Glycerol configurations of environmental GDGTs investigated using a selective *sn*2 ether cleavage protocol

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Abstract

The glycerol configurations of glycerol dialkyl glycerol tetraethers (GDGTs) in environmental samples were investigated using a selective *sn*2 ether cleavage protocol. Using this procedure, GDGTs with a parallel glycerol configuration afford two types of derivatives, diols and diallylethers, whereas only one kind, monoallylethers, originate from their antiparallel isomers. Isoprenoidal GDGTs from a marine sediment are shown to be predominately parallel based on the distributions of these ether cleavage products. Crenarchaeol and its so-called regioisomer both have parallel configurations with the cyclohexane ring located on the *sn*3,3 ether bonded tricyclic biphytanyl moiety. A Messel shale sample containing isoprenoidal GDGTs contributed mainly by methanogenic archaea has a substantial portion with the antiparallel configuration. Branched (non-isoprenoidal) GDGTs in both the Messel shale and the marine sediment are mainly antiparallel. This selective *sn*2 ether cleavage approach provides a potentially powerful analytical tool to investigate not only the exact molecular structures of GDGT constitutional isomers and their biosynthetic pathways but also to evaluate the heterogeneous inputs of sedimentary GDGT and their isotopic signatures, if different source species synthesize GDGTs with unique glycerol configurations. Further analyses of this type will reveal the glycerol configurations of the GDGTs of a broad range of microbial cultures and environmental samples.

Key words: GDGT, chemical degradation, glycerol configuration, crenarchaeol, marine sediment, methanogen

1. INTRODUCTION

Isoprenoidal GDGTs are distinctive bipolar membrane-spanning lipids exclusively synthesized by Archaea. The intact polar lipids of archaeal tetraethers comprise, in general, three molecular components, namely the polar head groups, the isoprenoidal hydrocarbon chains and the glycerol backbones (Fig. 1). Structural diversity of archaeal tetraethers is recognized in the variety of polar head groups; modifications - including hydroxylation, cyclization, unsaturation and methylation - to the isoprenoidal hydrocarbon chains; and the two types of glycerol arrangements, the parallel and antiparallel configurations (Fig. 1). The widely applied analytical method of liquid chromatography-mass spectrometry (LC–MS) combined with various ionization approaches, such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), can readily distinguish GDGTs with different polar headgroups and hydrocarbon chains, but is not capable of distinguishing between the parallel and antiparallel glycerol constitutional isomers.

Gräther and Arigoni (1995) designed a chemical degradation sequence of reactions to cleave ether bonds specifically at the *sn*2 glycerol carbon, so that the glycerol configurations of precursor GDGT could be deduced from the composition of the degradation products. By conducting this selective *sn*2 ether cleavage reactions on the acyclic isoprenoidal GDGT (GDGT-0) isolated from three archaeal cultures, *Methanobacterium thermoautotrophicum, Thermoplasma acidophilum* and *Sulfolobus solfataricus,* Gräther and Arigoni (1995) reported an approximately 1:1 ratio of the parallel and antiparallel GDGT-0 regioisomers further speculating that the biosynthesis of archaeal tetraethers involved no preference for either type of glycerol configuration.

However, our recent investigation on the ***natural degradation*** derivatives of environmental archaeal tetraethers indicated a predominantly parallel glycerol configuration in marine sediments and substantial antiparallel configuration in sediments that were strongly influenced by methanogenic activity (Liu et al., 2018). To further verify our detection of GDGT glycerol configurations in natural depositions, we adapted the ***chemical degradation*** protocol of Gräther and Arigoni (1995) to investigate the glycerol configurations of environmental GDGTs.

2. EXPERIMENTAL

2.1. Sample collection and preparation

A commercial mixture of glyco-GDGT-phosphoglycerol (gly-GDGT-PG), the main phospholipids of *Thermoplasma acidophilum*, was purchased from Matreya LLC (PA 16803, USA), and used as a representative sample to verify and improve the chemical degradation protocol reported by Gräther and Arigoni (1995). Around 1 μg of this intact polar GDGT mixture was first hydrolyzed with 10% HCl in methanol at 70 °C for two hours to yield the core GDGTs. Two environmental samples, a marine sediment from ODP201 1227A and the Messel shale (excavation site E 8/9, horizon 2.5–3.5 m), were dried and powdered and approximately 2 g of each sample was extracted following the modified Bligh and Dyer protocol described previously (Sturt et al., 2004) to yield total lipid extracts (TLEs).

2.2. Selective *sn*2 ether cleavage

An aliquot of each sample was transferred into a 2 mL glass vial and dried with a stream of nitrogen (N_2) for chemical degradation. We adapted the first sequence of

88 reactions described by Gräther and Arigoni (1995) for our investigation (reaction scheme
89 shown in Fig. 2). Briefly, core GDGTs were first tosylated with an excess amount (10
90 mg) of *p*-toluenesulfonyl chloride (Sigma-Aldrich) in 100 µL pyridine (Sigma-Aldrich) at
91 room temperature for three days. Then, 500 µL of 5 N aqueous HCl and 500 µL *n*-hexane
92 were added to the reaction vial and the two phases were mixed vigorously by vortexing.
93 After separation of the two phases, the organic phase, containing tosylated GDGTs, was
94 transferred into another 2 mL vial. The aqueous phase was extracted a further three times
95 with *n*-hexane and the organic extracts were combined and dried under a stream of N_2 for
96 further reaction. To cleave the *sn*2 ether bonds, 10 mg of sodium iodide (NaI, Sigma-
97 Aldrich) and 10 mg of zinc powder (purum grade, Sigma-Aldrich) were added to the
98 residue from the previous reaction and the mixture was suspended in 100 µL 1,2-
99 dimethoxyethane (DME, Sigma-Aldrich) and heated at 90 °C for two hours. Degradation
100 products were finally retrieved by liquid-liquid extraction using 500 µL of water and 500
101 µL *n*-hexane and then vortexing. The two phases were separated, and, as before, the
102 aqueous phase was extracted a further three times with *n*-hexane. Finally, the combined
103 organic phases were dried in a 2 mL vial for analysis.

104 **2.3. Lipid analysis with LC–MS**

105 Lipid analysis by LC–MS was performed on an Agilent 1290 series UPLC system
106 coupled to an Agilent 6530 qTOF mass spectrometer through an Agilent jet stream dual
107 electrospray ionization (AJS–ESI) interface. The ESI drying gas (N_2) temperature was set
108 at 300°C, the N_2 flow rate was 8 L min^{-1} and the nebulizer gas (N_2) pressure was 35 psi.
109 The qTOF parameters were set to: capillary voltage 3.5 kV, fragmentor voltage 175 V;
110 skimmer voltage 65 V and octopole voltage 750 V in auto MS/MS scanning mode with
111 MS^1 range of *m/z* 100-3000 and MS^2 mass range of *m/z* 50-3000.

112 Separation of compounds was achieved with a reverse phase LC method modified
113 from Zhu et al. (2013). Briefly, samples were dissolved in methanol and injected with a
114 volume of 10 μL onto an Agilent Zorbax Eclipse XDB-C18 column (1.8 μm, 4.6 × 100
115 mm; Agilent) maintained at 35 °C. Mobile phase flowed at a rate of 0.5 mL min^{-1}, first
116 isocratically with 100% A for 2 min, followed by a gradient to 40% B at 15 min, and to
117 90% B at 40 min, and then to 100% B at 41 min and hold for 14 min, and finally re-
118 equilibrated with 100% A for 10 min, where the eluent A was 100:0.04:0.10 of
119 methanol/formic acid/14.8 M NH_3(aq.) and B was 100:0.04:0.10 of 2-propanol/formic
120 acid/14.8 M NH_3(aq.).

121

122 **3. RESULTS**

123 **3.1. Lipid composition of samples prior to their chemical degradation**

124 Core GDGTs released by acid hydrolysis from the gly-GDGTs-PG of *T.*
125 *acidophilum* consist of GDGT-0 to GDGT-6 (Fig. 3A1). The TLE of marine sediment,
126 ODP201_1227A, is dominated by GDGT-0 and crenarchaeol (cren-a) with a small
127 proportion of GDGT-1 to -3, hydroxyl GDGTs (OH-GDGTs) and branched GDGTs. The
128 putative crenarchaeol regioisomer (cren-b) elutes prior to cren-a under reverse phase
129 condition. Substantial amounts of biphytanediols (bpdiols), which consist of mainly
130 bpdiol-0 and -cren, occur as natural degradation product of GDGTs (Fig. 3B1 and
131 Supplementary Fig. S1). The Messel shale extract exhibits a GDGT distribution that is
132 typical of sulfidic lacustrine settings (Liu et al., 2016) in which branched GDGTs are
133 relatively more abundant than isoprenoidal GDGTs and GDGT-0 is the major
134 isoprenoidal GDGT and accompanied by S-GDGTs as minor components (Fig. 3C1).

3.2. Detection of degradation derivatives

The major ionized forms of GDGTs and their corresponding degradation derivatives, the bpdiol and the monoallyl biphytanol monoether (mbpm), are their protonated molecular ions, $[M+H]^+$. However, the other type of degradation product, the diallyl biphytanyl diether (dbpd), is detected mainly in the form of its ammonium adducts, $[M+NH_4]^+$. All three categories of degradation derivatives, bpdiol, dbpd and mbpm, occur in the sample of *T. acidophilum* after ether cleavage, but each group has a different ring distribution (Fig. 3A2 and Supplementary Fig. S2). Three bpdiols with up to two rings are released by ether cleavage. A later eluting peak (labeled as '?') following the monocyclic bpdiol (bpdiol-1) occurs and partially co-elutes with bpdiol-2. The MS^2 fragmentation pattern of this compound is very different from those of bpdiols (Fig. S4), and it could not be identified as a bpdiol isomer. The monoallylether derivatives are the most abundant degradation products and consist of mbpm-0 to -3 (Supplementary Fig. S2). Dbpds contain however up to 4 rings. When subjected to chemical degradation isoprenoidal GDGTs in the Messel shale also produce all three types of degradation derivatives (Fig. 3C2 and Supplementary Fig. S3). So far no clear signal of S-GDGT related degradation derivatives can be distinguished for those of regular GDGTs. Degradation derivatives in both *T. acidophilum* and Messel shale exhibit a mbpm-dominated distribution pattern. However, GDGTs in the marine sediment, ODP201_1227A, produce almost exclusively bpdiol and dbpd as their degradation products except for the trace amount of mbpm-0 (Fig. 3B2). Acyclic and tricyclic derivatives (dbpd-0 and -cren-a) prevail over other dbpds in the chemically degraded marine sediment (Supplementary Fig. S1). Bpdiols contain up-to two rings with no tricyclic derivatives detected (Supplementary Fig. S1).

159 Given the high abundance of branched GDGTs in the Messel shale extracts, all
160 three types of degradation products (diols, monoallylethers and diallylethers) are detected
161 (Fig. 4A). Degradation derivatives of branched (non-isoprenoidal) GDGT in
162 ODP201_1227A are less abundant compared to those in Messel shale sample (Fig. 4B).
163 Monoallylethers prevail over diols and diallylethers in both samples, and the
164 monoallylethers in Messel shale and marine sediment are dominated by dimethyl and
165 trimethyl derivatives, respectively (Fig. 4).
166
167 **4. DISCUSSION**

168 **4.1. Reactions of selective *sn*2 ether cleavage**

169 In the process of chemical degradation the two free hydroxyl groups of core
170 GDGTs are first tosylated with *p*-toluenesulfonyl chloride in pyridine at room
171 temperature for three days. The tosylated GDGTs are then heated with sodium iodide and
172 zinc powder in DME at 90 °C for 2 hours. The tosylates are converted into the
173 corresponding iodides *in situ* (Fig. 2) and the iodides undergo zinc-mediated Boord
174 haloalkoxy elimination (Dykstra *et al.* 1930). In this way, the *sn*2 ether bonds are cleaved
175 and double bonds were formed affording an allyl substituent in the remaining ether (Fig.
176 2). As shown in Fig. 2, cleavage of the *sn*2 ether bonds on a GDGT with parallel glycerol
177 configuration gives two degradation products, the bpdiol that was originally ether-bonded
178 to the glycerol *sn*2 carbons and the remaining dbpd. However, *sn*2 ether cleavage on an
179 antiparallel GDGT results in two mbpms.
180 The ratio of mbpm/(bpdiol+dbpd) of chemically degraded *T. acidophilum* is
181 approximately 1:1, which indicates a mixture of parallel and antiparallel configured
182 precursor GDGTs in nearly equal abundance. Such a result is consistent with the previous

183 report on *T. acidophilum* by Gräther and Arigoni (1995) and validates this selective *sn*2
184 ether cleavage protocol.

185 **4.2. Antiparallel GDGT of methanogenic input**

186 The thermophilic methanogen, *M. thermoautotrophicum*, has been known to
187 produce both parallel and antiparallel GDGTs in ~1:1 ratio (Gräther and Arigoni, 1995).
188 Archaeal lipids preserved in the Messel shale are primarily derived from methanogenic
189 archaeal communities (Hayes et al., 1987; Bauersachs et al., 2014). The approximately
190 1:1 ratio of mbpm/(bpdiol+dbpd) in chemically degraded Messel shale extracts suggests
191 that mesophilic methanogens synthesize GDGTs similar to those of their thermophilic
192 relatives, with both parallel and antiparallel configurations. Our previous analysis on
193 natural degradation derivatives in seep carbonate (Liu et al., 2018) also points to the idea
194 that environments rich in contributions from Euryarchaeota, including methanogens, will
195 be dominated by antiparallel GDGTs..

196 **4.3. Predominant parallel glycerol configuration of marine archaeal GDGTs**

197 The remarkable abundances of bpdiol and dbpd with a minor mbpm-0 as the
198 degradation derivatives of ODP201_1227A show that the precursor GDGTs in marine
199 sediment possess a predominantly parallel configuration (Fig. 3B2). The predominance of
200 bpdiol and dbpd chemical degradation products confirms unambiguously what we have
201 observed with the distribution of natural degradation derivatives in various marine
202 subsurface sediments (Liu et al., 2018). Since isoprenoidal GDGTs in our previously
203 analyzed marine sediments (cf. Table 1 of Liu et al., 2018), especially those from open-
204 ocean slope settings, are primarily derived from planktonic species, mainly marine
205 *Thaumarchaeota* (Pearson et al., 2016), we can hypothesize that marine *Thaumarchaeota*

206 predominantly synthesize parallel glycerol configured GDGTs. The minor mbpm-0
207 represents a small proportion of antiparallel GDGT-0 either synthesized by
208 *Thaumarchaeota* or contributed by other archaeal taxa. We also noticed in our previous
209 investigation that the natural degradation product of antiparallel GDGT, *sn*2,3-GMGD
210 (glycerol monobiphytanyl glycerol diether), is either absent or present in very low
211 abundance in most sediments collected from regular marine deposits, but has been
212 observed in substantial amounts in one particular sample affected by anaerobic methane
213 oxidation (AOM) (Liu et al., 2018).

214 **4.4. The structures of crenarchaeol and its isomer**

215 Conventional, non site-specific, ether cleavage can only show that there is one
216 tricyclic biphytane originating from cren-a and a further two different tricyclic isomers
217 with distinct ring configurations originating from cren-b (Liu et al., 2018; Sinninghe
218 Damsté et al., 2018). However, our studies of degradation products from both natural
219 diagenesis and chemical degradation reveal not only the distinct ring configurations but
220 also the glycerol configuration and the location of the cyclohexyl ring. Bpdiol-cren-a and
221 -b exist in the TLE of marine sediment as natural degradation products (Fig. 3B1), but
222 there is no tricyclic bpdiol generated through the selective *sn*2 ether cleavage. The
223 occurrence of dbpd-cren-a and -b with equally abundant bpdiol-2 confirms our previous
224 deductions concerning the molecular structures of cren-a and -b. Degradation products of
225 cren-a and -b released from either natural diagenesis or selective *sn*2 ether cleavage
226 indicate that both are parallel in glycerol configuration, but with different *sn*3,3 ether-
227 bonded tricyclic biphytanes (see crenarchaeol structure illustrated in Fig. 3B1). Further
228 studies are still required to determine the precise ring structures in cren-a and -b.

229 **4.5. Glycerol configuration of branched GDGTs**

All three classes of degradation products, diols, monoallylethers and diallylethers, were detected after chemical degradation of branched GDGTs in Messel shale extracts and in the ODP201_1227A extract. Their co-occurrence suggests the existence of both parallel and antiparallel glycerol configured branched GDGTs in our analyzed samples. The much higher abundance of monoallylether relative to diols and diallylether suggests that branched GDGTs are mainly antiparallel in the glycerol configuration (Fig. 4) in these two types of depositions, marine and sulfidic lakes. It is shown that branched GDGTs in the analyzed marine sediment are dominated by hexamethylated (branched GDGT-1050, Fig. 3B1) while tetramethylated derivatives (branched GDGT-1022, Fig. 3C1) are found predominantly in Messel shale. Such a difference in methylation patterns is also reflected in their degradation derivatives, especially the monoallylethers, in which the trimethylated component derived from branched GDGT-1050 is more abundant in the marine sediment but the dimethylated representing branched GDGT-1022 is remarkable in the Messel shale extracts (Fig. 4). Interestingly, no trimethylated diol and diallylether are detected in the marine sediment sample, which implies that the branched GDGT-1050 in this marine subsurface sediment only in antiparallel form.

5. Conclusion and future applications

The glycerol configurations of GDGTs in both biological and environmental samples can be determined by the composition of their chemical degradation derivatives yielded by the selective *sn*2 ether cleavage protocol. Results of chemical degradation confirm our previous report of GDGT glycerol configurations based on the distribution of natural degradation derivatives. Combining data of natural and chemical degradation derivatives allows us to conclude: (1) GDGTs in marine sediments are predominantly

254 configured with parallel glycerols; (2) AOM-related archaeal communities may have
255 contributed the antiparallel GDGTs in the environmental samples we analyzed; (3)
256 crenarchaeol and its so-called 'regioisomer' are both parallel with *sn*3,3 ether bonded
257 tricyclic biphytanyl moieties. Natural degradation derivatives of branched GDGTs were
258 not discussed in our previous work, but their chemical degradation derivatives, identified
259 here, indicate antiparallel dominates over the parallel configuration.

260 Many previous studies of lipid distributions and their carbon isotopic
261 compositions have shown that mixed planktonic and benthic sources contribute GDGTs
262 in marine sediment (e.g. Liu et al., 2011; Pearson et al., 2016 and references cited
263 therein). However, to date, no technique has been reported as capable of differentiating
264 them. The glycerol configurations of GDGTs synthesized by a broader range of archaeal
265 species should be investigated to verify whether marine *Thaumarchaeota* synthesize the
266 parallel configuration predominantly. Potential proxies based on the relative abundance
267 of mbpm versus bpdiol and dbpd can then be developed to investigate the impact of
268 benthic contribution to the total sedimentary GDGT pool.

269 Compared to biphytanes released by conventionally applied general ether
270 cleavage, mbpm and dbpd obtained from the selective *sn*2 ether cleavage contain both
271 glycerol and isoprenoid carbons. Their carbon isotopic compositions can reflect more
272 precisely the original signal of precursor GDGTs. If antiparallel GDGTs in marine
273 sediment are solely contributed by benthic archaea rather than synthesized by
274 *Thaumarchaeota*, then the carbon isotope composition of mbpm-0 should be distinct
275 from that of bpdiols and dbpds. Thus, the application of selective *sn*2 ether cleavage to
276 marine sedimentary GDGTs, followed by compound specific isotope analysis on mbpm,

bpdiol and dbpd can be an approach to potentially disentangle the mixed surface water and sedimentary signals.

Acknowledgements

X.-L.L. was supported by the startup funding from the Department of Geology and Geophysics, University of Oklahoma. Research at MRC and MIT was funded by the Simons Foundation Collaboration on the Origins of Life (SCOL). We are grateful to the inspiring discussions and suggestions provided by Dr. John D. Sutherland. We thank the participating scientists and ship crews of the Ocean Drilling Program (ODP Legs 201) for providing the marine sediment sample, ODP201-1227A.

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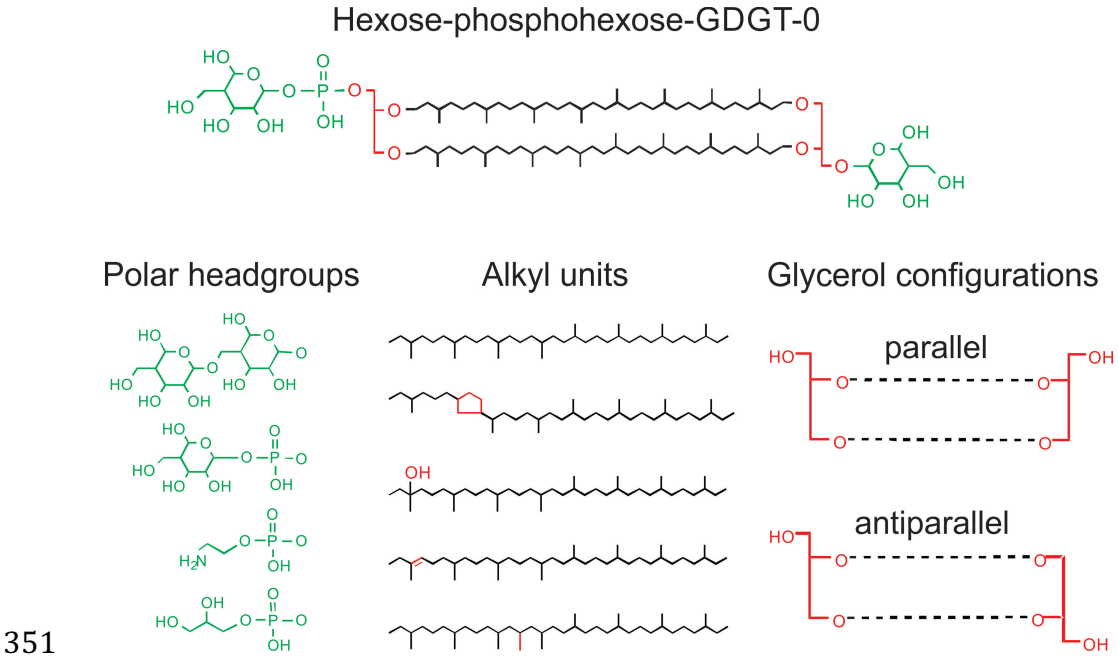
Figure captions

Fig. 1. Hexose-phosphohexose-GDGT-0 as an example of intact polar archaeal tetraethers showing the three general molecular components, namely, polar headgroups, alkyl units and glycerol backbones, and structure variation resulted from the modification of each component. Emphasized here are two types of glycerol configurations, the parallel and antiparallel configurations.

Fig. 2. This chemical degradation scheme illustrates the distinct reaction products generated by selective *sn*2 ether cleavage on GDGT-0 with different glycerol configurations. Parallel GDGT-0 yields acyclic biphytanediol (bpdiol-0) and diallyl biphytanyl diether (dbpd-0). Antiparallel GDGT-0, however, is degraded into two acyclic monoallyl biphytanol monoethers (mbpm-0).

Fig. 3. Combined extracted ion chromatograms of LC–MS showing the composition of GDGT core lipids in acid hydrolyzed intact polar lipids of *T. acidophilum* (A1) and TLEs of marine sediment ODP201-1227A (B1) and Messel shale (C1), and the degradation derivatives released via selective *sn*2 ether cleavage from *T. acidophilum* (A2), ODP201-1227A (B2) and Messel shale (C2).

Fig. 4. LC–MS extracted ion chromatograms combined to illustrate the composition of degradation derivatives of branched GDGTs in Messel shale (A) and ODP201-1227A (B).


Hexose-phosphohexose-GDGT-0
Polar headgroups
Alkyl units
Glycerol configurations
parallel
antiparallel

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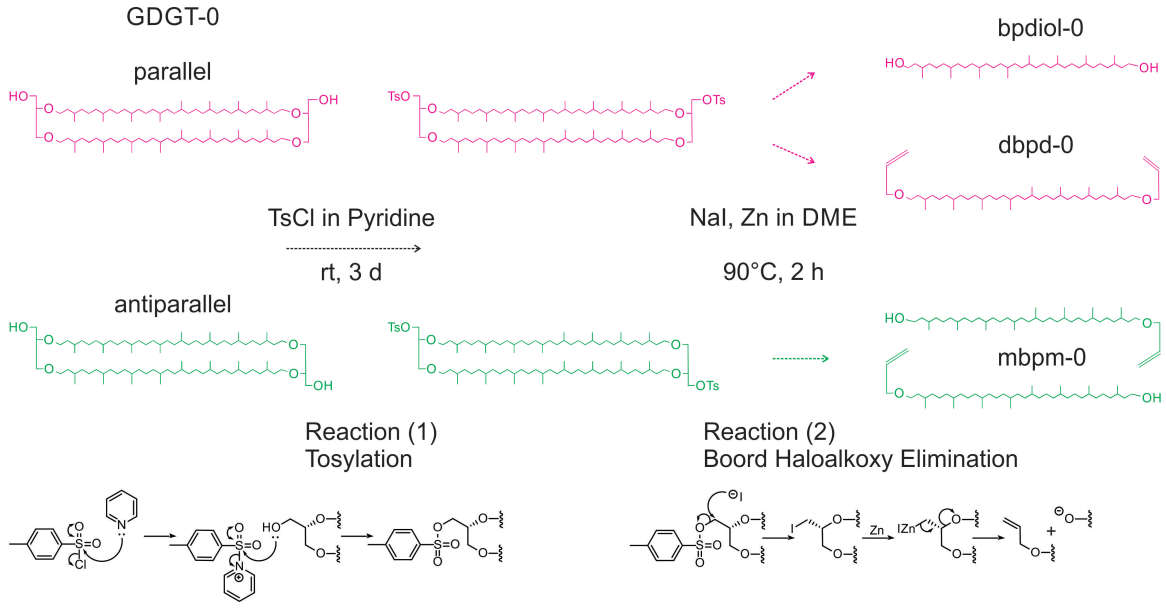
GDGT-0
parallel
bpdiol-0
dbpd-0
TsCl in Pyridine
rt, 3 d
NaI, Zn in DME
90°C, 2 h
antiparallel
mbpm-0
Reaction (1)
Tosylation
Reaction (2)
Boord Haloalkoxy Elimination

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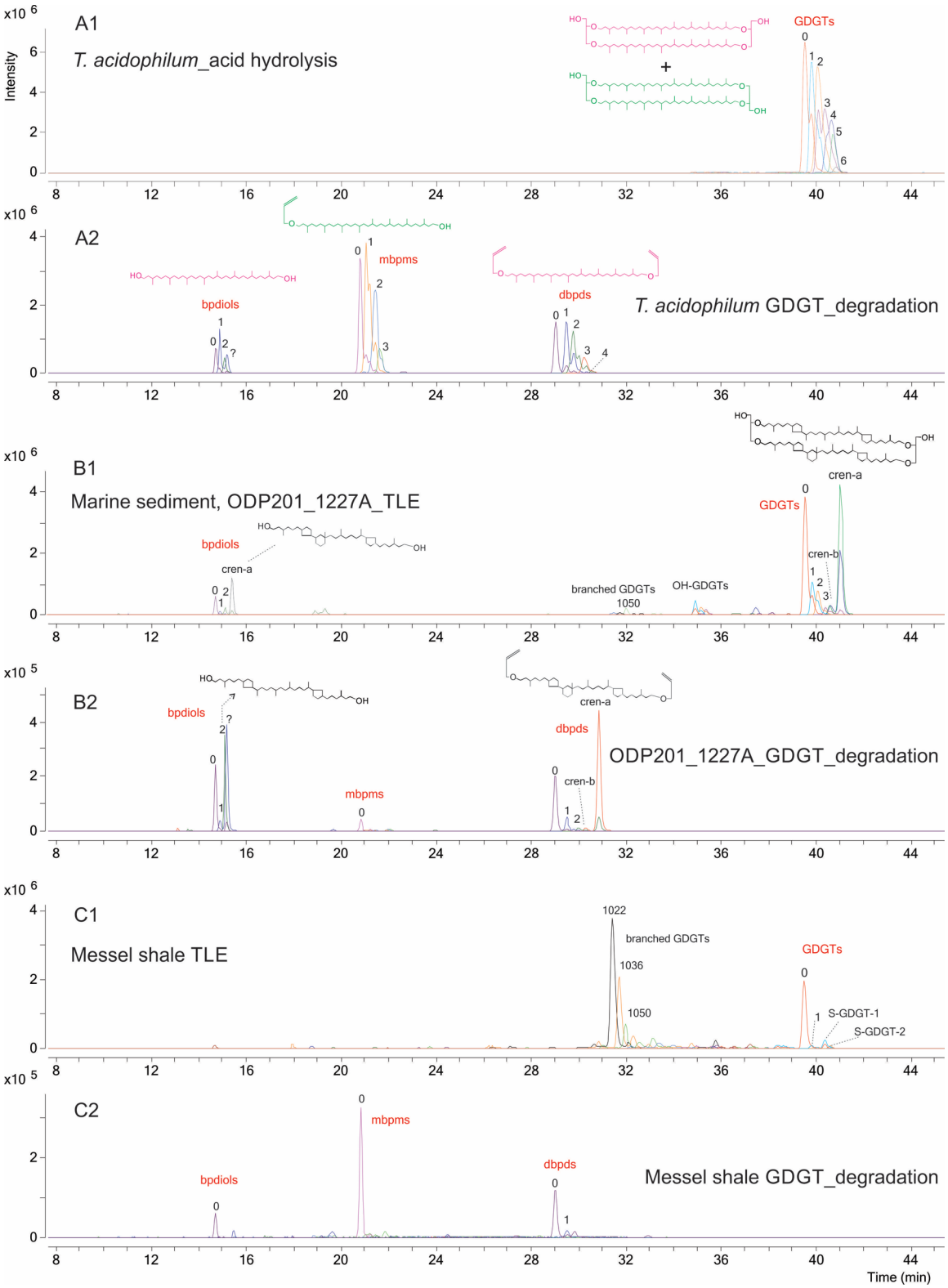
A1
T. acidophilum_acid hydrolysis
GDGTs
Intensity
A2
mbpms
bpdiols
dbpds
T. acidophilum GDGT_degradation
B1
Marine sediment, ODP201_1227A_TLE
cren-a
GDGTs
bpdiols
cren-b
branched GDGTs
1050
OH-GDGTs
B2
bpdiols
cren-a
dbpds
ODP201_1227A_GDGT_degradation
mbpms
cren-b
C1
Messel shale TLE
1022
branched GDGTs
1036
1050
GDGTs
S-GDGT-1
S-GDGT-2
C2
mbpms
bpdiols
dbpds
Messel shale GDGT_degradation
Time (min)

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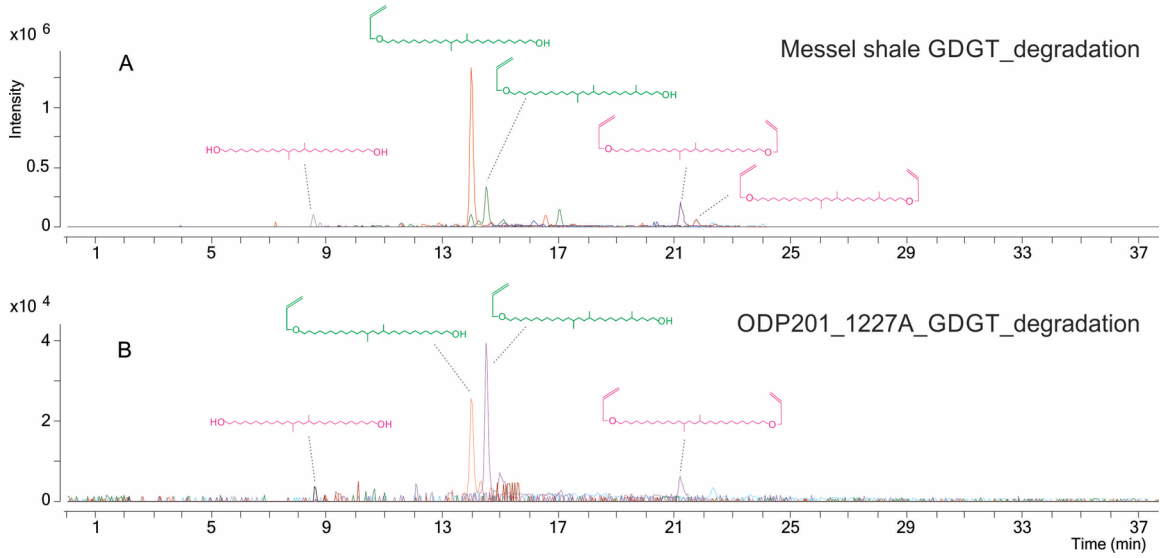

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