediments showed the different GDGT composition in the core lipids and intact polar lipids fractions (Liu et al., 2011), and suggested a careful evaluation of the applied proxies and further study of the biologic sources of isoprenoid GDGTs in marine sediments.

3.4. H-shaped isoprenoid GDGTs (H-GDGTs)

H-GDGT with two biphytanyl carbon skeletons covalently bound by a C-C bond was first demonstrated by Morii et al. (1998) as the archaeal membrane lipid in a hyperthermophilic methanogen, and then a series of H-GDGTs containing 0-4 cyclopentane rings and together with the IPL form of H-GDGT, phospho-glycerol H-GDGT (PG-H-GDGT), were observed in the thermoacidophilic euryarchaeote isolated from deep-sea hydrothermal vents (Schouten et al., 2008b). Schouten et al. (2008a) reported the detection of H-GDGT in the sediments from several non-extreme marine and lacustrine environments and suggested that non-thermophilic archaea may also be able to synthesize this type of ether lipids.

But as discussed in Chapter 5 that the same molecular mass, m/z 1300, and co-elution of H-shaped GDGT-0 and one dehydrated compound of OH-GDGT-0 points to the danger of identifying H-shaped GDGT only on the basis of retention time and molecular mass (e.g., Schouten et al., 2008a). MS^2 spectrum is required to be able to distinguish H-GDGT from dehydrated hydroxyl isoprenoid GDGT. Deferred from the normal isoprenoid GDGTs, in the MS^2 spectra, no fragments of losing one alkyl chain can be formed from H-GDGTs because of the covalent bond connected the two carbon chains. There are only the ions of $[M+H]^+$ and losing water and glycerol units (Fig. 6-7).

H-GDGTs were only detected in sample ODP201 1229A 22H1, which contains H-GDGT-0 with a low abundance of 0.4% of the total ethers (data was not shown in Table 6-1). In another a few sediments there are only trace level signal of m/z 1300 at the retention time of H-GDGTs (between crenarchaeol regioisomer. and branched GDGTs, cf. Schouten et al., 2008a), which was not able to generate good enough MS^2 spectra for identification. Therefore, we cannot confirm whether they are H-GDGTs or dehydrated products of hydroxyl GDGTs. On the other hand, we found in one hyperthermophilic methanogen species, *Methanopyrus kandleri*, a series of H-GDGTs showing the molecular mass of m/z 1314 and 1328, in which one and two additional methyl groups could be added to the carbon skeleton of H-GDGT-0 (Fig. 6-7).

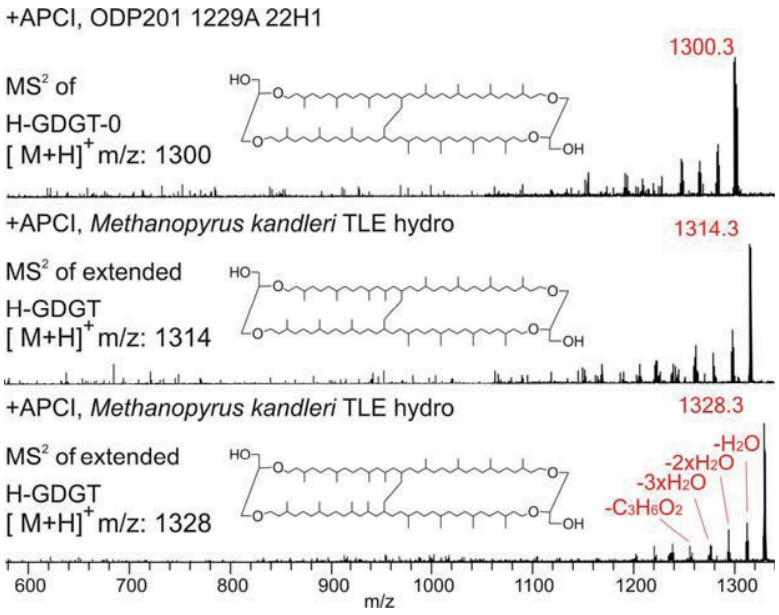

Figure 6-7. MS^2 mass spectra showing the detected H-shaped GDGTs in ODP201 1229A 22H1 and the hydrolyzed TLE of Methanopyrus kandleri, H-GDGT-0, $[M+H]^+$ m/z: 1300, extended H-GDGTs, [M+H]+ m/z: 1314, 1328, characteristic fragments of losing water and glycerol units.

The position of covalent bond in H-GDGTs was not determined in previous works (Morii et al., 1998; Schouten et al., 2008a). With NMR analysis Lutneas et al. (2006 and 2007) identified a series of C_{80}, C_{81} and C_{82} isoprenoid tetracids in which a covalent bond was located between two methyl groups on the two carbon chains and one and two extra methyl groups were added near the covalent bond of C_{81} and C_{82} isoprenoid tetracids, respectively. It is not clear whether these isoprenoid tetracids are degradation derivatives of H-GDGTs (Lutneas et al., 2006) or not, however, here we temporally assign the positions of covalent bond and extra methyl groups in the H-GDGTs, m/z 1300, 1314 and 1328, as the same as tetracids (see tentative molecular structures in Fig. 6-7).

The biosynthesis and function of H-GDGTs are still not clear, but discussed by previous studies (Morii et al., 1998; Sugai et al., 2004; Schouten et al., 2008b) that thermophilic archaea seem to be the major source of these lipids in the environment, and the form of covalent bond between the two biphytanes was supposed to be a reinforcement of the monolayer membrane to protect the cell against membrane lysis at high temperature (Schouten et al., 2008b).

3.5. Branched Glycerol Dialkyl Glycerol Tetraethers (branched GDGTs)

Branched GDGTs are non-isoprenoid GDGTs containing 13,16-dimethyl or 5,13,16-trimethyl octacosanyl moieties were identified in peats, coastal marine and lake sediments (Sinninghe Damsté et al., 2000; Schouten et al., 2000). Differed from the 2,3-di-O-alkyl-sn-glycerol configuration of isoprenoid GDGTs, which considered unique for Archaea (Kates, 1978), the 1,2-di-O-alkyl-sn-glycerol configuration of branched GDGTs made them possible bacterial rather than archaeal origin (Weijers et al., 2006). Two IPL precursors, glucosyl- and glucuronosyl- branched GDGT, were recently identified in a peat sample (Liu et al., 2010), but to date the biological source of branched GDGT have not been identified. Nevertheless, branched GDGT related proxies have been developed for the reconstruction of various palaeoenvironmental parameters such as soil input to marine sediments (Hopmans et al., 2004), mean annual air temperature and soil pH (Weijers et al., 2007).

In both marine and terrestrial environments there are three most dominant branched GDGTs, $[M+H]^+$ m/z: 1022, 1036 and 1050, which differ from each other by the number of methyl groups (see molecular structures in Fig. 6-8). The abundance of branched GDGTs in analyzed sediments ranged from 0.2% to 3.8% of the total glycerol ether lipids (Table 6-1). Branched GDGTs eluted after the H-GDGTs (Fig. 6-1), and were recognizable by their molecular ions and characteristic fragments (Fig. 6-9G-I). For each of these three branched GDGTs, compounds with one or two cyclopentane rings were less abundant (Schouten et al., 2000; Weijers et al., 2006). Besides, in this work detailed mass spectra interpretation revealed another series of novel branched GDGTs with both higher and lower degree of methylation than found in previously described branched GDGTs (Sinninghe Damsté et al., 2000), named here overly branched GDGTs (OB-GDGTs; cf. chromatogram in Fig. 6-8 and MS^2 spectra in Fig. 6-9), and sparsely branched GDGTs (SB-GDGTs, Fig. 6-8). These two groups of lipids are present as minor components of total ether lipids in the marine sediments, 0.5% for OB-GDGTs and 0.1% for SB-GDGTs in average (Table 6-1). Because of the similar tetraether structure of branched GDGTs and isoprenoid GDGTs, their MS^2 fragments patterns are also similar, showing the recognizable fragments of losing water (-18 m/z), glycerol unit ($C_3H_6O_2$, 74

Da) and one alkyl unit (Fig. 6-9). By comparing the characteristic fragments and chromatographic behavior (retention time) of OB-GDGTs and the known branched GDGTs tentative structures of OB-GDGTs were proposed in this work (Fig. 6-9). Unfortunately, due to the co-elution and low abundance of SB-GDGTs no MS^2 spectra of them were shown, but tentative structures with less methyl groups based on the known branched GDGTs were suggested (Fig. 6-8). It has been noticed that the biggest OB-GDGT, $[M+H]^+$ m/z 1134, displayed a nearly isoprenoid structure, including three isopranyl units, and the smallest SB-GDGT, $[M+H]^+$ m/z 966, was linear. The biological sources of OB- and SB-GDGTs and the synthetic mechanism of adding and reducing methyl are still not clear, however, the correlation demonstrated between the methylation level of branched GDGTs in soil and mean annual air temperature and soil *p*H (Weijers et al., 2007) implied that potential biogeological proxies providing similar information could be developed with these lipids.

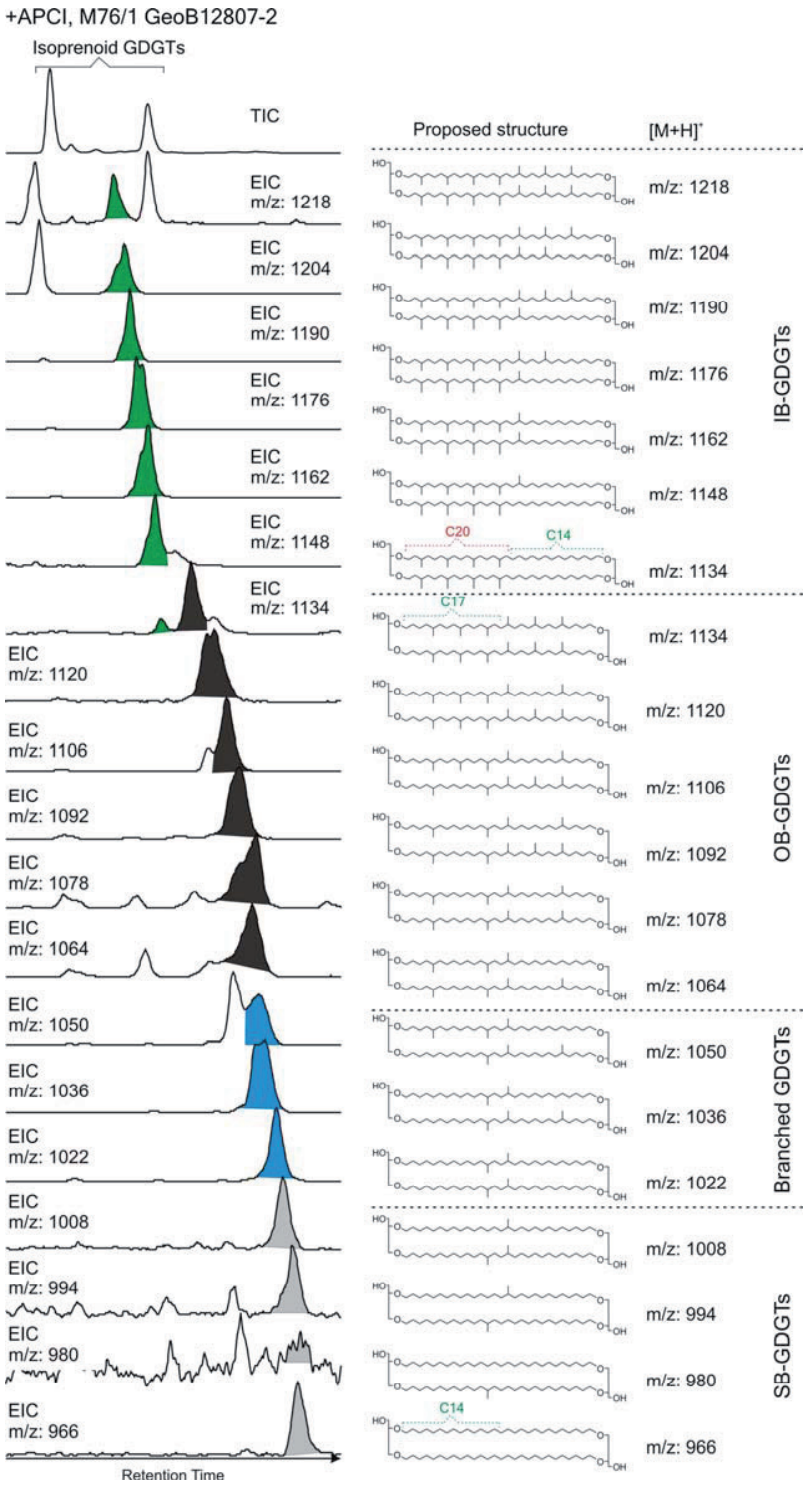

Figure 6-8. LC-APCI-MS (MSD) chromatogram showing the distribution of IB-GDGTs, OB-GDGTs, branched GDGTs and SB-GDGTs in one example sediment, M76/1 GeoB 12807-2. In the tentatively identified structure of IB-GDGTs hybrid carbon skeleton consists of half isoprenoid (C_{20}) and half branched (basic C_{14} back bone with adding methyl groups).

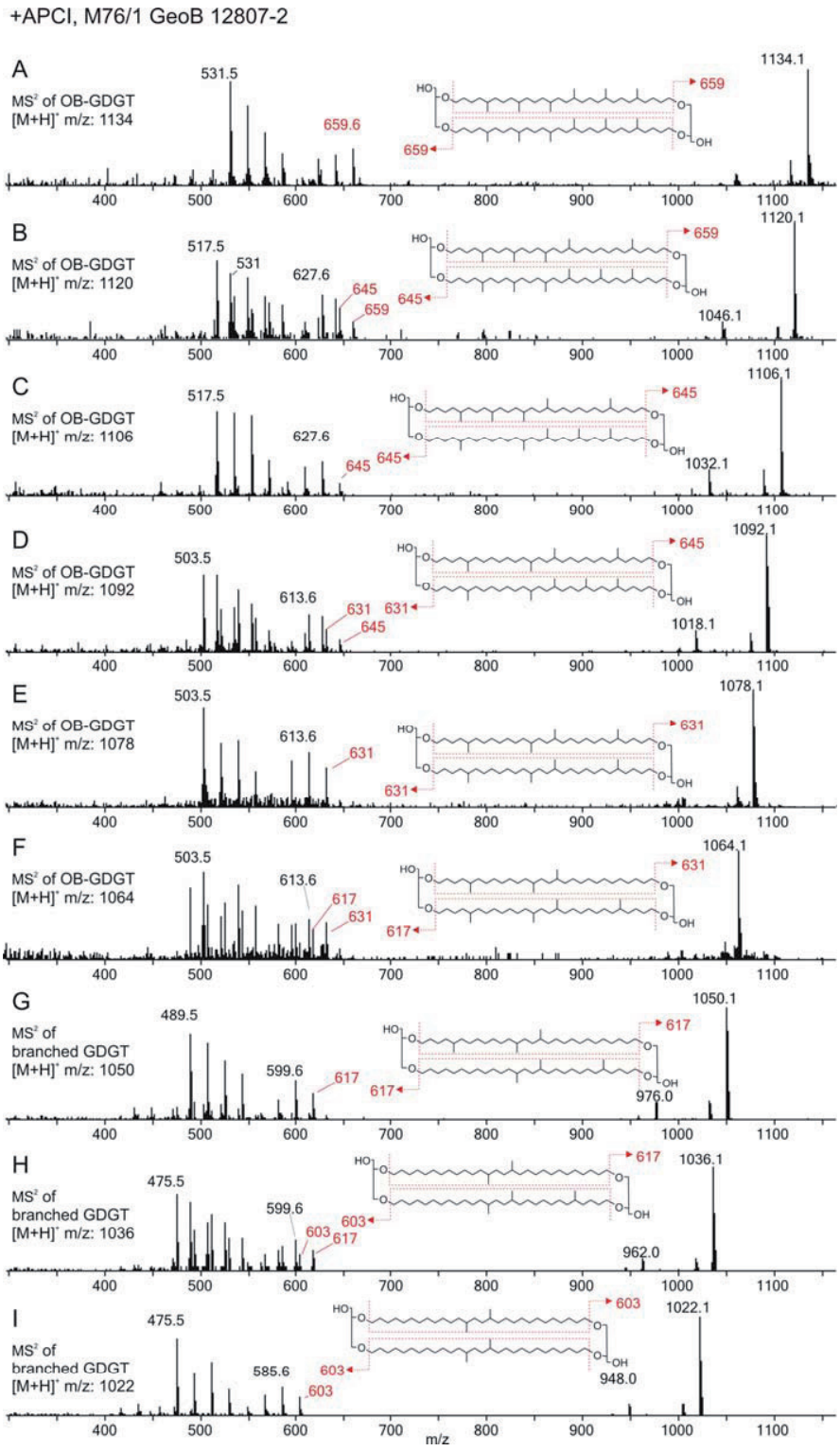

Figure 6-9. MS^2 (QTOF) mass spectra of OB-GDGTs (A~F) and branched GDGTs (G~I) showing the detailed fragmentation pattern, in which the characteristic fragments related to loss of one alkyl chain enable to verify the general structure (determination of size of alkyl moiety)

3.6. hybrid isoprenoid/branched GDGTs (IB-GDGTs)

IB-GDGTs were first detected in minor amounts in both marine and lacustrine sediments (Schouten et al., 2000), in which alkyl chains resemble partly those of archaea (phytanyl moiety) and of eubacteria (iso-alkyl moiety). With detailed LC-MS spectra, we revealed more IB-GDGT analogues with either higher or lower degree of methylation. In spite of the difficulty of detection caused by their low abundance, in average, 2% of total ether lipids in the analyzed sediments (Table 6-1) and close or even co-elution with isoprenoid GDGT-3 and crenarchaeol (Fig. 6-8), the ubiquitous distribution of IB-GDGTs was shown by their presence in all analyzed marine sediment.

Seven IB-GDGTs were detected in the marine sediments with the $[M+H]^+$ ion of m/z 1218, 1204, 1190, 1176, 1162, 1148 and 1134. Similar to the EB- and SB-GDGTs, the biggest molecule, $[M+H]^+$ m/z 1218, of IB-GDGTs has its branched half part nearly isoprenoid and the smallest molecule, $[M+H]^+$ m/z 1134, with its branched half part branchless. We also notice that the identical molecular ion, $[M+H]^+$ m/z 1134, of the smallest IB-GDGT and the biggest EB-GDGT, whereas attribute to two peaks with different retention times because of their different structures (EIC mass window of m/z 1134 in Fig. 6-8). Detailed fragmentation patterns of IB-GDGTs were interpreted for identifying their molecular structure (Fig. 6-10). We noted that the methylation pattern of IB-GDGTs was not the same as that of OB-GDGTs. In the molecule of OB-GDGTs additional methylation occurred on alternated alkyl chains from the lower mass compounds to the higher one, but demonstrated by the one alkyl chain less fragments of IB-GDGTs, from the compound of m/z 1162 to that of 1190 two steps of methylation continuously happened on one alkyl chain, see proposed molecular structures in Fig. 6-10. This result also consists with what has been reported by Schouten et al. (2000) that two ether cleavage released hydrocarbons from the unknown tetraether m/z 1190 showed the mass difference of 28 Da. Due to the low abundance of IB-GDGTs, m/z 1148 and 1134, and their co-elution with crenarchaeol, no satisfactory MS^2 spectra of these two compounds were able to be extracted in our data. Isolation of these compounds with improved LC method will be helpful for verifying the predicted structures. To date, the biological source of IB-GDGTs remains unknown. Further work is needed to have a

greater understanding of the origin of IB-GDGTs.

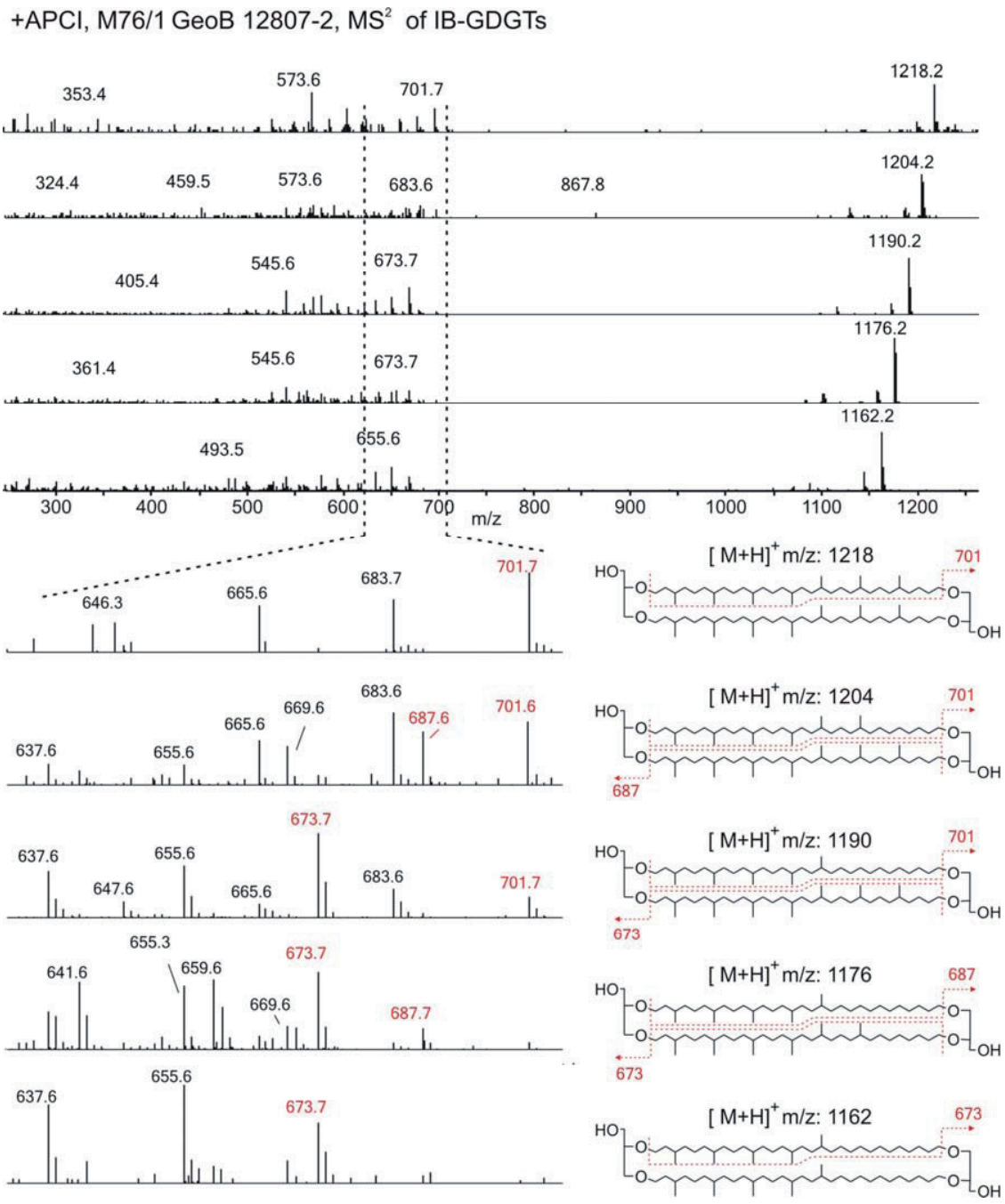

Figure 6-10. MS^2 (QTOF) mass spectra of IB-GDGTs showing the detailed fragmentation pattern, in which the characteristic fragments of losing one alkyl chain helped to identify the general structure of difference between the two alkyl chains in each molecule.

3.7. Hydroxylated isoprenoid GDGTs (OH-GDGTs)

With two-dimensional NMR and LC-MS several core lipids and IPLs of OH-GDGTs were recently indentified in various marine sediments and also in the culture of one thermophilic methanogen, *Methanococcus Thermolithotrophicus* (Chapter 5). Except for the ubiquitous distribution of OH-GDGTs in marine sediments, they also occurred as the second most abundant ether lipid groups following isoprenoid GDGTs, 8% in average (Table 6-1). Because of the higher polarity caused by the additional hydroxyl groups OH-GDGTs eluted several minutes later than the normal isoprenoid GDGTs in LC chromatogram (Fig. 6-1 and 6-11). With normal APCI-MS conditions these hydroxylated compounds yielded only dehydrated molecular ions, $[M+H]^+$-18 for monohydroxyl isoprenoid GDGTs and $[M+H]^+$-36 for dihydroxyl isoprenoid GDGT (Fig. 6-11). By comparing the MS^2 spectra of GDGT-0, OH-GDGT-0 and 2OH-GDGT-0 (Fig. 6-12), preferential loss of the hydroxylated biphytanes characterize the fragmentation pattern of hydroxylated isoprenoid GDGTs, and the structure with two hydroxyl groups on one biphytane can be determined for 2OH-GDGT-0.

According to the proposed synthetic pathway of isoprenoid GDGT that the biphytane skeleton was formed via head-to-head condensation of two archaeol units (Nemoto et al., 2003), hydroxylated isoprenoid GDGTs may be synthesized with two diethers including one or two hydroxyl archaeols. In all analyzed marine sediments the different ring composition of GDGTs and hydroxyl GDGTs implies that the biological source of hydroxyl GDGTs is possibly different from that of isoprenoid GDGTs. And, IPLs of hydroxyl GDGTs, such as 2Gly-OH-GDGT, had been detected in remarkable abundance in anoxic water column (Schubotz et al., 2009), methane seep sites (Rossel et al., 2010) and other subsurface sediments (Lipp and Hinrichs, 2009; Liu et al., Chapter 5), and also in the archaeal cultures of both Crenarchaeota (*Candidatus* Nitrosopumilus maritimus; Schouten et al., 2008c) and Euryarchaeota (*Methanococcus Thermolithotrophicus*; Chapter 5). This wide range distribution and high abundance of hydroxyl isoprenoid GDGTs in natural settings showed their potential of being used as taxonomic biomarkers or biogeological proxies.

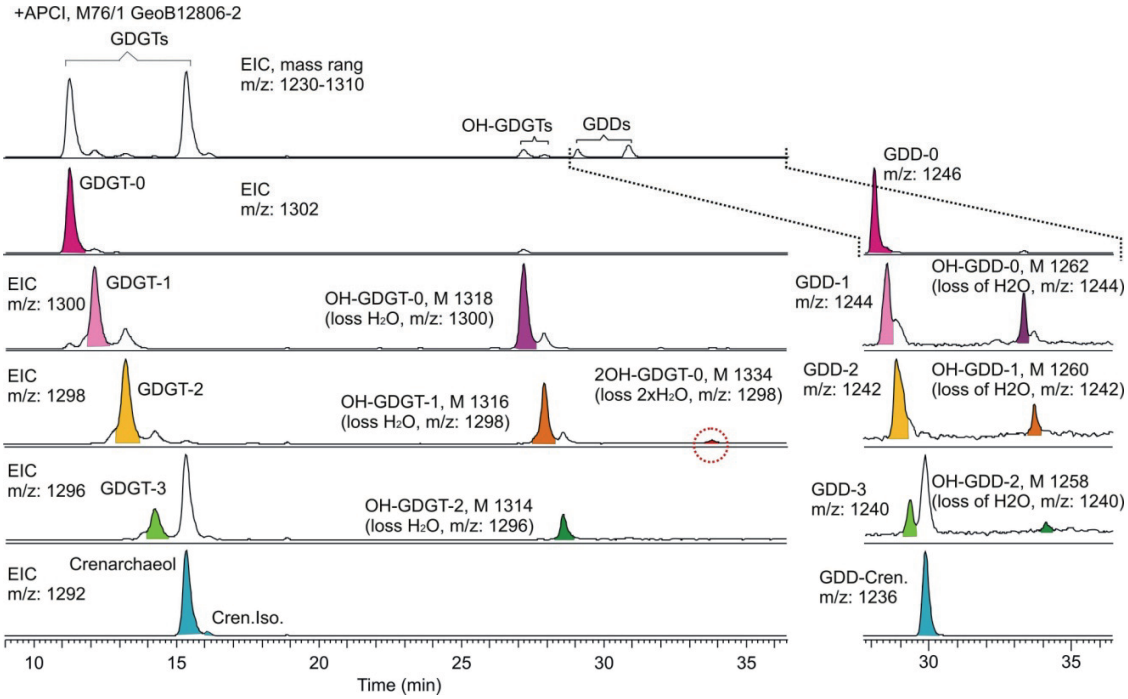

Figure 6-11. LC-APCI-MS (MSD) chromatogram showing the distribution of isoprenoid GDGTs and GDDs with their hydroxylated compounds (2OH-GDD-0 was detect in a very low abundance and not shown in this chromatogram) in marine sediment sample M76/1 GeoB 12806-2.

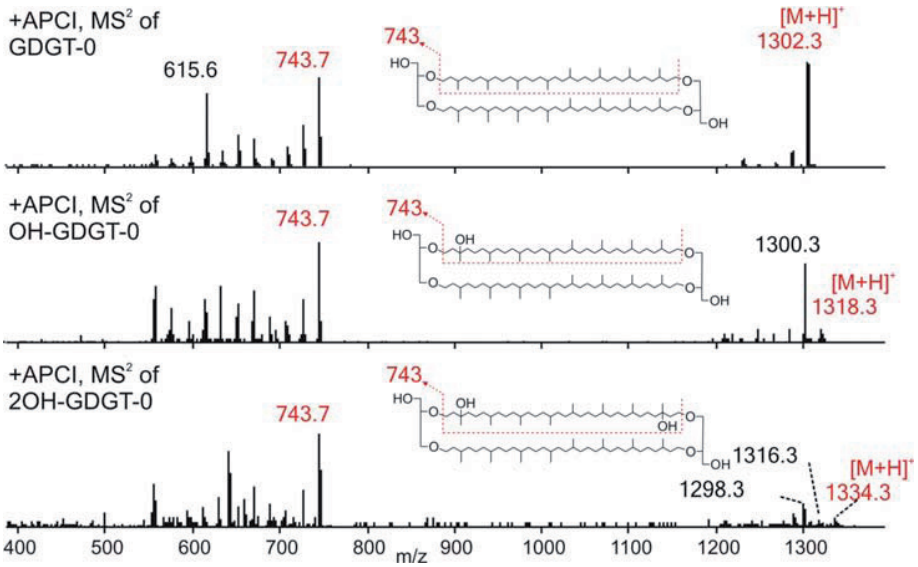

Figure 6-12. MS^2 (QTOF) spectra of isoprenoid GDGT-0, OH-GDGT-0 and 2OH-GDGT-0 in M76/1 GeoB12806-2 showing the characteristic fragments of losing one alkyl chain, which indicate the preferential loss of hydroxylated biphytane in OH-GDGT-0 and 2OH-GDGT-0, and the location of two hydroxyl groups on one biphytane of 2OH-GDGT-0.

3.8. Glycerol Dialkanol Diethers (GDDs)

GDDs are another series of novel lipids coexisting with GDGTs in both marine and terrestrial environments. Corresponding to each isoprenoid GDGT, in the analyzed marine sediments isoprenoid GDDs were identified to have the distinctive structure of one glycerol moiety (C_3H_4O) less compared to isoprenoid GDGTs (Fig. 6-11 and detail description in Chapter 4). Likewise, branched GDDs (Fig. 6-14) were detected in the peat bog samples dominated by branched GDGT. In hydrothermal vent sediments, where H-shaped GDGTs occurred in a relative high abundance, we even observed H-shaped GDDs (Kellermann et al., unpublished data). Hydroxylated GDDs (Fig. 6-11) were also recognized to yield dehydrated molecular ions during MS analysis.

GDDs have the higher polarity compared to their corresponding GDGTs, which results in higher retention times compared to hydroxylated GDGTs (Fig. 6-1). Characteristic fragments in the MS^2 spectra of GDD-Cren., GDD-0, OH-GDD-0 and 2OH-GDD-0 were shown in Fig. 6-13. Consist with the identification of 2OH-GDGT-0 (Chapter 5), the major fragment ion of 2OH-GDD-0, m/z 669, which presents one glycerol moiety with one saturated biphytane, indicates that the two hydroxyl groups are on one biphytane (Fig. 6-13). During the ionization and fragmentation process of hydroxyl GDDs there will be dehydration and preferential loss of the hydroxylated carbon chain.

As discussed in Chapter 4, similar ring distribution pattern of isoprenoid GDGTs and GDDs implied highly related biological sources of these two lipid groups. The unique bipolar structure and coexistence with their corresponding GDGTs in marine sediments and also in one archaeal culture, *Methanococcus Thermolithotrophicus*, made GDDs to be considered possible as both functional lipids and synthetic intermediates. The presence of hydroxyl isoprenoid GDDs minimized the possibility that GDDs were generated from isoprenoid GDGTs by diagenetic processes or artifact formation during sample preparation. It is unlikely to degrade the labile hydroxy isoprenoid GDGTs to hydroxy GDDs without losing the hydroxyl groups. The ubiquitous distribution and remarkable abundance of isoprenoid GDDs (in average 7% of total ethers, Table 6-1) in marine sediments also showed the potential of serving as biomarker or molecular proxies.

On the other hand, the existence of GDD also supports the lipids recycling hypothesis of benthic archaea (Takano et al., 2010), if GDDs are intermediates of recycling.

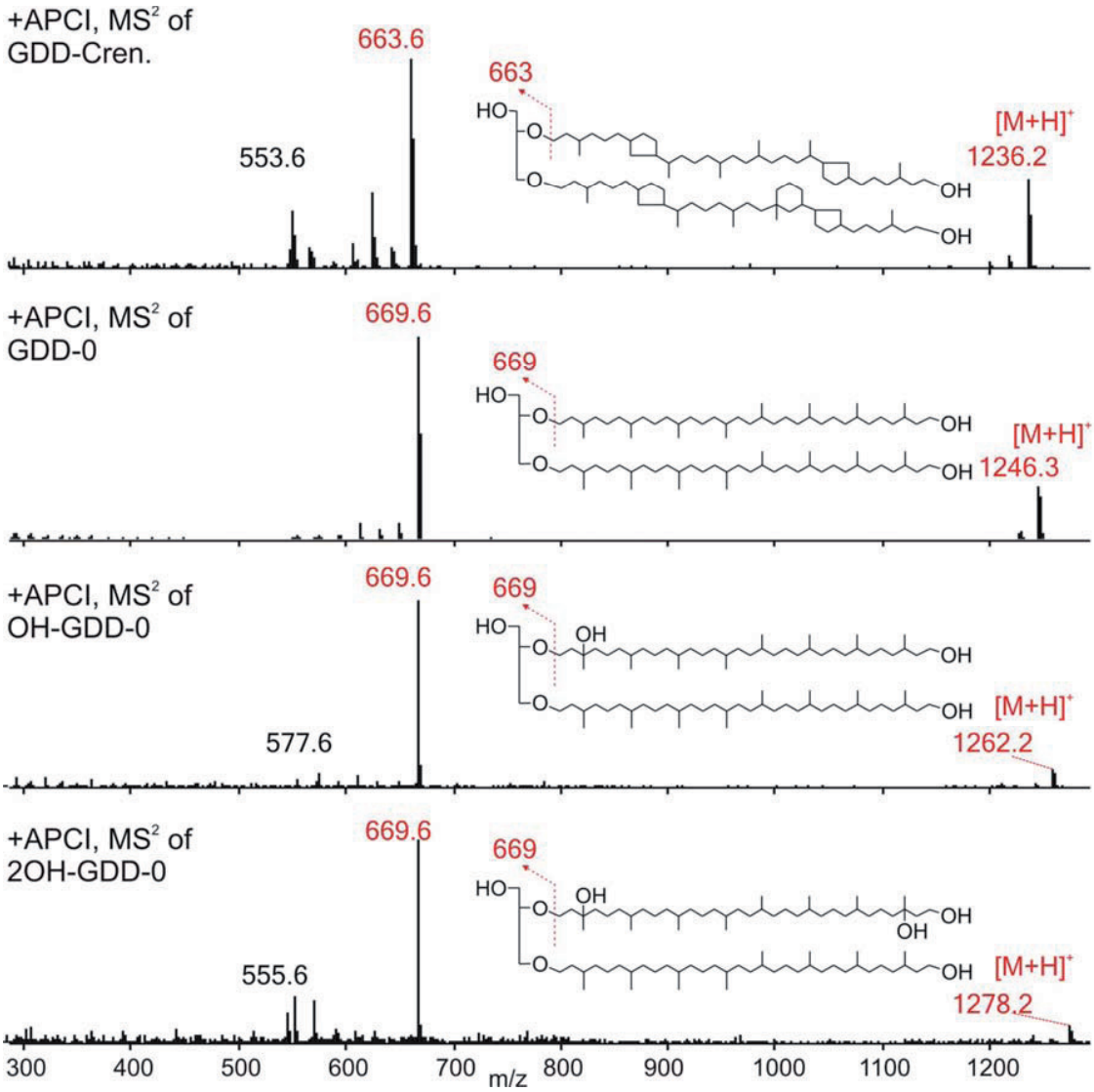

Figure 6-13. MS^2 (QTOF) mass spectra of isoprenoid GDD-Cren., GDD-0, OH-GDD-0 and 2OH-GDD-0 in M76/1 GeoB12806-2 showing the characteristic fragments of losing one carbon chain, which indicate the preferential loss of hydroxylated biphytane in OH-GDD-0 and 2OH-GDD-0, and the location of two hydroxyl groups on one biphytane of 2OH-GDD-0.

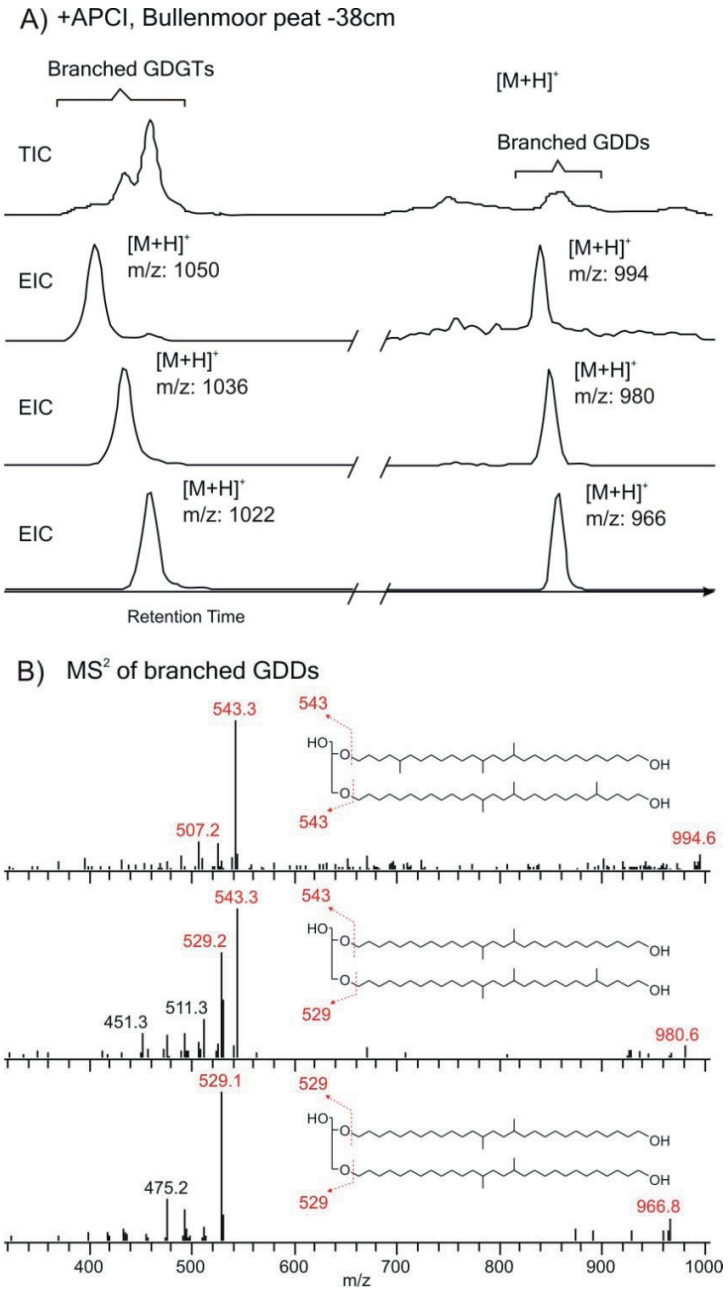

Figure 6-14. A) LC-APCI-MS (ion trap) chromatogram showing the distribution of branched GDGTs and branched GDDs in the sample of Bullenmoor peat -38 cm, detail sample information was published in Liu et al. 2010. B) MS^2 spectra of branched GDDs, $[M+H]^+$ m/z 994, 980 and 966, showing the characteristic fragments of losing one carbon chain.

3.9. Other unknown lipids

Except for these ether lipid groups discussed above, there are still numerous unknowns detected in the analyzed marine sediments (data was not shown). Among these unknowns a few compounds also occurred in all samples with remarkable abundance. For example, in the LC chromatogram one compound eluted out right before OH-GDGT-0 with the $[M+H]^+$ of m/z 1020 (Fig. 6-1) and the relative abundance of around 6.4% compared to the total ether lipids (H-1020 in Table 6-1). Detailed MS^2 spectrum analysis indicates a H-shaped GDGT structure, because the absence of loss of one alkyl chain ion and the characteristic fragment pattern resulting from loss of water and glycerol units (Fig. 6-15). The ubiquitous distribution in marine sediments and H-shaped structure of this unknown raised the interest of further structural identification in our future study.

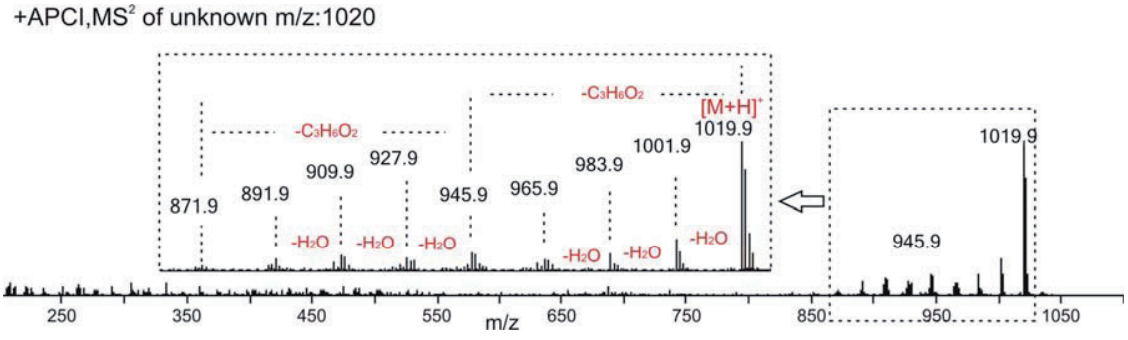

Figure 6-15. MS^2 (QTOF) mass spectra of unknown, $[M+H]^+$ m/z 1020, showing the characteristic fragments of losing waters and glycerol units ($C_3H_6O_2$) that indicates a general structure of H-shaped GDGT.

4. Summary and outlook

This survey of glycerol diether and tetraether lipids in marine subsurface sediments provided useful information for a better understanding of microorganism, especially Archaea, in marine environment. We discussed above the detection and implication of more than forty compounds in each structural group and in MS^2 spectra the characteristic fragment ions showed the fingerprint of each lipid that made them easily

recognizable. Some novel lipids were first reported in this work, such as the hybrid isoprenoid/branched GDGTs, overly branched GDGTs, sparsely branched GDGTs, hydroxylated GDDs and H-shaped unknown GDGT (m/z 1020), but the molecular structures of these lipids were not fully determined. Regarding the ubiquitous distribution further structural determination of extended H-shaped GDGTs, IB-GDGTs, OB-GDGTs, SB-GDGTs and the unknown H-shaped GDGT, m/z 1020, is needed. Extended H-shaped GDGTs, IB- GDGTs, OB-GDGTs and SB-GDGTs are characterized with a common feature of methylation series, containing compounds with one -CH_2- unit difference between each other. The distribution of these lipids with different level of methylation will provide molecular proxies indicating past environmental factors such as temperature and salinity which may have influenced the methylation level of lipids during synthesis in their source organism. The unknown H-shaped GDGT (m/z 1020), occurred in all marine sediments with very high abundance, 6.4% of all ethers, may represent some kind of widespread and yet unknown microbe.

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Samples		% of each group in total ethers										
		Ar	GTGT-0	Isoprenoid GDGTs	Hydroxy -GDGTs	Isoprenoid GDDs	Hydroxy-isoprenoid GDDs	Branched GDGTs	IB-GDGTs	OB-GDGTs	SB-GDGTs	H-1020
M76/1 (Namibia Margin)	GeoB 12806-2	1.0	0.3	62.6	*19.8	2.9	*3.8	0.2	0.9	0.1	0.008	8.4
	GeoB 12807-2	1.0	0.2	76.4	11.5	7.8	0.1	0.7	1.8	0.2	0.04	0.1
DP201 (Peru Margin)	1229D 4H4	1.0	0.2	58.9	11.4	*12.1	1.7	1.1	1.5	0.4	0.1	11.6
	1229A 22H1	*13.1	0.2	53.4	6.5	6.7	0.6	*3.8	*8.5	1.3	0.1	6.0
ODP201 (Equatorial Pacific)	1226B 10H3	0.5	0.3	77.8	7.7	7.4	1.1	1.0	1.3	0.4	0.1	2.4
	1226E 20H3	1.1	0.2	81.3	4.0	7.9	0.3	0.9	0.3	0.2	0.02	3.7
ODP204 (Hydrate Ridge)	1250D 6H5	1.9	0.2	73.4	9.4	9.3	0.8	0.5	2.0	0.5	0.02	1.9
	1250D 12H5	0.6	0.1	64.3	8.2	7.0	0.7	0.8	2.5	*1.7	0.02	13.9
IODP311 (Cascadia Margin)	1327C 10H5	1.9	0.2	73.3	6.9	8.9	1.1	1.0	3.0	0.7	0.04	2.8
	1327C 13X6	1.1	0.2	76.4	5.9	9.2	0.8	1.3	2.1	0.5	0.05	2.5
ODP160 (Mediterranean Sapropel)	966C 5H02	0.3	0.2	74.8	4.0	2.7	0.2	0.6	0.7	0.3	0.05	*16.2
	966C 7H04	1.2	0.2	79.2	3.5	6.5	0.2	0.9	0.8	0.3	0.1	7.0
Average		2.1	0.2	71.0	8.2	7.4	1.0	1.1	2.1	0.5	0.1	6.4

Table 6-1. The relative abundance of 11 ether lipid groups in 12 analyzed marine sediments. Abbreviations of lipids: archaeol (Ar), glycerol trialkyl glycerol tetraether without ring (GTGT-0), hybrid isoprenoid/branched GDGTs (IB-GDGTs), overly branched GDGTs (OB-GDGTs), sparsely branched GDGTs (SB-GDGTs), H-shaped unknown GDGT, $[M+H]^+$ m/z 1020 (H-1020).
* Indicates biggest values far beyond the average.
Detail sampling information has been published in Liu et al. (2011).

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Chapter 7

Conclusions and perspectives

1. Conclusions

In this PhD project, a more complete understanding of the distribution of glycerol ether lipids in marine sediments has been achieved through identification of a multitude of novel compounds. Several identified novel core lipids illustrate the high structural diversity of glycerol ether lipids in marine sediments and imply a high phylogenetic diversity of their microbial sources. Furthermore, the identification of intact precursors of previously known core lipids represents an important step for the search of their source organisms.

Branched GDGTs are known to be widespread in both marine and terrestrial environments (Schouten et al., 2000; Sinninghe Damsté et al., 2000; Hopmans et al., 2004). A detailed survey of the distribution of glycerol ether lipids in 12 marine sediments indicated that the average abundance of branched GDGTs is 1% of total ether lipids. The structural features and natural distribution of branched GDGTs implied an origin from soil bacteria (Weijers et al., 2006), but the compounds still remain so-called orphan lipids. Despite the lack of knowledge about the microbial source organism, molecular proxies based on branched GDGTs have been developed, such as the BIT index as tracer for soil input into marine sediment (Hopmans et al., 2004), and the CBT and MBT indices for reconstructing soil pH and mean annual air temperature, respectively (Weijers et al., 2007). However, application of molecular proxies without knowing the biological sources of the compounds is risky. We studied a soil depth profile of the Bullenmoor peat bog in order to identify for the first time the intact polar precursors of the branched GDGTs in order to further constrain their biological producers. Two IPLs of branched GDGT, glucosyl- and glucuronosyl-branched GDGT, were identified. These two compounds account for 4-7% of the total detected IPLs, suggesting

that the branched GDGT producers represent a sizeable but not dominant component of the microbial community in the peat bog. Among cultured bacterial phyla that are prominent in soils, glucuronosyl lipids have been found in the Actinobacteria and the Alphaproteobacteria. The identification of IPL precursors will open up investigative avenues for narrowing down the identity and function of their source organism, e.g., via stable-isotope probing experiments.

In sub-surface sediments at a site with high methane flux in the Black Sea it was observed that *in-situ* production of glycosidic GDGTs by methanotrophic archaea goes along with their relatively rapid hydrolysis and production of corresponding core GDGTs, suggesting that *in-situ* production will have an impact on the sedimentary record of fossil GDGTs and thus on proxies such as the TEX_{86}. Archaeal IPLs in 12 selected marine sediments were dominated by 2Gly-GDGTs and 2Gly-OH-GDGTs, with proportions of >70%, followed by 1Gly-GDGT and 3Gly-GDGT. The proportion of glycosidic GDGTs in total GDGTs ranged from 4% to 11%, similar to the range reported by Lipp and Hinrichs (2009). Such high concentrations of glycosidic IPLs could potentially lead to a significant contribution to the core lipid pool after hydrolysis.

A systematic comparison of the ring distribution of core GDGTs and intact polar GDGTs in a wide range of marine sub-surface sediments revealed distinct differences. Acyclic GDGT-0 was generally more abundant in the intact polar GDGT pool, while crenarchaeol was more abundant in the core GDGT pool. On the other hand, GDGT-2 and GDGT-3 are relatively more abundant as intact lipid, which results in somewhat higher TEX_{86} values calculated for the intact polar GDGT pool. Despite these significant differences in ring distribution, a correlation was expressed between the TEX_{86} values of both pools. The recently proposed recycling of fossil GDGTs from planktonic archaea by the benthic archaeal community (Takano et al., 2010) was considered as a potentially important process leading to this relationship.

Our knowledge of the diversity of glycerol ether lipids in marine sediments benefited from the identification of GDDs and hydroxy-GDGTs. These two groups of lipids constitute on average 7% and 8%, respectively, of total ether lipids in the analyzed sediments. Despite their wide distribution and remarkable abundance in marine sediments, the origin and significance of GDDs and hydroxy-GDGTs remains unclear.

Both lipid families were detected in the TLE of *Methanococcus thermolithotrophicus*, indicating a biological origin as a potential input of these compounds in sediments. Moreover, intact glycosidic hydroxy-GDGTs were detected in the same archaeal culture and in numerous sediments, confirming the archaeal membrane lipid origin of hydroxy-GDGTs. However, no IPLs with GDD as the core lipid have been found during lipid screening. GDDs were found co-existing with their corresponding GDGTs in all analyzed sediments, which implied the highly related or even identical source of GDD and GDGT. Diagenesis of GDGTs, i.e., biodegradation by benthic archaea, could account for this observation. However, the distinctive ring composition of GDGTs and hydroxy-GDGTs suggested different biological sources.

Some other novel lipids were also first reported in this work, such as the extended H-shaped GDGTs, hybrid isoprenoid/branched GDGTs (IB-GDGTs), overly branched GDGTs (OB-GDGTs), sparsely branched GDGTs (SB-GDGTs), hydroxylated GDDs and an unknown H-shaped GDGT (m/z 1020), but the molecular structures of these lipids have not yet been fully determined. All detected glycerol ether lipids can be classified into 11 structural groups. Each lipid group shows characteristic fragment ions in MS^2 spectra, which can be used as fingerprints for recognizing lipids. The ubiquitous distribution of these ether lipids in marine sediments indicates the phylogenetic diversity and ecological significance of their source organisms. Novel phylogenetic biomarkers and biogeological proxies can be developed with these newly identified lipids.

2. Perspectives

This work explored the diversity of glycerol ether lipids in marine sediments, but the biological sources of known branched GDGTs and the newly identified lipids are still unknown. Furthermore, a better understanding of the sedimentary input of isoprenoid GDGTs from benthic archaea and their mechanism of lipids synthesis is needed. Future studies should focus on the following aspects:

1) Searching for the microorganisms producing branched GDGTs.

By analyzing the distribution of lipids and 16S rRNA genes in a peat bog setting, Weijers et al. (2009) proposed that the branched GDGT-producing microbe was possibly related to *Acidobacteria*. However, their screening of pure and enriched acidobacterial cultures did not find branched GDGTs. Based on the identification of branched GDGT IPLs in this PhD thesis work, the activity and abundance of branched GDGT-producing bacteria can be determined in natural settings. Selected samples with higher abundance of intact branched GDGTs can be collected for cultivation experiments to enrich the branched GDGT-producing bacteria. Subsequent genetic analysis and microbial screening may result in determination or isolation of the bacteria. Previous studies of the stable carbon isotopic composition of branched GDGTs from various settings indicated that the source microorganism is heterotrophic and assimilates compounds derived from degradation of bulk organic carbon in sediment for lipid synthesis (Weijers et al., 2010). Using stable isotope labeled substrates for the incubation experiments and subsequent analysis of isotopic compositions of branched GDGT IPLs will provide a better understanding of the metabolic features of their source organisms.

2) Exploring the distribution of GDDs and identification of the intact precursors of GDDs

The newly identified GDD core lipids co-occurred with corresponding GDGTs in all analyzed sediments, but the implication of their occurrence in sediment and their exact origin is still unknown. Exploring the distribution of GDDs in a depth profile will provide

more insight into the origin and fate of GDD lipids in marine sediments. A comparison of the GDD distribution in selected environmental settings, such as seep vs. non-seep, water column vs. sediment, coastal marine vs. open ocean, will potentially enable development of novel molecular proxies. Another approach to determine the origin of GDDs is searching for their intact precursors. Although a brief screening for potential candidate molecules was done in some of the sediments and cultures, no IPLs giving the typical core lipid signal of GDDs were detected. A further screening in a wide range of samples is therefore suggested. Fragmentation features of GDD lipids under LC-ESI-MS conditions are still unknown and it needs to be considered to search for signals of other fragments than the whole GDDs.

3) Exploring the distribution of hydroxy-GDGTs

Hydroxy-GDGTs were also detected to be widespread in marine sediments. Their IPLs, glycosidic hydroxy-GDGTs, have been previously reported as a major unknown component in archaeal cultures (Schouten et al., 2008c), sediments (Lipp and Hinrichs, 2009; Rossel et al., 2011; Pitcher et al., 2011) and the water column (Schubotz et al., 2009). The widespread occurrence of IPLs of hydroxy-GDGTs suggests an important contribution of certain archaeal phyla with high activity in a wide range of environmental settings. A more detailed study of their distribution in certain environments will help to unravel the distribution of source organisms and related metabolic activities.

The experiments leading to identification of hydroxy-GDGTs also demonstrated the presence of putative regioisomers and dehydrated compounds in the hydrolyzed TLE of *Methanococcus thermolithotrophicus*. The relative abundance of two OH-GDGT-0 isomers is nearly equal in *Methanococcus thermolithotrophicus*, but the earlier-eluting isomer is barely detectable in marine sediments. Identification of hydroxy-GDGT regioisomers in environmental samples is suggested for understanding the input and preservation in sediment. Chromatograms of environmental samples show small earlier eluting peaks corresponding to the molecular masses of GDGT-1, -2, and -3 and were previously proposed to be their isomers (e.g., Pitcher et al., 2009; 2011). However, these compounds more likely represent dehydrated hydroxy-GDGTs. After confirmation of this hypothesis by further structural analysis, an index based on the ratio of these compounds

and hydroxy-GDGTs can be used as a molecular proxy to reflect maturity of sedimentary organic matter.

4) Structural determination of novel lipids

Further structural determination of extended H-shaped GDGTs, IB-GDGTs, OB-GDGTs, SB-GDGTs and the unknown H-shaped GDGT is necessary because of their ubiquitous distribution in marine sediments. Extended H-shaped GDGTs, IB-GDGTs, OB-GDGTs and SB-GDGTs are characterized with a common feature of methylation series, containing compounds with one -CH_2- unit difference between each other. Future work exploring the distribution of these lipids with different level of methylation can possibly lead to the development of molecular proxies indicating past environmental factors such as temperature and salinity which may have influenced the methylation level of lipids during synthesis in their source organism.

The unknown H-shaped GDGT occurred in all marine sediments with high abundance and accounts for 6.4% of all ether lipids. H-shaped GDGTs are usually restricted to hyperthermophiles (Morii et al., 1998; Schouten et al., 2008b), even though the detection of H-shaped GDGT with very low abundance in sediments from several non-extreme marine and lacustrine environments suggested that non-thermophilic archaea may also be able to synthesize this type of ether lipids (Schouten et al., 2008a). This highly abundant unknown H-shaped GDGT must represent some kind of widespread and yet unknown microorganism. Knowledge of its structural features, such as isoprenoid or non-isoprenoid, will further constrain the microbial source.

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Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

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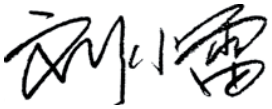

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