Analysis of non-derivatized bacteriohopanepolyols using UHPLC-HRMS reveals great structural diversity in environmental lipid assemblages

HOPMANS, Ellen C, SMIT, Nadine T, SCHWARTZ-NARBONNE, Rachel <http://orcid.org/0000-0001-9639-9252>, SINNINGHE DAMSTÉ, Jaap S and RUSH, Darci

Available from Sheffield Hallam University Research Archive (SHURA) at:
http://shura.shu.ac.uk/28851/

This document is the author deposited version. You are advised to consult the publisher's version if you wish to cite from it.

Published version

HOPMANS, Ellen C, SMIT, Nadine T, SCHWARTZ-NARBONNE, Rachel, SINNINGHE DAMSTÉ, Jaap S and RUSH, Darci (2021). Analysis of non-derivatized bacteriohopanepolyols using UHPLC-HRMS reveals great structural diversity in environmental lipid assemblages. Organic Geochemistry, p. 104285.

Copyright and re-use policy

See http://shura.shu.ac.uk/information.html
Analysis of non-derivatized bacteriohopanepolyols using UHPLC-HRMS reveals great structural diversity in environmental lipid assemblages

Ellen C. Hopmans a,*, Nadine T. Smit a, Rachel Schwartz-Narbonne b, Jaap S. Sinninghe Damsté a, c, Darci Rush a, b

a NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Microbiology and Biogeochemistry, P.O. Box 59, 1790 AB Den Burg, Texel, the Netherlands
b School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne NE1 7RU, United Kingdom
c Utrecht University, Faculty of Geosciences, Department of Earth Sciences, P.O. Box 80.121, 3508 TA Utrecht, the Netherlands

* Corresponding author.
E-mail address: ellen.hopmans@nioz.nl (E.C. Hopmans).

Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem

ARTICLE INFO

Keywords:
Non-derivatized bacteriohopanepolyols
UHPLC-HRMS
novel composite BHPs
Adenosylhopanes

ABSTRACT

Bacteriohopanepolyols (BHPs) are lipids with great chemotaxonomic potential for microbial populations and biogeochemical processes in the environment. The most commonly used methods for BHP analysis are chemical degradation followed by gas chromatography-mass spectrometry (MS) or derivatization followed by high performance liquid chromatography (HPLC)-atmospheric pressure chemical ionization/MS. Here we report on significant advances in the analysis of non-derivatized BHPs using UHPLC-electrospray ionization-high resolution MS2. Fragmentation mass spectra provided information on the BHP core, functionalized side chain, as well as the conjugated moiety of composite BHPs. We successfully identified the common bacteriohopanepolyol and their (di)methylated and (di)unsaturated homologues, aminoBHPs, and composite BHPs (e.g., cyclitol ethers and methylcarbamate-aminoBHPs) in biomass of several known BHP-producing bacteria. To show how the method can be exploited to reveal the diversity of BHPs in the environment, we investigated a soil from an active methane seep, in which we detected ca. 130 individual BHPs, including a complex distribution of adenosylhopanes. We identified the nucleoside base moiety of both adenosylhpane type-2 and type-3. Adenosyl hpane type-3 contains a methylated adenine as its nucleobase, while type-2 appears to contain a deaminated and methylated adenine, or N1-methylinosine. In addition, we detected novel adenosylhopanes. Furthermore, we identified a series of novel composite BHPs comprising of bacteriohopanepolyols conjugated to an ethenolamine moiety. The novel ethenolamineBHPs as well as aminohBHPs were also detected acylated to fatty acids. The analytical approach described allows for simultaneous analysis of the full suite of IPLs, now including BHPs, and represents a further step towards environmental lipidomics.

1. Introduction

Bacteriohopanepolyols (BHPs) are membrane lipids with great chemotaxonomic potential with regards to microbial populations as well as biogeochemical processes in the environment. BHPs are composed of a pentacyclic triterpenoid ring system with an extended side chain containing 4, 5, or 6 functional groups (Fig. S1) comprised of either all hydroxyl moieties (bacteriohopanetetrol (BHT), bacteriohopanepentol (BHPentol), and bacteriohopanhexol (BHHexol); Rohmer et al., 1984; Talbot et al., 2003a; Talbot and Farrimond, 2007) or 3, 4, or 5 hydroxyl moieties and a single terminal amino-group (i.e., aminotriol, -tetrol, and -pentol; e.g., Neunlist and Rohmer, 1985b; Rohmer, 1993). The basic, unmodified ring system with a linear side chain is usually the dominant BHP in both culture and environmental samples (see also review of BHP sources in Talbot and Farrimond, 2007, and Talbot et al., 2008). BHPs can be further modified by methylation at the C-2 (2MeBHPs; e.g., Talbot et al., 2008 and references therein), C-3 (3MeBHPs; e.g., Cvejic et al., 2000a), both C-2 and C-3 (2,3diMeBHPs; Sinninghe Damsté et al., 2017), C-31 (Simonin et al., 1994), or C-12 (Costantino et al., 2000) positions. BHPs can also include unsaturation in the ring system located at Δ6, Δ11 or both (Talbot et al., 2007b and references therein). A BHP with an unsaturation in the side chain was identified in a methanotrophic Methylovulum bacterium (van Winden et al., 2012). A large variety of so-called composite BHPs, where the terminal functional group is bound to complex, often polar moieties, have also been identified (Talbot et al., 2007a and references therein), such as the...
Advances followed with the application of atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (ESI)-high resolution dual-stage MS (HRMS²). Using an approach, which has been successfully applied to the analysis of intact polar lipids (IPLs), we analyzed a number of bacterial species, a.o. ‘Candidatus Scalindua profunda’, ‘Ca. Methylospirillum oxysphaera’, Methylococcus capsulatus, and Methylaminococcus vadi, as well as a soil from an active terrestrial methane seep. We discuss elution and fragmentation behaviour of a wide range of known BHPs, including N-containing BHPs and composite BHPs, and the tentative identification of an extensive set of novel BHPs.

2. Materials and Methods

2.1. Sample description

Komagataeibacter xylinus strain R-2277 (formerly Gluconacetobacter xylinus and Acetobacter aceti ssp. xylinus) was obtained as frozen cells in culture medium from an industrial culture (Hoffmann-La Roche, Basel). This culture has been used in previous BHP studies (Peiseler and Rohmer, 1992; Schwartz-Narbonne et al., 2020). An enrichment culture of the bacterium ‘Ca. Methylospirillum oxysphaera’ was obtained from a bioreactor operated under conditions described previously by Ettwig et al. (2009). The bioreactor population consisted of ca. 67% ‘Ca. M. oxysphaera’, while the remainder was composed of a mix of ANME-2d archaea and different minor bacteria phyla (see Smit et al. (2019) for details). This culture has been used in previous BHP studies by Kool et al. (2014). *Methylococcus capsulatus* (strain Bath) was obtained from the University of Warick culture collection (as described in Talbot et al., 2001). *M. capsulatus* has been studied in previous BHP studies by Neunlist and Rohmer (1985b) and Talbot et al. (2001). An enrichment culture of ‘Ca. Scalindua profunda’ was grown in a sequencing batch reactor at room temperature (ca. 20 °C) as described by van de Vosenberg et al. (2008) and consisted of 80–90% ‘Ca. S. profunda’. BHPs have been previously studied in this ongoing enrichment culture by Rush et al. (2014) and Schwartz-Narbonne et al. (2020). Methylaminococcus vadi (strain IT-4) was isolated from a microbial mat of a shallow (–23 m water depth) marine hydrothermal system in a coral reef off Taketomi Island, Okinawa, Japan (Hirayama et al., 2007; 2013). Cultivation of this strain was performed at JAMSTEC, Japan, using M2 medium pH 6.6 at 37 °C. This culture has been used in previous BHP studies (Rush et al., 2016).

The Fuoco di Censo seep (37°37′30.1″N, 13°23′15.0″E), in the mountains of Southwestern Sicily, Italy, is a typical example of a natural ‘Everlasting Fire’ (Etiöpe et al., 2002; Grassa et al., 2004; Smit et al., 2021). The Censo seep gas consists of mainly thermally generated methane (76–86%). A soil sample was recovered from a horizon 5–10 cm below soil surface directly at the main gas seep (Censo 0 m). The soil sample was stored in a clean geochemical sampling bag and kept frozen at –20 °C until freeze drying and extraction. Further details can be found in Smit et al. (2021).

2.2. Lipid extraction

Freeze-dried bacterial biomass and the soil from the Censo seep were extracted using a modified Bligh and Dyer method (Bligh and Dyer, 1959; Bale et al., 2013). The samples were ultrasonically extracted (10 min) with a solvent mixture containing methanol (MeOH), dichloromethane (DCM) and phosphate buffer (2:1:0.8, v:v:v). Solvent was collected after centrifugation and the residues re-extracted twice. A biphasic separation was achieved by adding additional DCM and phosphate buffer to the combined extracts in a ratio of MeOH, DCM and...
phosphate buffer (1:1:0.9, v:v:v). After the DCM layer was collected, the aqueous layer was washed twice with DCM. Combined DCM layers were dried under a continuous flow of N$_2$ gas (N$_2$ pressure, 300 mbar; S-lens 70 V). Detection was achieved using positive ion ESI analysis with a Thermo Scientific Pierce LTQ Velos Orbitrap MS for untargeted detection and identification of BHPs with data dependent MS$^2$ analysis using HRMS, similar to the approach that has successfully been applied to the analysis of IPLs (Moore et al., 2016; Besseling et al., 2018; Bale et al., 2019). For non-targeted IPL analysis,

### 3. Results and Discussion

#### 3.1. UHPLC method development

The suitability of C$_{18}$ columns for the retention and separation of several non-derivatized BHPs was previously demonstrated by Talbot et al. (2016). Two types of C$_{18}$ column were tested in that study: an Acquity BEH UHPLC column and an Ace base-deactivated Excel UHPLC column, of which the latter showed the best chromatographic behavior, particularly for N-containing BHPs. Previously, the Acquity BEH C$_{18}$ column was selected by Wörmer et al. (2013) in a new UHPLC-ESI/MS method for the analysis of IPLs. As BHPs, like IPLs, consist of a large apolar moiety and a polar functionalized side chain with or without an additional, often polar, moiety or head group, we set out to test whether the chromatographic system, combined with positive ion ESI, as described by Wörmer et al. (2013) for IPLs, is suitable for the analysis of non-derivatized BHPs. For this purpose, we investigated extracts of biomass of various known BHP-producing bacteria. The exact distribution of the detected BHPs and their fragmentation spectra are discussed in detail in section 3.3. Here we only discuss the general features of the chromatographic method. BHT was easily identified in several extracts in detail in section 3.3. Here we only discuss the general features of the chromatographic method. BHT was easily identified in several extracts in detail in section 3.3. Here we only discuss the general features of the chromatographic method.

#### 3.2. MS method development

MS detection of derivatized BHPs, in general, has been done by using positive ion APCI combined with ion trap MS$^2$ (Talbot et al. 2003a, b, 2007a, b). For non-derivatized BHPs, Talbot et al. (2016) employed APCI combined with MRM using a triple quadrupole MS. Here we explored the use of positive ion ESI combined with a quadrupole-Orbitrap MS for untargeted detection and identification of BHPs with data dependent MS$^2$ analysis using HRMS, similar to the approach that has successfully been applied to the analysis of IPLs (Moore et al., 2016; Besseling et al., 2018; Bale et al., 2019). For non-targeted IPL analysis.
we apply a stepped normalized collision energy of 15, 22.5 and 30 to produce informative and diagnostic spectra (e.g., Bale et al., 2019; Sollai et al., 2020). Although the separation of different stereoisomers of non-derivatized BHTs (i.e., BHT-34 and BHT-22) was analyzed to study the protonated ([M+H]+, base peak), ammoniated ([M+NH4]+) and sodiated ([M+Na]+) of BHT (m/z 151, 547.472, 546.499, m/z 569.454, respectively) revealed the presence of multiple isomers of BHT (Fig. 1A). The two peaks at 20.8 and 21.1 min (peaks b and c) match the retention time for BHT with the 22 \( \Delta^6 \) isomer, respectively, as established by Schwarz-Narbonne et al. (2020). The most abundant isomer of BHT (i.e., labeled a), eluting early at 19.3 min, may be BHT-22 \( \Delta^6 \), which was reported in relatively high abundance (13% of all BHTs) in K. xylinus by Peiseler and Rohmer (1992). In addition to these abundant isomers, there are several isomers present at trace levels (not visible at the scale of Fig. 1) eluting after both the early eluting BHT isomer and the BHT-22 \( \Delta^6 \) isomer.

3.3. Commonly detected BHPs in various microorganisms

Talbot et al. (2016) reported MS\textsuperscript{2} spectra under various fragmentation conditions for non-derivatized BHT. However, no spectra were reported for methylated BHTs, unsaturated BHTs or BHpentol and BHhexol. To establish the chromatographic behavior and fragmentation of various BHPs we analyzed biomass of several known BHP-producing bacteria. 

3.3.1. Bacteriohopanetetrols

Biomass of K. xylinus, which has been reported to produce multiple (3Me)BHT isomers with up to two unsaturations at \( \Delta^6 \) and/or \( \Delta^{11} \) (Peiseler and Rohmer, 1992; Talbot et al., 2007b), was analyzed to study the various isomers and homologues of BHT. Schwartz-Narbonne et al. (2020) already showed the separation of different stereoisomers of non-derivatized BHTs (i.e., BHT-34S, BHT-34R and BHT-x) using the method described here. Analysis of biomass of K. xylinus provided further evidence that various stereoisomers of BHT are (partially) separated under the UHPLC conditions in this study (Fig. 1). Table S1 lists all BHPs discussed below and provides details on preferred ionization, in-source fragmentation and diagnostic fragments.

The summed mass chromatogram of the calculated exact masses of \( \Delta^6 \) (peak f, Fig. 1) and \( \Delta^{11} \) (peak g, Fig. 1) isomer and the BHT-22 \( \Delta^6 \) abundant isomer of BHT (i.e., labeled a), eluting early at 19.3 min, may be BHT-22S,34S, which was reported in relatively high abundance (13% of all BHTs) in K. xylinus by Peiseler and Rohmer (1992). In addition to these abundant isomers, there are several isomers present at trace levels (not visible at the scale of Fig. 1) eluting after both the early eluting BHT isomer and the BHT-22S,34S/R.

All the BHT isomers described above had similar MS\textsuperscript{2} spectra, therefore we only discuss the MS\textsuperscript{2} spectrum of BHT-22S,34S (peak b, Fig. 1A), obtained from the ammoniated molecule (m/z 564.5; Fig. 2A). The MS\textsuperscript{2} spectrum obtained here is similar to that reported by Talbot et al. (2016) for non-derivatized BHT upon MS\textsuperscript{2} fragmentation of m/z 529 (formed after in source loss of H\textsubscript{2}O from protonated BHT ([M+H-H\textsubscript{2}O]+)). Here, m/z 529.461 (C\textsubscript{20}H\textsubscript{33}O\textsubscript{3}, \( \Delta \) ppm = 0.65) is the base peak. Fragment ions at m/z 511.45 (C\textsubscript{20}H\textsubscript{31}O\textsubscript{2}, \( \Delta \) ppm = 1.56), m/z 493.437 (C\textsubscript{20}H\textsubscript{29}O\textsubscript{2}, \( \Delta \) ppm = 6.73), and m/z 475.430 (C\textsubscript{20}H\textsubscript{26}O\textsubscript{2}, \( \Delta \) ppm = 0.80) represent consecutive losses of hydroxyl moieties as H\textsubscript{2}O (see Table S1 for assigned elemental composition (AEC) of fragments). In the lower mass range, m/z 163.148 (C\textsubscript{4}H\textsubscript{8}O, \( \Delta \) ppm = 0.17) is the dominant
fragment, but the universal diagnostic fragment ion for hopanoids (m/z 191.179, C_{36}H_{70}, Δ ppm -0.56) (Peters and Moldowan, 1993) is clearly present and more dominant than observed in the spectrum of Talbot et al. (2016). Unlike electron ionization (EI) fragmentation, collision-induced dissociation (CID) fragmentation typically does not produce radicals but one protonated fragment and a neutral. Any cleavage results in proton rearrangements and the formation of a double bond equivalent in one of the two fragments (for discussed fragmentation pathways and fragments, see Fig. 3). In case of BHT, the m/z 191 fragment represents the protonated A and B ring of the hopanoid structure with 2 double bonds (unlike the structure proposed by Talbot et al. (2003b)), generated by the double cleavage at C-9/C-11 and C-8/C-14 following fragmentation pathway A. Additional diagnostic fragments are found at m/z 369.352 (complete hopanoid ring system after loss of side chain; C_{36}H_{68}, Δ ppm -0.21) and at m/z 283.242 (C_{21}H_{31}, Δ ppm -1.58), m/z 301.251 (C_{21}H_{33}O^+, Δ ppm -3.63), 319.264 (C_{21}H_{35}O^-, Δ ppm 3.52), and 337.273 (C_{21}H_{37}O^-, Δ ppm -1.19). These latter C_{21} fragments are complementary to the fragment at m/z 191 and represent the D and E rings and side chain with 0 to 3 hydroxyl moieties remaining (fragmentation A’, Fig. 3). Talbot et al. (2003a, b) observed the equivalent fragments for acetylated BHT.

3.3.2. Methylated BHTs

The distribution of the 3MeBHTs in K. xylinus in general followed the distribution of the BHTs and they elute roughly 2 min after their non-methylated BHT-counterparts, but were ca. an order of magnitude less abundant. Two stereoisomers of 3MeBHT were detected (peaks k and l, Fig. 1D) and based on their relative retention time were identified as methylated BHT-counterparts, but were ca. an order of magnitude less abundant. The MS spectra of the ammoniated molecules (m/z 562.5) associated with peaks e and f (e.g., Fig. 2B) are almost identical. The HPLC-MS analysis of acetylated unsaturated BHTs was extensively discussed by Talbot et al. (2007b), but not for non-derivatized 3MeBHTs. We observed several isomers of unsaturated BHTs in biomass of K. xylinus (Fig. 1B). The summed mass chromatogram of the calculated exact masses of the protonated, ammoniated, and sodiated molecules of unsaturated BHT (m/z 545.456 + 562.483 ÷ 567.438) showed two main peaks at 18.5 and 18.7 min (e and f), as well as a minor early eluting isomer at 17.8 min (d) and a pair of late eluting isomers at 19.5 and 19.6 min (g and h). Peiseler and Rohmer (1992) reported two isomers of Δ^6- BHT (22R,34S and 22R,34R) in relatively high abundance in K. xylinus (20 and 36% respectively of total BHTs). Based on this distribution it is likely that peaks e and f represent Δ^6-BHT-22R,34S and Δ^6-BHT-22R,34R, respectively. The MS^2 spectra of the ammoniated molecules (m/z 562.5) associated with peaks e and f (e.g., Fig. 2B) are almost identical. The HPLC-MS analysis of acetylated unsaturated BHTs was extensively discussed by Talbot et al. (2007b), but not for non-derivatized BHTs (Talbot et al. 2016). The unsaturation in Δ^6-BHT-22R,34R (peak f, Fig. 2B) is clearly observed in several fragments formed after consecutive losses of H_2O from the side chain, that are offset by 2 Da from the spectrum of BHT, e.g., m/z 527.466 (C_{30}H_{58}O_3, Δ ppm -0.36), 509.435 (C_{29}H_{56}O_2, Δ ppm -0.37), 491.421 (C_{28}H_{54}O_2^+, Δ ppm -6.76) and 473.415 (C_{28}H_{52}, Δ ppm 1.88). Interestingly, and

Fig. 3. Fragmentation pathways and structures for proposed diagnostic fragments after collision induced fragmentation for BHT, Δ^6-BHT, Δ^{11}-BHT, Δ^6,^{11}-BHT, and their 3Me-counterparts.
similar as described by Talbot et al. (2007), no such offset is observed in the diagnostic fragment ion at m/z 191.179 (C_{11}H_{22}Δ, Δ ppm –0.88). While in case of BHT the fragment at m/z 191 is produced by cleavage at C-9/C-11 and C-8/C-14 and a proton rearrangement resulting in two double bonds in the A and B Ring fragment (as discussed above), it appears that the presence of a pre-existing double bond at Δ results in an alternative proton rearrangement: only one additional double bond is formed in the A and B ring fragment, thus generating the m/z 191 also seen in BHT, and the other double bond is formed in the neutral loss fragment (fragmentation A, Fig. 3). Talbot et al. (2007b) also observed evidence for an alternative fragmentation pathway in acetylated BHT, with cleavage of the C-11/C-12 and C-8/C-14 bonds (pathway B, Fig. 3). With proton rearrangement to form two additional double bonds on the A and B ring fragment, this fragmentation would produce the here observed m/z 203.179 (C_{11}H_{23}, Δ ppm –0.233; Fig. 3). A complementary series of C_{20} fragments with 1–3 hydroxy moieties are observed at m/z 287.237 (C_{20}H_{21}O^+, Δ ppm 0.41), 305.247 (C_{20}H_{22}O_2, Δ ppm –0.42) and 323.258 (C_{20}H_{23}O_2, Δ ppm –0.44) and represent the D and E rings with the (partially dehydroxylated) side chain.

The MS^2 spectra of peaks g and h (Fig. 1B) are largely similar to those of peaks d-f, however there are some notable differences in the middle mass range (Fig. 2C), which shows a series of C_{20} to C_{28} fragments with up to three hydroxyl moieties. It is possible that the presence of a double bond in the C ring leads to a multitude of alternative fragmentation pathways involving the A and B rings. For example, the larger fragments (>C_{24}) appear to originate from cleavage of the C-9/C-10 bond in combination with cleavage at C-5/C-6, C-6/C-7, or C-7/C-8 (Fig. 3, pathways C, D, E). We, therefore, tentatively assign the location of the unsaturation in peaks g and h to Δ11. Peiseler and Rohmer (1992) did not report Δ11-BHT in K. xylinus, perhaps due to lesser sensitivity of the method used, however both Δ11-3MeBHT-22,34S and Δ11-2MeBHT-22,34R were reported in this microorganism, showing its capability to produce an unsaturation at that position. Based on this and the typical pair-wise elution pattern observed here for the 22R,34S and 22S,34R stereoisomers of BHT and Δ11-BHT, we tentatively identify peaks g and h as Δ11-BHT-22S,34S and Δ11-BHT-22R,34R.

The MS^2 spectrum of the early eluting isomer (d) is almost identical to those of peaks e and f (Δ5-BHT-22R,34S and Δ5-BHT-22R,34R), suggesting that this isomer contains a double bond at Δ5. If that is the case, and based on the retention time offset between the BHT-22S,34R and Δ5-BHT-22R,34R pair and the Δ6-BHT-22R,34S/R pair, its fully saturated BHT counterpart would be one of the very minor BHT isomers with unknown stereochemistry eluting between peaks a and b.

The summed mass chromatogram revealing the distribution of di-unsaturated BHTs (Fig. 1C) shows two main peaks at 17.5 and 17.7 min (peaks i and j), most likely the 34- and 35-BHTs, to the B-ring and assigned a trimethylbenzene structure.
fragment. The fragment at m/z 189 likely represents the A and B rings. Although formation of an A and B ring fragment with three double bonds does not appear to be favored in case of a Δ^6^-unsaturation, an additional unsaturation at Δ^11^- is apparent due to the presence of a Δ^11^-unsaturation. The fragments in the middle mass range in the MS² spectrum of the di-unsaturated BHT are less abundant (<2%; e.g. m/z 257.266 [C_19H_28O_2, Δ ppm -0.38], m/z 269.226 [C_20H_29O_2, Δ ppm -0.08], m/z 283.242 [C_21H_30, Δ ppm -1.65], m/z 319.264 [C_22H_30O_2, Δ ppm 2.08], m/z 337.274 [C_21H_29O_2, Δ ppm 1.77], m/z 389.305 [C_22H_29O_2, Δ ppm 1.10], m/z 415.321 [C_22H_30O_2, Δ ppm 0.21], m/z 429.336 [C_22H_29O_2, Δ ppm 0.02], m/z 441.336 [C_22H_29O_2, Δ ppm 0.29]) and appear to represent a combination of the fragment pathways observed for the Δ^6^- and Δ^11^-unsaturation.

3.3.4. Unsataturated methylated BHTs

The summed mass chromatogram for the unsaturated 3MeBHT showed two pairs of peaks at 20.0 and 20.2 min (peaks m and n) and at 21.2 and 21.4 min (peaks p and q) (Fig. 1E). The MS² spectra of peaks m and n (Fig. S2B) showed very similar fragmentation to the Δ^6^-unsaturated BHTs, with the expected +14 Da offsets in the A and B ring fragments. The fragments in the middle mass range were largely absent. The spectra of peaks p and q (Fig. S2C) showed the same series of C_20 to C_26 fragments as observed for the two unsaturated BHT peaks (g and h) designated as Δ^11^-unsaturated. Based on the relative retention times and similarities of their spectra to the unsaturated BHTs, we identify these peaks as (m) Δ^6^-3MeBHT-22R,34S, (n) Δ^6^-3MeBHT-22R,34R, (p) Δ^11^-3MeBHT-22R,34S, and (q) Δ^11^-3MeBHT-22R,34R. All but Δ^6^-3MeBHT-22R,34R were also reported by Peiseler and Rohmer (1992) in K. xylinus.

We also detected two isomers of di-unsaturated 3MeBHT at 19.00 and 19.12 min (Fig. 1F, peaks q and r). Peiseler and Rohmer (1992) also detected two isomers of Δ^6,11^-3MeBHT in K. xylinus, differing in stereochemistry at the C-34. Based on this and the relative retention times we identify peaks q and r as Δ^6,11^-3MeBHT-22R,34S and Δ^6,11^-3MeBHT-22R,34R, respectively. The observed fragmentation spectrum (Fig. S2D) closely matched those of Δ^6,11^-BHT. It is noteworthy that the fragment at m/z 203 (analogous to m/z 189 in the MS² spectrum of Δ^6,11^-BHT) is diagnostic for a di-unsaturated MeBHT and not for Δ^6^-BHT as in the case with EI ionization. However, the m/z 203 fragment alone is not sufficient to identify a Δ^6,11^-MeBHT as a fragment with identical m/z is also present in the fragmentation spectrum of Δ^6^-BHT, although it is generated via a different fragmentation pathway.

3.3.5. Bacteriohopanepentols and –hexols

In order to establish elution and fragmentation patterns for bacteriohopanepentol and -hexol (BHpentol and BHhexol, respectively), as well as their 3Me-homologues, we analyzed an extract of Ca. M. oxyfera (Kool et al., 2014). Fig. 4A shows summed mass chromatograms revealing the presence of BHT (peak a), BHpentol (peak b) and BHhexol (peak c) and their methylated analogues (peaks d, e, and f). The BHPs elute in reversed order of number of hydroxylations on the side chain. Whereas ammoniation is the preferred ionization for BHT, the balance shifts towards protonation for BHhexol (Table S1). Fig. 4D shows the MS² spectrum of the protonated molecule ([M+H]⁺) of BHhexol. The observed fragmentation pattern is comparable to the one described above for BHT, with major fragments representing losses of 1 to 5 hydroxyl moieties at m/z 561.451 [C_26H_29O_2, Δ ppm -0.86], 543.441 [C_26H_29O_2, Δ ppm -0.14], 525.431 [C_26H_29O_2, Δ ppm 1.65], 507.421 [C_26H_29O_2, Δ ppm 0.16], and 489.406 [C_26H_29O_2, Δ ppm -6.65], respectively. The base peak is the diagnostic ion for BHPs at m/z 191.179 [C_14H_21, Δ ppm -1.50]. The fragment at m/z 369.353 [C_20H_29O_2, Δ ppm -2.51] representing the ring system after loss of the side chain is
only present in very low abundance (<5%), but instead a fragment at $m/z$ 397.383 ($C_{29}H_{49}^+$, $\Delta$ ppm 0.31) is formed with the cleavage apparently occurring at the C-22/C-30 bond. Similar to what was observed for BHT, we observe several fragments at $m/z$ 297.222 ($C_{21}H_{29}O$, $\Delta$ ppm 3.56), $m/z$ 315.233 ($C_{21}H_{31}O_2$, $\Delta$ ppm 2.96), $m/z$ 333.243 ($C_{21}H_{33}O_2$, $\Delta$ ppm 1.59) and $m/z$ 351.253 ($C_{21}H_{35}O_4$, $\Delta$ ppm −0.33), which based on their assigned elemental composition, appear to be complimentary to the ion at $m/z$ 191 (A and B ring) and represent the remainder of the ring system and the side chain with 0 to 4 hydroxyl moieties.

The MS$^2$ spectrum of the ammoniated molecule for BHpentol (Fig. 4 C) shows the same characteristics as discussed for BHT and BHhexol. 3MeBHpentol and 3MeBHhexol elute ca. 2 min after and fragment similarly to their non-methylated counterparts, generating a.o. the diagnostic fragment at $m/z$ 205 and with all ions containing the A-ring shifted by +14 Da (see Fig. S3 for mass spectra).

### 3.3.6. Aminobacteriohopanepolyols

Mass chromatograms of the protonated molecules of aminotriol, -tetrol, and -pentol (Fig. 5A; peaks a, b, c, and d, respectively), as well as their 3Me-counterparts (Fig. 5B; peaks e, f, and g, respectively) show their distribution in *M. capsulatus*, a well-studied bacterium producing these aminoBHPs (e.g., Neunlist and Rohmer, 1985b; Talbot et al., 2001). As expected, the aminoBHPs elute in reversed order of the number of functional groups. The 3Me-aminoBHPs elute ca. 1 to 2.5 min after their corresponding non-methylated counterparts (see Table S1 for exact retention times). Fig. 5A shows a partially resolved, late-eluting isomer of aminotetrol (peak c). Although Talbot et al. (2001) observed a late-eluting isomer to aminotetrol in *Methylocystis parvus*, it has to the best of our knowledge not been observed in *M. capsulatus*.

Talbot et al. (2016) discussed the fragmentation characteristics of non-derivatized aminotriol only and, based on those observations,
predicted suitable MRM target ions for (3Me)aminotriol and (3Me)aminopentol. Here we show MS² spectra of aminopentol (Fig. 5C) and 3Me-aminopentol (Fig. 5D) (additional spectra for the aminotriols and -tetros are shown in Fig. 5E; the late eluting isomer of aminotetrol shows similar fragmentation as the main isomer). As was observed by Talbot et al. (2003a) for acetylated aminotriol and Talbot et al. (2016) for non-derivatized aminotriol, fragmentation of the amino-BHPs only occurs with increased collision voltage. Under the conditions used here, i.e., with a stepped collision energy, fragmentation was still limited, but yielded sufficient diagnostic features for positive identification of the full series of aminoBHPs. As is the case with the previous discussed BHPs, the hydroxyl moieties in the side chain are readily lost as H₂O, yielding a series of fragments at m/z 560.467 (C₃₀H₄₀O₁₁N⁺, Δ ppm 0.65), m/z 542.455 (C₂₉H₄₀O₁₀N⁺, Δ ppm –2.99), m/z 524.448 (C₂₈H₃₈O₁₀N⁺, Δ ppm 0.48), and m/z 506.437 (C₂₈H₃₈O₁₀N⁺, Δ ppm 2.19) for aminopentol and m/z 574.482 (C₂₉H₄₀O₁₁N⁺, Δ ppm –1.09), m/z 556.471 (C₂₈H₃₈O₁₀N⁺, Δ ppm –1.98), m/z 538.462 (C₂₈H₃₈O₁₀N⁺, Δ ppm –0.12), and m/z 520.450 (C₂₈H₃₈O₁₀N⁺, Δ ppm –2.58) for 3Me-aminopentol. In the lower mass range the diagnostic fragments at m/z 191.179 (C₁₀H₁₃O₄, Δ ppm –3.75) for BHPs and m/z 205.195 (C₁₁H₂₀O₅, Δ ppm –1.40) for MeBHPs are relatively abundant. In addition, there are several N-containing fragments in the mass range between m/z 300 and 400 (Fig. 5C and D, Table S1) that represent the D and E rings with side chain, equivalent to the fragmentation observed for BHPs described above.

3.4. Composite BHPs

3.4.1 Cyclitol ether bacteriohopanetetrol. BHT-CE is a commonly detected, so-called composite BHP (Talbot et al., 2007a). Composite BHPs consist of a linear functionalized side chain bound to a more complex, often polar moiety or head group. In case of BHT-CE, the BHT is ether bound to an amino sugar on the C-35 position (Fig. 6B). Here we analyzed cell material of 'Ca. Scalindua profunda', in which BHT-CE was previously detected by Rush et al. (2014), to establish elution and fragmentation of this BHP. Fig. 6A shows the mass chromatogram for the intact headgroup (m/z 708.541). Whereas Rush et al. (2014) detected three isomers of BHT-CE in 'Ca. S. profunda', eluting closely together, we detected two isomers (Fig. 6A, peak a and b), which could be due to different growing conditions. Fig. 6B shows the MS² spectrum of the most abundant isomer, peak b. Talbot et al. (2016) also discussed the fragmentation of non-derivatized BHT-CE, which is very similar to the fragmentation pattern observed here. Fragmentation is limited, but several fragments resulting from loss of up to three hydroxyl moieties are observed at m/z 690.529 (C₄₁H₆₂O₁₁N⁺, Δ ppm –1.32), m/z 672.529 (C₄₁H₆₀O₁₁N⁺, Δ ppm –0.65) and m/z 654.508 (C₄₁H₆₀O₁₁N⁺, Δ ppm –2.60). In the lower mass range several fragments, representing the intact headgroup (m/z 180.087 (C₈H₄O₂N⁺, Δ ppm –0.72)) and the headgroup after loss of several hydroxyl moieties (m/z 162.076 (C₈H₁₂O₂N⁺, Δ ppm –1.45), m/z 144.066 (C₈H₁₀O₂N⁺, Δ ppm –0.62) and m/z 126.065 (C₆H₆O₂N⁺, Δ ppm 0.28)), are observed. Fragments at...
m/z 222.097 (C_8H_{15}O_6N^+, \Delta \text{ppm} -1.37) and m/z 204.086 (C_8H_{15}O_6N^+, \Delta \text{ppm} -0.98) appear to consist of the headgroup with two additional carbon atoms from the side chain after fragmentation at the C-33/C-34 bond.

### 3.4.1. Methylcarbamate-aminoBHPs

A more recently described series of composite BHPs are the methylcarbamate-aminoBHPs (MC-aminoBHPs; Rush et al., 2016). Fig. 7A shows the series detected in M. vadi. The MS² spectrum of MC-aminotriol (Fig. 7B; spectra of MC-aminotetrol and -pentol are shown in Fig. S5) showed the predicted losses of up to three hydroxyl moieties as H_2O generating ions at m/z 586.483 (C_{38}H_{64}O_4N^+, \Delta \text{ppm} 0.23), 568.474 (C_{38}H_{62}O_3N^+, \Delta \text{ppm} 2.38), and 550.461 (C_{38}H_{60}O_2N^+, \Delta \text{ppm} 2.99). A loss of 32 Da (CH_3OH, representing the methoxy moiety) from

---

Fig. 8. Partial mass chromatograms of adenosylhopanes, with increasing number of methylations, in a soil from a terrestrial methane seep (Censo 0 m). Each trace is labeled with the exact mass used for searching, and the intensity of the highest peak in arbitrary units (AU). MS² spectra are shown in Fig. 9 if discussed in text; additional MS² spectra are shown in Fig. S7. Peaks labeled with ‘*’ are potential isomers, but have no MS² spectrum associated.

---

Fig. 9. Adenosylhopanes detected in Censo 0 m soil. A: General structure of adenosylhopanes, B through H: MS² spectra of adenosylhopanes. Proposed structure of the nucleobase is shown with each spectrum. Spectra of peak f (panel D) and peak g (panel E) appear to be mixed spectra of 2 co-eluting adenosylhopanes with identical elemental composition. The nomenclature and structure shown with those spectra is the proposed nucleobase of the major component. Placement of methylations on the nucleobase is arbitrary as the position is unknown.
the ion at \( m/z \) 586 to produce \( m/z \) 554.456 (C\(_{29}\)H\(_{50}\)O\(_2\)N\(^+\), \( \Delta \) ppm –1.64) was also observed. Losses of 75 Dalton (C\(_2\)H\(_3\)O\(_2\)N\(^-\)), representing the methylcarbamoyl moiety, were observed from the mass peaks at \( m/z \) 568 or 550 leading to fragment ions at \( m/z \) 493.438 (C\(_{28}\)H\(_{47}\)O\(^-\), \( \Delta \) ppm –4.81) and \( m/z \) 475.430 (C\(_{27}\)H\(_{45}\)O\(^-\), \( \Delta \) ppm 0.17). These losses were also observed by Rush et al. (2016) after fragmentation of acetylated MC-aminoBHPs. N-containing product ions were observed at \( m/z \) 76.040 (C\(_2\)H\(_8\)O\(_2\)N\(^+\), \( \Delta \) ppm 9.40), 88.040 (C\(_4\)H\(_8\)O\(_2\)N\(^+\), \( \Delta \) ppm 5.85) and 118.050 (C\(_6\)H\(_{10}\)O\(_2\)N\(^+\), \( \Delta \) ppm 1.61) and likely represent the methylcarbamate moiety after fragmentation between the nitrogen and C-35, at C-34/C-35, and at C-33/C-34, respectively.

### 3.5. BHPs in a soil from a terrestrial methane seep

To further examine the performance of the UHPLC-HRMS method for environmental samples with more complex matrices than biomass, we analyzed a soil from a terrestrial methane seep in Sicily (Censo 0 m). A base peak chromatogram is shown in Fig. 8c. We were able to detect several of the above discussed BHPs. All detected BHPs are listed in Table S2 and additional MS\(^2\) spectra of BHPs that are not further discussed in the text are shown in supplemental figures. Here we will focus on the description of not previously discussed and new composite BHPs.

#### 3.5.1. AdenosylBHPs

Adenosylhopanes occur ubiquitously in soils (Cook et al., 2008a; 2009b), yet, adenosylhopane is also an intermediate in BHP side chain synthesis (Bradley et al., 2010) and the adenosyl-BHPs seem to also have an in situ origin in oxygen minimum zones of the oceans (Kusch et al., 2021). Their occurrence and relative abundance have been used to trace soil organic matter during riverine transport and deposition into the marine environment (Zhu et al., 2011). Three types of adenosylhopanes have been described: type-1, -2, and -3 (see Fig. 9A for general structure; Talbot et al., 2007a; Rethemeyer et al., 2010) of which the only polar head group of adenosylhopane type-1 has been fully identified (Neunlist and Rohmer, 1985a) as adenine. Adenosylhopane type-2 and –3 show similar fragmentation behavior to type-1, but have an unknown, presumably, nucleoside-type polar head group. Upon fragmentation of adenosylhopanes, a diagnostic fragment, representing the nucleoside, is formed with \( m/z \) 136 for type-1 (adenine), \( m/z \) 151 for type-2, and \( m/z \) 150 for type-3 (Talbot et al., 2016). Methylated homologues have been observed for each of the three adenosylhopane types (Talbot and Bartram, 2007; Rethemeyer et al., 2010). Adenosylhopane type-1 and its (di)methylated homologues are the only adenosylhopanes with a known elemental composition and, therefore, searchable based on their exact mass. For adenosylhopanes type-2 and -3, we initially mined the data and represented the methylated adenine in Fig. 9C for proposed structure; placement of the methylation is arbitrary.

Fig. 8C shows the mass chromatogram of \( m/z \) 690.532 (EC = C\(_{42}\)H\(_{78}\)O\(_2\)N\(^+\)) and shows one dominant peak at 25.5 min (h) and two minor earlier eluting peaks (f and g). The MS\(^2\) spectrum of peak f (Fig. 9D) contains two fragments related to the head group, i.e., the base peak at \( m/z \) 150.077 (C\(_{19}\)H\(_{21}\)N\(_2\), \( \Delta \) ppm –0.01) and a fragment at \( m/z \) 164.093 (C\(_{19}\)H\(_{21}\)N\(_2\), \( \Delta \) ppm –0.07). This suggests that this peak represents a co-elution of Me-adenosylhopane type-3 (and a minor) adenosylhopane type-3 with a second methylation on the adenine. Talbot et al. (2016) used an MRM transition from \( m/z \) 690 to 150 and detected a single peak in a sediment from the river Tyne. It is likely that this BHP is similar to the here observed peak f. The MS\(^2\) spectrum of peak g (Fig. 9E) showed a base peak at \( m/z \) 136.061 (EC = C\(_{19}\)H\(_{21}\)N\(_2\)) and a minor fragment at \( m/z \) 150.077 (C\(_{19}\)H\(_{21}\)N\(_2\)). We, therefore, propose peak g to represent a co-elution of diMe-adenosylhopane type-1 and Me-adenosylhopane type-3. Peak h again appears to be an adenosylhopane type-3, with an additional methylation on the adenine, based on the product ion at \( m/z \) 164.093 (C\(_{19}\)H\(_{21}\)N\(_2\); \( \Delta \) ppm –0.99; Fig. 9F), similar to the minor co-elution in peak f.

The mass chromatogram of adenosylhopanes with an EC of C\(_{42}\)H\(_{78}\)O\(_2\)N\(_2\) (\( m/z \) 704.547; Fig. 8D) revealed a series of peaks, i, j and k. The MS\(^2\) spectra of all three peaks (shown for peak i in Fig. 9G) show a single fragment at \( m/z \) 164.093, and therefore we tentatively identify these BHPs as methyl-adenosylhopane type-3, with an additional methylation on the adenine head group. Peak j also shows minor fragments related to fragmentation in the hopanoid ring system, including \( m/z \) 191 (Fig. 5A). These BHPs appear to be the core-methylated homologues of peak h, and based on the offset in retention times to peak h, are tentatively identified as having a methylation at C-2 (peak i), methylation at unknown position (peak j), and a methylation at C-3 (peak k); a similar distribution as observed for the MeBHTs and the Me-adenosylhopane type-1 peaks.

A search for adenosylhopanes with an EC of C\(_{44}\)H\(_{72}\)O\(_3\)N\(_2\) (\( m/z \) 718.563) showed a series of peaks (Fig. 8E) with relative low abundance (two orders of magnitude less than the adenosylhopanes with \( m/z \) 690.532). Only peak l had an associated MS\(^2\) spectrum, which showed one fragment ion related to the head group at \( m/z \) 164.093 and minor fragments related to fragmentation in the ring structure (Fig. 7H). We have tentatively identified this BHP as dimethyl-adenosylhopane type-3 with an additional methylation on the adenine head group. Evidence for BHPs methylated at both C-2 and C-3 was previously seen in ‘Ca. Koribacter versatilis’, isolated from a pasture soil (Simininger et al., 2017). Adenosylhopanes with \( m/z \) 732.579 (EC C\(_{46}\)H\(_{80}\)O\(_2\)N\(_2\)) were not detected.

As, in fact, it appears that all adenosylhopanes discussed above are adenosylhopane type-1 with one or two methylations either on the BHP core and/or on the adenine head group, we propose the following nomenclature for this extended family of adenosylhopanes: methylations on the BHP core are indicated as “(di)Me-adenosylhopane”, while methylations on the adenine head group are indicated by a subscript. Adenosylhopane type-1 would thus be simple be named adenosylhopane. Adenosylhopane type-3 would be named adenosylhopaneHG-Me.

Organic Geochemistry 160 (2021) 104285

E.C. Hopmans et al.
(where the subscript HG refers to head group). The Me-adenosylhopanes with an additional methylation on the adenine (peaks i, j, and k) would be named Me-adenosylhopane\(^{HG-diMe}\), and peak l would be named diMe-adenosylhopane\(^{HG-diMe}\).

As the elemental composition of adenosyl type-2 is unknown, we searched the MS\(^2\) data for the diagnostic fragment ion with a nominal \(m/z\) of 151 (Talbot et al., 2016). Several signals were found, mostly associated with amino acid lipids such as ornithines, but one MS\(^2\) spectrum (Fig. 10B) clearly showed an adenosylhopane signature, with a single dominant fragment ion at \(m/z\) 151.061 (C\(_6\)H\(_7\)ON\(_4^+\), \(\Delta ppm\) —0.38 ppm). Interestingly, this elemental composition matches the EC for N1-methylinosine, which is formed from adenine via inosine in transfer

![Fig. 10. Adenosylhopane type-2 in Censo 0 m soil. A: Partial mass chromatograms, (within 2 ppm mass accuracy) of the tentatively identified N1-methylinosylhopane (peak a), 2-methyl-N1-methylinosylhopane (peak b), and inosylhopane (peak c), from a soil from an active terrestrial methane seep in Sicily (Censo 0 m). Each trace is labeled with the exact mass used for searching, and the intensity of the highest peak in arbitrary units (AU). B: MS\(^2\) spectrum of N1-methylinosylhopane. C: MS\(^2\) spectrum of inosylhopane. Proposed structures of the inosine based headgroups are shown.](image)

![Fig. 11. Novel N-containing composite BHPs in Censo 0 m soil. A. Partial mass chromatograms of a series of novel composite BHPs (a, b, c). Each trace is labeled with the exact mass used for searching, and the intensity of the highest peak in arbitrary units (AU). B. MS\(^2\) spectrum associated with peak b. (mass peak labeled with \* results from co-isolation of a co-eluting compound and is not related to this BHP). Also shown is the proposed structure of ethenolamine-BHpentol with diagnostic fragmentations indicated.](image)
C. molecule of adenosylhopane type-2 was determined to be C\textsubscript{archaea} (Grosjean and Constantinesco, 1996). The EC of the protonated spectrum showing the predicted head group fragment at 24.24 min from which the MS from Censo 0 m soil extract with the relatively low abundance peak at (RNAs, and is found in the RNA of eukaryotes and halo- and thermophilic archaea (Grosjean and Constantinesco, 1996). The EC of the protonated molecule of adenosylhopane type-2 was determined to be C\textsubscript{archaea} (m/z 677.500). Fig. 10A shows the mass chromatogram of m/z 677.500 from Censo 0 m soil extract with the relatively low abundance peak at 24.24 min from which the MS\textsuperscript{2} spectrum was derived. A homologue of the tentatively identified N1-methylinosylhopane, methylated on the BHP core (m/z 691.516, C\textsubscript{archaea}N\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}), was detected at 24.30 min (peak e, Fig. 10A). Minor fragments at m/z 617.494 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}), was detected at 24.30 min (peak -e). Based on the retention time, we tentatively identify this BHP as 2Me-N1-methylinosylhopane. As N1-methylinosine is formed from adenosine via an initial hydrolytic deamination to inosine, we also searched for the proposed intermediate between adenosylhopane and N1-methylinosylhopane, i.e., inosylhopane (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}; m/z 663.484). At 20.63 min a peak was identified with an associated MS\textsuperscript{2} spectrum showing the predicted head group fragment at m/z 137.0458 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}, Δ ppm −0.20; peak c, Fig. 10A). Minor fragments at m/z 529.461 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}) and m/z 511.450 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}) further confirmed the anhydro-BHT core structure (Fig. 10C).

3.5.2. A novel composite BHP with an N-containing moiety
During a broad search for known BHPs in the Censo 0 m soil, two peaks matching the exact mass and EC of protonated MC-aminotriol and -tetroil (m/z 604.494, C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}, respectively) were encountered (Fig. 11A, peaks a and b). However, these peaks elute later than the equivalent BHPs described for M. vadi (see above). Although the MS\textsuperscript{2} spectra of the peaks detected in the Censo 0 m soil share many characteristics with those of the MC-aminobHPs, there are several distinct differences. The MS\textsuperscript{2} spectrum of peak b (Fig. 11B) is characterized by a series of initial losses of H\textsubscript{archaea} (18) from the protonated molecule producing ions at m/z 586.483 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}, Δ ppm −0.33), m/z 568.474 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}, Δ ppm 2.23), m/z 550.462 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}, Δ ppm −0.23), and m/z 532.453 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}N\textsubscript{archaea}N\textsubscript{archaea}, Δ ppm 2.64). However, instead of the characteristic loss of 75 Da, representing the loss of the methylcarbamate, losses of 41 Da (C\textsubscript{archaea}H\textsubscript{archaea}N\textsubscript{archaea}) and 59 Da (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}) were observed here, generating fragment ions at m/z 491.424 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}, Δ ppm −2.43) and m/z 473.414 (C\textsubscript{archaea}H\textsubscript{archaea}, Δ ppm −1.41), respectively. Dominant N-containing product ions are observed at m/z 60.045 (C\textsubscript{archaea}H\textsubscript{archaea}O\textsubscript{archaea}, Δ ppm 1.90). The middle m/z region shows similar minor fragments,
representing the D and E ring and the side chain after loss of the functionalities, as observed for BHPentol. Based on the assigned EC and the fragmentation pattern, we propose this BHP is a composite BHP based on BHPentol bound to an ethenolamine moiety (C_2H_2N) via an ether bond. To the best of our knowledge this composite BHP has not been observed before.

After having tentatively identified this ethenolamine-BHPentol, we identified the BHT and BHhexol homologues (peak a and c, respectively, in Fig. 11A), based on calculated exact mass and MS^2 fragmentations (Table S3, Figs. S8A and B). Methylated ethenolamine-BHPs were not detected in Censo 0 m, however a BHP matching the calculated exact mass and EC of methylated ethenolamine-BHT (C_{38}H_{59}O_3N^+) was detected at 21.44 min (for details see Table S3). The fragmentation pattern was almost identical to what was observed for ethenolamine-BHT (Fig. 11B). However, both diagnostic N-containing product ions were offset by +14 Da resulting in fragments at m/z 74.061 (C_2H_2N^+) and m/z 116.071 (C_3H_5O_2N^+, Δ ppm 3.575) (Fig. S8C). No diagnostic losses could be observed in this case. This BHP was, therefore, tentatively identified as a propenolamine-BHT. A butenolamine homologue was not detected.

### 3.5.3. Acylated ethenolamines

Using the mass of the most common diagnostic ion for BHPs, i.e., m/z 191.179 (C_{36}H_{57}O), the MS^2 data was investigated for other potential unknown BHPs. This revealed the presence of a series of late eluting (35–40 min) compounds with m/z values > 800 (Fig. S9). One of the most abundant of these unknowns was a compound with m/z 840.743 and an assigned EC of C_{38}H_{59}O_3N^+ (Δ ppm ±1.54). A mass chromatogram for this EC showed a cluster of peaks consisting of at least eight isomers (peaks a to h, Fig. 12A) with near identical MS^2 spectra. Fig. 12B shows the MS^2 spectrum of the most abundant peak e. Two losses of H_2O are observed from the parent ion resulting in fragment ions at m/z 822.733 (C_{38}H_{59}O_3N^+, Δ ppm ±0.26) and m/z 804.720 (C_{38}H_{58}O_2N^+, Δ ppm ±0.46). In the middle region of the mass spectrum a cluster of fragment ions are observed, which are formed after an initial loss of 252.246 (C_7H_2O^+, Δ ppm 0.81) from the parent ion and further losses of −18 Da (H_2O) and, interestingly, −41 Da (C_2H_3N) and −59 Da (C_2H_5ON), similar to the fragmentation pattern observed for the earlier described ethenolamine BHPs. Indeed, the diagnostic product ions of this novel class of BHPs at m/z 60 and 102 were also present. Based on the resemblance of the MS^2 spectrum to that of ethenolamine-BHT and the loss of a C_{12}H_2O moiety, we tentatively identified this compound as a C_{17:0}-N-acyl-ethenolamine-BHT (Fig. 12B). The fatty acid moiety can, based on the data here, only be identified to the level of carbon number and double bond equivalent. Several causes are possible for the multitude of isomers. The head group can be bound to the polyol tail of the BHP at different positions (C-32, C-33, C-34, or C-35), and/or by isomery within the BHT core structure, or by structural differences (linear vs. branched) in the fatty acid tail.

In addition to the C_{17:0}-N-acyl-ethenolamine-BHTs, we detected complex distributions of C_{15:0} to C_{18:0}-N-acyl-ethenolamine-BHTs (Fig. S9). C_{14:0} and C_{15:0}-N-acyl-ethenolamine-BHTs appeared only present at trace levels and could not be confirmed by obtaining MS^2 spectra. A full listing by retention time, for those isomers confirmed by MS^2, is given in Table S2. Ethenolamine-BHTs bound to C_{15:0} and C_{17:0} fatty acids were most abundant in the Censo 0 m soil, but the C_{16:0} bound ethenolamine-BHTs showed the most complex distribution with 12 isomers confirmed by MS^2 spectra. A search for unsaturated homologues resulted in the detection of ethenolamine-BHPTs bound to C_{17:1} and C_{18:1} fatty acids (Table S2, Fig. S10), which were only present at trace levels. Acylated ethenolamine-BHTs comprising an unsaturated hopanoid core were not detected. Acylated ethenolamine-BHPs based on BHPentol and BHhexol were also detected (Table S2, Fig. S10) and comprised C_{15:0} to C_{18:0} and C_{17:1} and C_{18:1}-N-bound acyl moieties, i.e., comparable with the distribution of the ethenolamine-BHTs. Interestingly, many more isomers were identified for ethenolamine-BHHexol than for ethenolamine-BHPentol, while BHHexol itself was not detected.

### 3.5.4. Acylated aminotriols

A further search for acylated BHPs revealed a series of N-acyl-aminotriols in the Censo 0 m soil (Fig. S11). HPLC-MS detection of derivatized N-acyl-aminotriols was previously reported by Talbot et al. (2007a) in *Nitrosomonas europaea* and *Rhodococcus vannielli*. However, the MS^2 spectrum (shown for C_{14:0:0}-N-acyl-aminotriol, Fig. 13) of
the non-derivatized molecule proves to be much more diagnostic than that produced from the derivatized molecule. Here, three losses of H₂O are observed from the parent ion resulting in fragment ions at \( m/z \) 738.676 (\( \text{C}_{16}\text{H}_{30}\text{O}_6\text{N}^+ \), Δ ppm 0.04), \( m/z \) 720.663 (\( \text{C}_{16}\text{H}_{28}\text{O}_2\text{N}^+ \), Δ ppm −3.078) and \( m/z \) 702.658 (\( \text{C}_{16}\text{H}_{26}\text{ON}^+ \), Δ ppm −3.17). Loss of 210.199 Da (\( \text{C}_4\text{H}_4\text{O}_2 \), Δ ppm 4.07) leads to a product ion at \( m/z \) 546.486 (\( \text{C}_{15}\text{H}_{24}\text{O}_2\text{N}^+ \), Δ ppm 4.07), matching the \([\text{M}+\text{H}]^+\) of aminoBHP. For illustration, the \([\text{M}+\text{H}]^+\) of an aminoBHT bound to a \( \text{C}_{14} \) \( \text{FA} \) has an elemental compositions of \( \text{C}_{55}\text{H}_{98}\text{O}_4\text{N}_2\text{O}^+ \), which is identical to that of an ethenolamineBHT bound to a \( \text{C}_{15} \) \( \text{FA} \). It is, therefore, important to evaluate the fragmentation of each detected compound.

### 3.5.5. Multi-conjugated composite aminotriols

While charting the full inventory of \( \text{N} \)-acyl-aminotriol-BHPs, we also encountered several compounds that were clearly related to the \( \text{N} \)-acyl-aminotriols as evident from their \( m/z \) 932.719 spectra (e.g., Fig. 14B). The most abundant of these was a compound revealed in a mass chromatogram of \( m/z \) 932.719 (Fig. 14A, peak a) and an assigned EC of the protonated molecule \( \text{C}_{15}\text{H}_{30}\text{O}_3\text{N}^+ \) (Δ ppm −1.07). An initial loss of 176 \( \text{C}_4\text{H}_4\text{O}_2 \) yields a base peak at \( m/z \) 756.685 with an assigned EC matching that of an ethenolamineBHT bound to a \( \text{C}_{15} \) \( \text{FA} \) and the \( \text{C}_{14} \) fatty acid moiety. Further fragmentation was identical to that observed for \( \text{C}_{14} \) \( \text{N} \)-acyl-aminotriol, revealing the aminotriol core at \( m/z \) 546 and the \( \text{C}_{14} \) fatty acid moiety at \( m/z \) 228. The \( m/z \) spectrum of peak b is identical to that of peak a, and peak b thus probably reflects an isomer. Although glucuronic acid is not a very common head group in intact polar lipids, it...
has been observed in bacteria, fungi, and plants (e.g., Bosak et al., Burugupalli et al., 2020; Fontaine et al., 2009; Hözl and Dormann, 2007; Wang et al., 2020). The stereochemistry of the glucuronic acid was not confirmed here and therefore we tentatively identify these compounds as C_{14:0}-N-acyl-glycuronyl-aminotriol (Fig. 14B). In addition to the C_{14:0}-N-acyl-glycuronyl-aminotriol, a C_{15:0P}-N-acyl-glycuronyl-aminotriol was also detected (Table S2). To the best of our knowledge this is the first report of BHPs with conjugations on more than one position on the BHP core.

4. Conclusions

We have shown the applicability of UHPLC-ESI/HRMS$^2$ for the analysis of non-derivatized BHPs in both microbial cultures as well as environmental samples. The chromatographic system used here allows separation of a broad range of BHPs, ranging from the relatively simple BHPs to nitrogen-containing BHPs and complex composite BHPs. Furthermore, isomers are readily separated. Identification is achieved based on diagnostic spectra, that contain information on the BHP core structure, the functionalized tail, as well as bound moieties. For the first time, we established the elemental composition of the nucleobase of adenosylhopanoids type-2 and type-3, showing that in fact, all adenosylhopanoids identified so far are modifications of adenosylhopane type-1 either by one or two methylations on the adenine head group (type-3) or by deamination followed by methylation (type-2). Furthermore, we have demonstrated the usefulness of HRMS in the identification of novel composite BHPs. We have tentatively identified several new composite BHPs in a soil (e.g., the (N-acyl)-ethenolamine-BHPs), showing a previously unobserved diversity and complexity in existing BHP structures. The analytical approach described here allows for simultaneous analysis of the full suite of IPIs, now including BHPs, and represents a further step towards environmental lipidomics. With this method a more complete view of the full assembly of BHPs will be possible. Connecting specific intact BHPs to specific sources and/or geological cycles will further aid in the interpretation of their diagenetic products, the geo-hopanoids, in the geological record. Future work will aim to establish a quantitative protocol for this method using isolated BHPs as well as synthetic internal standards.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the associate editor for handling this manuscript and Dr. Kusch and Dr. Lipp for their helpful comments and suggestions. This work was funded by Netherlands Earth System Science Center (NESSC) through a Gravitation grant to JSSD (grant no. 024.002.001) from the Dutch Ministry for Education, Culture and Science, NWO middelgroot grant no. 834.13.004 to ECH and a Natural Environment Research Council (NERC; United Kingdom) grant to DR (project ANAMMARKS (NE/N011112/1)). This project also received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation program (grant agreement No 694569-MICROLIPIDS) to JSSD. Cultures were kindly provided by H. Hirayama (JAMISTEC), G.H.L. Nuijten, O. Rasigraf, and M.S.M. Jetten (Radboud University), M. Rohmer and P. Schaeffer (Strasbourg University). We thank F. Grassa (INGV) for assistance to NS during field work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2021.104285.
Peters, K.E., Moldowan, J.M., 1993. The Biomarker Guide. Prentice Hall, Englewood

Rohmer, M., Dastillung, M., Ourisson, G., 1980. Hopanoids from C

Ricci, J.N., Coleman, M.L., Welander, P.V., Sessions, A.L., Summons, R.E., Spear, J.R.,

Peisler, B., Rohmer, M., 1992. Prokaryotic triterpenoids of the hopane series.

Moore, E.K., Hopmans, E.C., Rijpstra, W.I.C., Villanueva, L., Sinninghe Damst

Schwartz-Narbonne, R., Schaeffer, P., Hopmans, E.C., Schenesse, M., Charlton, E.A.,

E.C. Hopmans et al.

Newman, D.K., 2014. Diverse capacity for 2-methylhopanoid production correlates

Mollenhauer, G., 2010. Distribution of polar membrane lipids in permafrost soils and

Journal of Chemical Research – Part S 9, 289–299.

Peters, K.E., Moldowan, J.M., 1993. The Biomarker Guide. Prentice Hall, Englewood

Cliffs, NJ, USA.

Rathemeyer, J., Schulzof, F., Talbot, H.M., Cooke, M.P., Hinrichs, K.U.,

Mollenhauer, G., 2010. Distribution of polar membrane lipids in permafrost soils and sediments of a small high Arctic catchment. Organic Geochemistry 41 (10), 1130–1145.

Ricci, J.N., Coleman, M.L., Welander, P.V., Sessions, A.L., Summers, R.E., Spear, J.R.,

Newman, D.K., 2014. Diverse capacity for 2-methylhopanoid production correlates with a specific ecological niche. The ISME Journal 8, 675–684.

Rohmer, M., Castillung, M., Ourisson, G., 1980. Hopanoids from C30 to C34 in Recent mud - chemical markers for bacterial activity. Naturwissenschaften 67 (9), 456–458.

Rohmer, M., Bouvier-Nave, P., Ourisson, G., 1984. Distribution of hopanoid triterpenes in Prokaryotes. Journal of General Microbiology 130, 1137–1150.

Rohmer, M., Ourisson, G., 1986. Unsaturated bacteriohopanepolyol from Acetobacter aceti sp. xylinum. Journal of Chemical Research. Synopses 10, 356–357.

Rohmer, M., 1993. The biosynthesis of triterpenoids of the hopane series in the Eubacteria: A mine of new enzyme reactions. Pure and Applied Chemistry 65, 1293–1298.

Rush, S., Sinninghe Damste, J.S., Poulton, S.W., Thambdrup, B., Garside, A.L., Gonzalez, J.A., Schouten, S., Jetten, M.S.M., Talbot, H.M., 2014. Anaerobic ammonium-oxidizing bacteria: A biological source of the bacteriohopanepentetol stereoisomer in marine sediments. Geochimica et Cosmochimica Acta 140, 50–64.

Rush, D., Osborne, K.A., Birgel, D., Kappler, A., Hirayana, H., Feckmann, J., Poulton, S.W., Nickel, J.C., Mangelsdorff, K., Kalyuzhnyaya, M., Sidgwick, F.R., Talbot, H.M., 2016. The bacteriohopanepolypentol inventory of novel aerobes methane oxidizing bacteria reveals new biomarker signatures of aerobic methanotrophy in marine systems. PLoS ONE 11, e0160535.

Säenz, J.P.W., Wakeham, S.G., Eglinton, T.I., Summers, R.E., 2011. New constraints on the provenance of hopanoids in the marine geologic record: Bacteriohopanepolys in marine suboxic and anoxic environments. Organic Geochemistry 42 (11), 1351–1362.

Schulenberg-Schell, H., Neus, B., Sahn, H., 1989. Quantitative determination of various hopanoids in microorganisms. Analytical Biochemistry 181, 120–124.

Schertel-Narbonne, E., Alebasker, P., Hopmans, E.C., Schersenne, M., Charlton, E.A., Jones, D.M., Sinninghe Damste, J.S., Ul Haque, M.F., Jetten, M.S.M., Lengger, S.K., Murrell, J.C., Normand, P., Nuijten, G.H.L., Talbot, H.M., Rush, D., 2020. A unique bacteriohopanepentol stereoisomer of marine anammox. Organic Geochemistry 143, 103994.

Sessions, A.L., Zhang, L., Welander, P.V., Doughty, D., Summers, R.E., Newman, D.K., 2013. Identification and quantification of polyfunctionalized hopanoids by high temperature gas chromatography–mass spectrometry. Organic Geochemistry 56, 120–130.

Simonin, P., Tindall, B., Rohmer, M., 1994. Structure elucidation and biosynthesis of 31-

Smit, Nadine T., Villanueva, Laura, Rush, Darc, Grasa, Fausto, Witkowsky, Cathryn R., Holzheimmer, Mira, Minnaard, Adrian J., Sinninghe Damste, Jaap S., Schouten, Stefan, 2019. Novel hydrocarbontutilizing soil mycobacteria synthesise unique mycorrhizic acids at a Sicilian everlasting fire. Biogeosciences 16 (4), 1463–1479.

Sollai, Martina, Villanueva, Laura, Hopmans, Ellen C., Reichart, Gert-Jan, Sinninghe Damste, Jaap S., 2019. A combined lipidomic and 16S rRNA gene amplicon sequencing approach reveals archaenal sources of intact polar lipids in the stratified Black Sea water column. Geobiology 17 (1), 91–109.

Sturt, Helen F., Summons, Roger E., Smith, Kristin, Elvert, Marcus, Hinrichs, Kai-Uwe, 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high performance liquid chromatography/ electrospray ionization mass spectrometry – New biomarkers for biogeochemistry and microbial ecology. Rapid Communications in Mass Spectrometry 18 (6), 617–628.

Talbot, H.M., Watson, D.F., Murrell, J.C., Carter, J.F., Farrimond, P., 2001. Analysis of intact bacteriohopanepolyol from methanotrophic bacteria by reversed-phase high-performance liquid chromatography/negative pressure chemical ionization mass spectrometry. Journal of Chromatography A 921, 175–185.

Talbot, Helen M., Squier, Angela H., Keely, Brendan J., Farrimond, Paul, 2003a. Atmospheric pressure chemical ionisation reversed-phase liquid chromatography/ ion trap mass spectrometry of intact bacteriohopanepolyol. Rapid Communications in Mass Spectrometry 17 (7), 728–737.

Talbot, H.M., Summons, R., Jahnke, L., Farrimond, P., 2003b. Characteristic fragmentation of bacteriohopanepolyol during atmospheric pressure chemical ionization liquid chromatography/ion trap mass spectrometry. Rapid Communications in Mass Spectrometry 17, 2798–2796.

Talbot, Helen M., Watson, Diane F., Pearson, Emma J., Farrimond, Paul, 2003c. Diverse bacteriohopanepolyol compositions of non-marine sediments. Organic Geochemistry 34 (10), 1353–1371.

Talbot, H.M., Rohmer, M., Farrimond, P., 2007a. Rapid structural elucidation of composite bacterial hopanoids by atmospheric pressure chemical ionization liquid chromatography/ion trap mass spectrometry. Rapid Communications in Mass Spectrometry 21, 880–892.

Talbot, H.M., Rohmer, M., Farrimond, P., 2007b. Structural characterisation of unsaturated bacterial hopanoids by atmospheric pressure chemical ionization liquid chromatography/ion trap mass spectrometry. Rapid Communications in Mass Spectrometry 21, 1613–1622.

Talbot, Helen M., Farrimond, Paul, 2007. Bacterial populations recorded in diverse sedimentary bacteriohopanoid distributions. Organic Geochemistry 38 (8), 1212–1225.

Talbot, Helen M., Summons, Roger E., Jahnke, Linda L., Cockell, Charles S., Rohmer, Michel, Farrimond, Paul, 2008. Cyanoabacterial bacteriohopanepolyp signatures from cultures and natural environmental settings. Organic Geochemistry 39 (2), 232–263.

Talbot, H.M., Sidgwick, F.R., Bischoff, J., Osborn, K.A., Rush, D., Sherry, A., Spencer-Jones, C.L., 2016. Analysis of non-derivatized bacteriohopanepolyp by ultrahigh-performance liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry 30, 2087–2098.

van de Vossenberg, J., Rattray, J.E., Geerts, W., Kartal, B., van Niftrik, L., van Dongen, E.G., Sinninghe Damste, J.S., 2012. A mine of new enzyme reactions. Pure and Applied Chemistry 65, 1293–1298.

van de Vossenberg, J., Rattray, J.E., Geerts, W., Kartal, B., van Niftrik, L., van Dongen, Elvert, Marcus, Hinrichs, Kay-Uwe, 2015. Quantitative hopanoid analysis enables robust pattern detection and comparison between laboratories. Geobiology 13, 391–407.

Wang, H-Y, Tatituri, R.V.V., Goldner, N.K., Dantas, G, Hsu, F-F., 2020. Unveiling the biochemistry of lipid secretome in the nitrosomonas. Geochimica et Cosmochimica Acta 77 (2012), 5279–5296.

Wang, J., Tao, H., Gu, Y., Xia, Y., Tian, B., 2015. Distribution of bacteriohopanepolyp signatures from cultures and natural environmental settings. Organic Geochemistry 39 (2), 232–263.

Wang, H-Y, Tatituri, R.V.V., Goldner, N.K., Dantas, G, Hsu, F-F., 2020. Unveiling the biochemistry of lipid secretome in the nitrosomonas. Geochimica et Cosmochimica Acta 77 (2012), 5279–5296.