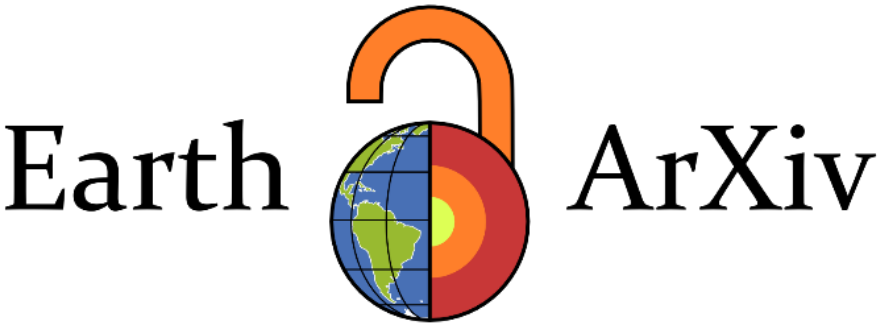


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# Occurrence of tetraester and mixed ether/ester-bound *iso*-diabolic acid membrane-spanning lipids in mineral soils

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**Highlights**

- Mixed ether/ester-bound membrane spanning lipids identified in mineral soils
- Different compounds released upon hydrolysis suggest distinct synthesis pathways
- Our findings support both current hypothesized pathways for brGDGT synthesis

**Abstract**

Branched glycerol dialkyl glycerol tetraethers (brGDGTs) are a suite of membrane lipids that are widely used as empirical proxies for past temperature and pH. Although the stereochemistry of their glycerol moiety suggests that they are produced by bacteria, the exact producers and the biosynthetic pathway of brGDGTs remain unclear. Here we report the occurrence of tetraester and mixed ester/ether membrane-spanning lipids with a backbone consisting of *iso*-diabolic acid (*iso*-DA) containing up to two additional methyl groups in mineral soils from Nepal and Rwanda. These compounds are presumed intermediate products during brGDGT synthesis but had not been detected in cultures or the environment before. Interestingly, while acid hydrolysis of the polar fraction releases *iso*-DA in the soil from Nepal, monoalkyl glycerol ethers with *iso*-$C_{15}$ and *iso*-$C_{17}$ chains are released in the soil from Rwanda. These results support both current hypotheses that brGDGT synthesis can occur via tail-to-tail condensation of two *iso*-$C_{15}$ fatty acids to form *iso*-DA, as well as through the reduction of diesters to diethers and subsequent carbon-carbon linking as shown to occur during the synthesis of archaeal GDGTs.

**Keywords:** branched GDGTs, *iso*-diabolic acid, mixed ether/ester lipids, soil

## 1. Introduction

Branched glycerol dialkyl glycerol tetraethers (brGDGTs) are a suite of membrane lipids that are widely used as paleoenvironmental proxies in paleoclimate studies due to their empirical relations with temperature and pH. BrGDGTs can vary in the number (4-6) and position (C-5, C-6, C-7) of methylations attached to their alkyl backbone, and can also have 0-2 internal cyclisations (Sinninghe Damsté et al., 2000; Weijers et al., 2006; De Jonge et al., 2013; Ding

et al., 2016). The stereochemistry of their glycerol moiety suggests that they are produced by bacteria (Weijers et al., 2006). *Acidobacteria* were proposed as likely producers due to their ubiquitous occurrence in soils and peats (Jones et al., 2009). Screening of lipid profiles of the 46 *Acidobacteria* strains that are currently available in culture indeed revealed the presence of small amounts of brGDGT-Ia in two strains from subdivision (SD) 1 (Sinninghe Damsté et al., 2011; 2014; 2018). More recently, "*Ca.* Solibacter usitatus" from SD3 was found to produce a wider variety of brGDGTs under changing temperature, oxygen, and pH conditions, albeit still not covering the entire range of brGDGTs found in the environment (Chen et al., 2022; Halamka et al., 2023).

Nevertheless, several strains of SD1, 3, 4, and 6 contain the membrane-spanning lipid 13, 16-dimethyl octacosanedioic acid (*iso*-diabolic acid; *iso*-DA), which has since been presumed to be a building block of brGDGTs (Sinninghe Damsté et al., 2011, 2014, 2018). Although *iso*-DA occurs ester-bound to a glycerol moiety in SD1, 3, and 6, *iso*-DA occurs as part of monoalkyl glycerol ethers (MGEs) in strains from SD4 (Sinninghe Damsté et al., 2018). Since SD4 contains the gene cluster responsible for the formation of ether bonds in bacteria (i.e., elbB-elbE; Lorenzen et al., 2014), this has led to the suggestion that diether/diester lipids composed of two esterified *iso*-DA MGEs could be an intermediate compound in the brGDGT synthesis pathway (Sinninghe Damsté et al., 2018, and molecular structures therein). However, the existence of mixed ether/ester-bound *iso*-DA membrane spanning lipids is merely hypothetical, as they have not yet been detected in cultures or the environment.

Here we report the occurrence of tetraester and mixed ether/ester membrane-spanning lipids with a backbone consisting of *iso*-DA containing up to two additional methyl groups in mineral soils from Nepal and Rwanda. We subsequently identify the compounds released

after acid hydrolysis of the GDGT fractions to obtain clues on the pathway of brGDGT biosynthesis.

## 2. Material and methods

### *2.1 Soil selection and GDGT analysis*

A mineral surface soil from Bremathang in Nepal (28.0768247 °N, 85.552645 °E; 3836 m above sea level (masl), pH = 4.4) was freeze dried, ground and sieved at 1 mm. Lipids were extracted by Accelerated Solvent Extraction (Dionex, ASE 350) in dichloromethane (DCM):methanol (MeOH) (9:1) with one 15 min extraction cycle. The total lipid extract (TLE) was eluted over aminopropyl using DCM:isopropanol (2:1) to obtain a neutral fraction. This fraction was further separated over a $SiO_2$ column (5% deactivated with $H_2O$), with hydrocarbons eluting with hexane and the remainder was eluted with DCM and MeOH to obtain the GDGTs. The GDGT fraction was dissolved in hexane:isopropanol (99:1) and passed over a 0.45 µm PTFE filter. Together with the polar fraction ("extract H") of the Rwanda soil ("soil C") from the recent brGDGT Round Robin study (1908 masl, pH = 3.2; De Jonge et al., 2024), GDGTs were analyzed on an Agilent 1260 ultra high performance liquid chromatograph (UHPLC) coupled to an Agilent 6130 single quadrupole mass spectrometer (MS) with settings according to Hopmans et al. (2016) at Utrecht University.

The Nepal polar fraction was analyzed by UHPLC - high resolution MS (HRMS) using an Agilent 1290 Inifinity II equipped with thermostatted auto-injector and column compartment coupled to a Q Exactive Plus (Quadrupole Orbitrap hybrid MS) MS equipped with ion max source with APCI probe (Thermo Fisher Scientific, USA) at Royal NIOZ. Positive-ion APCI settings were as described in Baxter et al. (2019). Chromatography was as in Hopmans et al. (2016) but with the following elution method: isocratic elution for 25 min

with 18% B, followed by a linear gradient to 30% B at 50 min, then a linear gradient to 100% B at 80 min which was held isocratically until 120 min, where A is hexane and B is hexane:isopropanol (9:1, v/v). Flow rate was 0.2 ml $min^{-1}$ and the total run time was 140 min including a 20 min re-equilibration.

*2.2 Acid hydrolysis of polar fractions and analysis of released compounds*

Polar fractions were hydrolysed in 1.5M HCl in MeOH for 2 hours at 70 °C. After cooling, $H_2O$ and DCM were added and the organic phase was collected. The aqueous phase was extracted (2x) with DCM. The combined DCM fraction was passed over a $Na_2SO_4$ column and dried under a gentle $N_2$ stream. An aliquot was methylated using trimethylsilyldiazomethane, passed over a $SiO_2$ column using ethyl acetate (EtOAc), and then silylated using *bis*(trimethylsilyl)trifluoracetamide in pyridine at 60 °C for 20 min. The derivatised aliquots were dissolved in EtOAC and injected on-column on an Agilent 7890B gas chromatograph (GC) coupled to a 5977B MSD using a CP-sil 5CB fused silica column (30 m x 0.32 mm i.d., film thickness 0.10 µm). The GC-MS was operated at a constant flow of 1.0 mL $min^{-1}$ with helium as the carrier gas. The oven was programmed starting at 70 °C to rise to 130 °C at a rate of 20 °C $min^{-1}$ and then to 320 °C at a rate of 4 °C $min^{-1}$, followed by an isothermal hold for 20 min. The MS was operated in Full Data Acquisition mode, scanning ions from *m/z* 50 - 800 at 70 eV. Aliquots of the polar fraction taken before and after hydrolysis were analyzed using UHPLC-MS as above, with the addition of *m/z* 1064.3, 1078.3, 1092.3, 1106.3 and a window of 1.0 to the original selected ion monitoring (SIM) method.

## 3. Results and discussion

*3.1 Identification of tetraester and mixed ether/ester-bound membrane lipids in mineral soils*

UHPLC-MS chromatograms of the soils from Nepal and Rwanda obtained using the original settings revealed an additional peak in the *m/z* 1050 trace, eluting at 70.4 mins, ~28 mins later than brGDGT-IIIa (Fig. 1). After screening UHPLC-MS chromatograms of soils previously analyzed at Utrecht University, the expression of this peak was tentatively linked to the low pH and/or relatively high elevation of the soils. To identify the compound represented by this peak, an accurate mass spectrum was generated using UHPLC-HRMS (Fig. 2; brGDGT-Ia+2esters). The obtained mass spectrum showed similarities with those of tetraester, tetraether, and mixed ether/ester lipids generated from cell material of different species from the order of *Thermatogales* (Sinninghe Damsté et al., 2007). Although the lipids of *Thermatogales* contain a core derived from diabolic acid rather than *iso*-DA, the close resemblance in fragmentation products with the mass spectrum generated here suggests a similar mixed ether/ester configuration of the core lipid. The exact mass of this ion at *m/z* 1049.970 indicates a $[M+H]^+$ ion with formula $C_{66}H_{129}O_8$, which fits with a brGDGT-Ia in which two ethers are replaced by esters (Table 1). Notably, the HRMS spectra revealed the presence of several other compounds with comparable fragmentation patterns that, together, suggest that the soils contain membrane-spanning lipids to which the *iso*-DA backbones are attached to glycerols with one, two, or four ester bonds (Fig. 2). In addition, the *iso*-DA can have one or two additional methylations, as in brGDGT-IIa and IIIa (Fig 1, Table 1). To confirm the presence of ester bonds, aliquots of the GDGT fractions were analyzed before and after acid hydrolysis using UHPLC-MS with the addition of ions representing brGDGTs Ia, IIa, and IIIa in which all four ethers are replaced by esters to the original method, i.e., *m/z* 1064, 1078, 1092, and 1106. Hydrolysis resulted in the disappearance of all peaks representing the suspected tetraester and mixed ether/ester lipids in the UPLC-MS chromatogram.

### *3.2. Implications for brGDGT synthesis*

In the soil from Nepal, three sets of Ia, IIa, and IIIa-tetraesters with virtually identical mass spectra but different retention times are present (Fig. 1; Table 1), which could be linked to the configuration of the glycerol units, or the position of the ester moiety. To possibly obtain insight in the position of the ester moieties in the mixed ether/ester lipids, the hydrolyzed GDGT fractions were analyzed using GC-MS. Interestingly, whereas hydrolysis released *iso*-DA from the Nepal GDGT fraction, this is not detected in the hydrolyzed Rwanda GDGT fraction. Instead, this fraction contains *iso*-$C_{15}$ and *iso*-$C_{17}$ MGEs, as also found in cell material from strains from SD4 (Sinninghe Damsté et al., 2014, 2018). This suggests that the mixed ether/ester lipids in these two soils could be produced by *Acidobacteria* from distinct SDs that follow different biosynthesis pathways: by tail-to-tail linkage of two *iso*-$C_{15}$ fatty acids to form *iso*-DA, or through the reduction of diesters into diethers and subsequent carbon-carbon linking.

## 4. Conclusions

The occurrence of tetraester and mixed ether/ester-bound *iso*-DA membrane-spanning lipids in the environment confirms their existence as hypothesized intermediates during brGDGT synthesis. Differences in compounds released after acid hydrolysis of the GDGT fractions support both current hypothesized biosynthesis pathways. However, both pathways contain steps for which the responsible enzymes or gene clusters have not (yet) been identified, complicating the identification of the organisms producing brGDGTs though bioinformatics.

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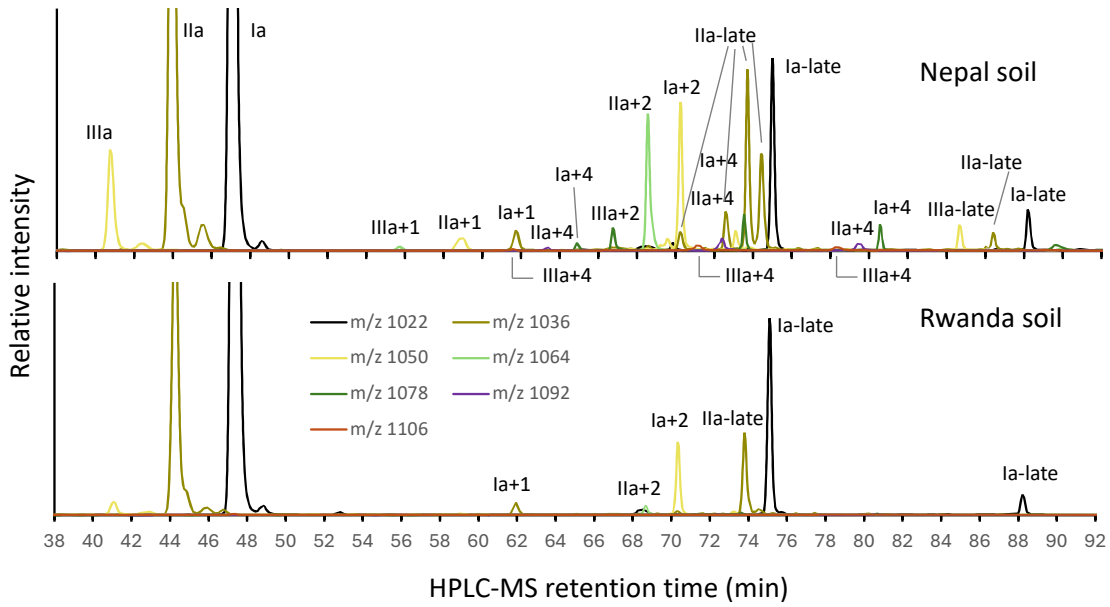


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224 Figure 1. HPLC-MS chromatograms of brGDGTs in mineral soils from Nepal and Rwanda with

225 peaks assigned as in Table 1.

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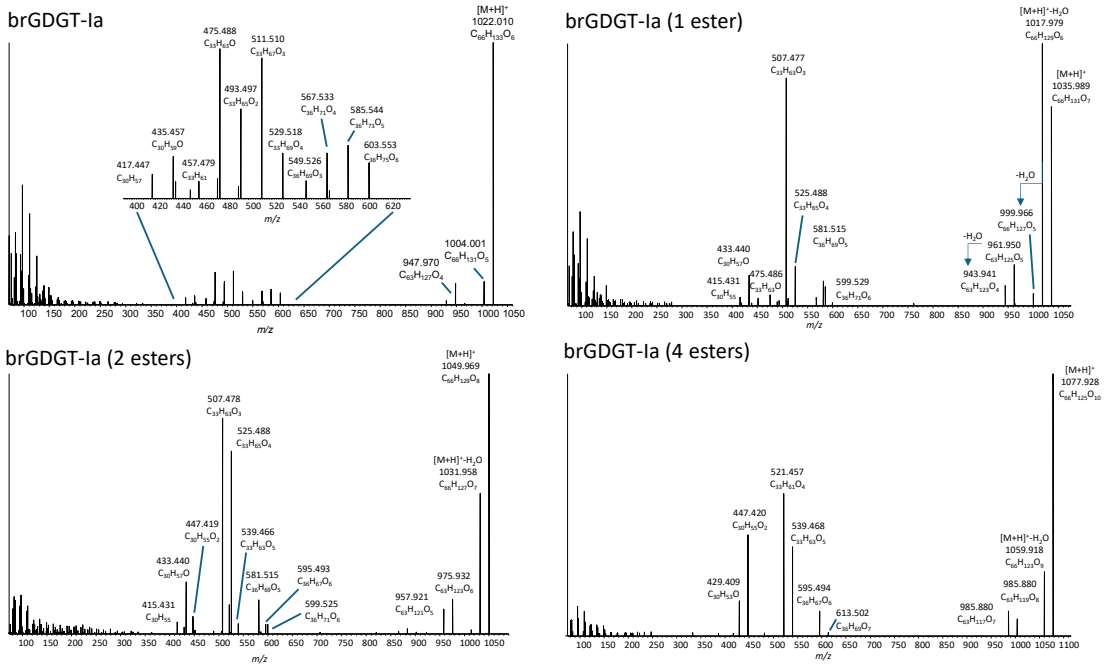


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228 Figure 2. UHPLC-HRMS mass spectra of brGDGT-Ia, mixed ether/ester, and tetraester-bound

229 *iso*-DA membrane spanning lipids in the Nepal soil based on accurate mass.

Table 1. Compounds observed in this study. AEC = assigned elemental composition, Δ mmu = (measured mass – calculated mass) x 1000.

| Assignment | Retention time (min) in Fig. 1 | MS nominal mass ($[M+H]^+$) | HRMS accurate mass ($[M+H]^+$) | | | |
|---|---|---|---|---|---|---|
| | | | Calculated | Observed | AEC | Δ mmu |
| brGDGT-Ia | 47.2 | 1022 | 1022.010 | 1022.010 | $C_{66}H_{133}O_6$ | 0.0 |
| brGDGT-Ia late eluting | 75.5 | 1022 | 1022.010 | 1022.012 | $C_{66}H_{133}O_6$ | -2.0 |
| brGDGT-Ia late eluting | 88.3 | 1022 | 1022.010 | 1022.013 | $C_{66}H_{133}O_6$ | -3.0 |
| brGDGT-Ia (1 ester) | 62.0 | 1036 | 1035.989 | 1035.991 | $C_{66}H_{131}O_7$ | -2.0 |
| brGDGT-Ia (2 esters) | 70.4 | 1050 | 1049.968 | 1049.970 | $C_{66}H_{129}O_8$ | -2.0 |
| brGDGT-Ia (4 esters) | 64.9 | 1078 | 1077.927 | 1077.929 | $C_{66}H_{125}O_{10}$ | -2.0 |
| brGDGT-Ia (4 esters) | 73.7 | 1078 | 1077.927 | 1077.928 | $C_{66}H_{125}O_{10}$ | -1.0 |
| brGDGT-Ia (4 esters) | 80.7 | 1078 | 1077.927 | 1077.931 | $C_{66}H_{125}O_{10}$ | -4.0 |
| brGDGT-IIa | 45.0 | 1036 | 1036.025 | 1036.024 | $C_{67}H_{135}O_6$ | 1.0 |
| brGDGT-IIa late eluting | 70.7 | 1036 | 1036.025 | 1036.027 | $C_{67}H_{135}O_6$ | -2.0 |
| brGDGT-IIa late eluting | 72.9 | 1036 | 1036.025 | 1036.028 | $C_{67}H_{135}O_6$ | -3.0 |
| brGDGT-IIa late eluting | 73.4 | 1036 | 1036.025 | 1036.028 | $C_{67}H_{135}O_6$ | -3.0 |
| brGDGT-IIa late eluting | 74.9 | 1036 | 1036.025 | 1036.027 | $C_{67}H_{135}O_6$ | -2.0 |
| brGDGT-IIa late eluting | 86.4 | 1036 | 1036.025 | 1036.029 | $C_{67}H_{135}O_6$ | -4.0 |
| brGDGT-IIa (1 ester) | 59.2 | 1050 | 1050.005 | 1050.007 | $C_{67}H_{133}O_7$ | -2.0 |
| brGDGT-IIa (2 esters) | 69.1 | 1064 | 1063.984 | 1063.984 | $C_{67}H_{131}O_8$ | 0.0 |
| brGDGT-IIa (4 esters) | 63.5 | 1092 | 1091.942 | 1091.945 | $C_{67}H_{127}O_{10}$ | -3.0 |
| brGDGT-IIa (4 esters) | 72.5 | 1092 | 1091.942 | 1091.945 | $C_{67}H_{127}O_{10}$ | -3.0 |
| brGDGT-IIa (4 esters) | 79.6 | 1092 | 1091.942 | 1091.944 | $C_{67}H_{127}O_{10}$ | -2.0 |
| brGDGT-IIIa | 41.1 | 1050 | 1050.041 | 1050.040 | $C_{68}H_{137}O_6$ | 1.0 |
| brGDGT-IIIa late eluting | 85.1 | 1050 | 1050.041 | 1050.044 | $C_{68}H_{137}O_6$ | -3.0 |
| brGDGT-IIIa (1 ester) | 55.8 | 1064 | 1064.020 | 1064.024 | $C_{68}H_{135}O_7$ | -4.0 |
| brGDGT-IIIa (2 esters) | 66.8 | 1078 | 1077.999 | 1078.002 | $C_{68}H_{133}O_8$ | -3.0 |
| brGDGT-IIIa (4 esters) | 61.8 | 1106 | 1105.958 | 1105.961 | $C_{68}H_{129}O_{10}$ | -3.0 |
| brGDGT-IIIa (4 esters) | 71.3 | 1106 | 1105.958 | 1105.959 | $C_{68}H_{129}O_{10}$ | -1.0 |
| brGDGT-IIIa (4 esters) | 78.5 | 1106 | 1105.958 | 1105.961 | $C_{68}H_{129}O_{10}$ | -3.0 |