

1 **Polyfunctionalised bio- and geohopanoids in the Eocene Cobham Lignite**

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24

25 **Abstract**

26 We investigated the bacteriohopanepolyol (BHP) distribution in the Cobham Lignite
27 sequence (SE England) deposited across the Palaeocene-Eocene boundary including part
28 of the Palaeocene-Eocene Thermal Maximum (PETM) as shown previously by a negative
29 carbon isotope excursion (CIE). A variety of BHPs were identified, including the commonly
30 occurring and non-source specific biohopanoid bacteriohopanetetrol (BHT) and 32,35-
31 anhydroBHT which was the most abundant polyfunctionalised geohopanoid in the majority of
32 samples. BHPs with a terminal amine functionality, diagnostic biomarkers for methanotrophic
33 bacteria were identified throughout the sequence, with similar distributions in both the lower
34 laminated and upper blocky lignite except that 35-aminobacteriohopanepentol (aminopentol)
35 indicative of Type I methanotrophs (gammaproteobacteria) was generally more abundant in
36 the upper section within the CIE.

37
38 The diagenetic fate of these compounds is currently poorly constrained, however, we also
39 identified the recently reported N-containing transformation product anhydroaminotriol and
40 several tentatively assigned novel N-containing structures potentially containing ketone
41 functionalities. Although present throughout the section, there is a sharp peak in the
42 occurrence of these novel compounds correlated with the onset of the CIE and highly
43 isotopically depleted hopanes in the upper part of the laminated lignite, both also correlate
44 well with peak abundance of aminopentol. The significant abundance of these compounds
45 suggests that 35-amino BHPs have their own specific diagenetic pathway, potentially
46 providing an alternative method allowing methanotroph activity to be traced in older samples
47 even if the original biohopanoid markers are no longer present.

48 At this time we cannot preclude the possibility that some or all of these BHPs have been
49 produced by more recent subsurface activity, post deposition of the lignite to date; however,
50 that would not be expected to generate the observed stratigraphic variability and we suggest
51 that unprecedented observations of a range of highly functionalised biohopanoids in
52 samples of this age could significantly extend the window of their known occurrence.

53

54 **1. Introduction**

55

56 Bacteriohopanepolyols (BHPs) are highly functionalised pentacyclic triterpenoids derived
57 from a wide range of prokaryotes (e.g. Rohmer et al., 1984; Pearson et al., 2007, 2009). In
58 their biological form (i.e. biohopanoids), they comprise a diverse suite of structures (e.g.
59 Rohmer, 1993; See Appendix for examples) which have been linked to cellular membrane
60 adaptation, regulating fluidity and permeability in response to environmental stress (e.g.
61 Kannerberg and Poralla, 1999; Welander et al., 2009; Sáenz et al., 2012; Kulkarni et al.,
62 2013). Typically they contain four, five or six functional groups (termed tetra-, penta- and
63 hexafunctionalised respectively) at the C-30 to 35 positions of the side chain although
64 cyclised side chains are also known. For example, adenosylhopane (**lp**) is the precursor for
65 all other side chain extended BHPs (Bradley et al., 2010) via a pathway involving the
66 intermediate ribosylhopane (Liu et al., 2014; Bodlenner et al., 2015). Biohopanoids are also
67 the precursors of the ubiquitous geohopanoids (hopanols, hopanoic acids, hopanes) found
68 in geological materials (e.g. Ourisson et al., 1987; Ourisson and Albrecht, 1992; Farrimond
69 et al., 2004).

70

71 Biohopanoids can provide useful information about certain source organisms,
72 biogeochemical processes and environmental conditions (e.g. Talbot and Farrimond, 2007).
73 One such group are BHPs with an amine functionality at the C-35 position (collectively
74 termed aminoBHPs herein; e.g. Talbot et al., 2014; Wagner et al., 2014; Spencer-Jones et
75 al., 2015) which include 35-aminobacteriohopane-32,33,34-triol (aminotriol from herein, **lf**),
76 35-aminobacteriohopane-31,32,33,34-tetrol (aminotetrol, **lg**) and 35-aminobacteriohopane-
77 30,31,32,33,34-pentol (aminopentol, **lh**). Sources of aminopentol are thought to be restricted
78 to Type I aerobic methane oxidising bacteria (Gammaproteobacteria; e.g. Neunlist and
79 Rohmer, 1985; Cvejic et al., 2000; van Winden et al., 2012a), whilst aminotetrol is produced

80 by both Type I and II (Alphaproteobacteria) methanotrophs. The only known additional
81 source of the penta- and hexafunctionalised compounds are some species of *Desulfovibrio*
82 sulphate reducing bacteria (SRB; Blumenberg et al., 2006, 2009), although aminopentol was
83 only reported from one species at trace levels (Blumenberg et al., 2012). Further, when
84 observed in *Desulfovibrio* sp., the ratio of aminotriol to aminotetrol (**lf/lg**) was in the range of
85 20 to 100 (Blumenberg et al., 2006, 2009, 2012) whilst it is significantly lower in most
86 methanotrophs, with the pentafunctionalised compound often more abundant (e.g. Janke et
87 al., 1999; Talbot et al., 2001). Aminotriol is less diagnostic as it is produced by a range of
88 other prokaryotes (including other proteobacteria and some cyanobacteria; Talbot et al.,
89 2008 and references therein) in addition to all Type II and some Type I methanotrophs
90 (Talbot et al., 2001; van Winden et al., 2012a; Banta et al., 2015).

91

92 AminoBHPs have been reported from a wide range of environments including soils (e.g.
93 Cooke et al., 2008a; Xu et al., 2009; Pearson et al., 2009; Rethemeyer et al., 2010; Kim et
94 al., 2011; Zhu et al., 2011), peats (e.g. van Winden et al., 2012a,b), marine, river and
95 lacustrine sediments (e.g. Talbot et al., 2003a; Talbot and Farrimond, 2007; Coolen et al.,
96 2008; Blumenberg et al., 2010; Zhu et al., 2010, 2011; Blumenberg et al., 2013). Aminotetrol
97 (**lg**) and aminopentol (**lh**) are therefore of particular interest and have been used to identify
98 material sourced from sites of intense aerobic methane oxidation such as river estuaries
99 (Zhu et al., 2010), tropical wetlands (Talbot et al., 2014; Wagner et al., 2014; Spencer-Jones
100 et al., 2015) and water columns (e.g. Blumenberg et al., 2007; Wakeham et al., 2007;
101 Berndmeyer et al., 2013). Other markers for Type I aerobic methanotrophs include
102 hopanoids methylated at the C-3 position (e.g. Cvejic et al., 2000), but non-methanotrophs
103 can also be potential sources of this structural feature (e.g. Welander and Summons, 2012).
104 Typically therefore, these studies also rely on analysis of compound specific carbon isotope
105 ratios with strong isotopic depletion expected for methanotroph-derived lipids (e.g. Collister
106 et al., 1992). However, the absence of C-3 methylated pseudohomologues of these
107 aminoBHP compounds does not preclude either the Type II methanotrophs or Type I

108 organisms such as *Methylomonas* sp. or *Methylovulum* sp. (e.g. Rohmer et al., 1984; van
109 Winden et al., 2012a) which do not produce the methylated homologues (see discussion in
110 Talbot et al., 2014).

111
112 Originally considered to be rapidly transformed to geohopanoids at the earliest stages of
113 diagenesis (both free and bound), evidence is now growing that polyfunctionalised
114 biohopanoids may be more stable than originally thought. The oldest reported
115 polyfunctionalised biohopanoid is bacteriohopane-32,33,34,35-tetrol (BHT; **1c**) in a sample of
116 marine Palaeogene cores from Tanzania dating to 50.4-49.7 million years ago (Ma; van
117 Dongen et al., 2006). This compound is the most frequently reported BHP structure,
118 facilitated by the fact that it is amenable to analysis by gas chromatography mass
119 spectrometry (GCMS) as well as liquid chromatography mass spectrometry (LCMS; e.g.
120 Talbot et al., 2003a). It has a wide range of potential prokaryotic sources meaning that it
121 cannot be considered as indicative of any particular group of bacteria or set of environmental
122 conditions (e.g. Talbot et al., 2008). Additional early degradation products retaining multiple
123 functional groups derived from BHT or more complex precursors such as BHT cyclitol ether
124 (**1m**) or BHT glucosamine (**1n**) include 32,35-anhydrobacteriohopane-32,33,34,35-tetrol
125 (anhydroBHT; **1a**) and its C-2 methylated homologue (**1la**; Schaeffer et al., 2008, 2010) and
126 they have been reported in samples up to Jurassic in age (Bednarczyk et al., 2005). Multiple
127 isomers of the related compound derived from a pentafunctionalised precursor i.e.
128 anhydrobacteriohopanepentol (anhydroBHPentol; **1d**) have been reported from geothermal
129 sinters (Talbot et al., 2005; Gibson et al., 2014).

130
131 Recently we have identified aminoBHPs including aminopentol (**1h**) in samples from the
132 Congo deep-sea fan aged up to 1.2 Ma and the source of the material is proposed as
133 continental wetland environments (Talbot et al., 2014; Spencer-Jones et al., 2015; Spencer-
134 Jones, 2016). Burhan et al. (2002) reported aminotriol from the Be'eri sulfur deposit
135 (Pleistocene age sandstones of the Southwestern Mediterranean Coastal Plain of Israel),

136 however, it is uncertain if it represents a Pleistocene age signal or one from sub-
137 contemporary bacteria feeding on seeping methane. These findings suggest that BHP
138 compounds produced in aerobic systems can be preserved in the geological record when
139 conditions are favourable and could therefore be useful in examining methane cycling in
140 more ancient settings.

141

142 To explore the potential for BHP preservation in ancient sediments, we investigated the BHP
143 signature of a well preserved, immature lignite sequence from southern England. The
144 Cobham Lignite is an exceptional example of a terrestrial lacustrine/mire deposit associated
145 with the Palaeocene-Eocene thermal maximum (PETM; Collinson et al., 2003, 2007, 2009).
146 Previously, a negative carbon isotope excursion (CIE) indicated by a sharp depletion in $\delta^{13}\text{C}$
147 of ~ 1 ‰ in the bulk organic carbon within the section has been interpreted as being the
148 negative CIE characteristic of the PETM onset, although its magnitude is markedly lower
149 than the total extent of the CIE observed in other terrestrial settings (McInerney and Wing,
150 2011). Furthermore, isotopically light hopanoids have also been reported from this section,
151 suggesting an increase in the methanotroph population resulting from enhanced methane
152 production, likely driven by hydrological changes towards a warmer and wetter climate
153 (Pancost et al., 2007). These changes are also manifested as a lithological change from
154 laminated to blocky lignite. Hopanoids at this site are present in exceptional abundance
155 relative to other biomarkers and are relatively immature based on observation of the
156 biological $17\beta,21\beta(H)$ configuration and lack of compounds with $22S$ stereochemistry
157 (Pancost et al., 2007). We therefore considered this site favourable for investigation of the
158 potential for preservation of polyfunctionalised biohopanoids.

159

160 **2. Methods**

161

162 *2.1 Site and samples*

163

164 The ca. 2 m thick early Paleogene Cobham Lignite sequence used for this study was
165 sampled from a temporary exposure, which became available near Cobham, Kent, southern
166 England, when a cutting was made through a hill for construction of the Channel Tunnel Rail
167 Link during 1999-2000. In order to procure a complete sequence of the lignite with intact
168 stratigraphy, surrounding sediment was excavated to produce pillars (10-15 cm in depth and
169 width) free on three sides. The three exposed sides were enclosed in a plaster jacket to
170 prevent breakage and pillars were removed from the exposure at the fourth side using
171 spades. The fourth side was not enclosed. These plaster-jacketed pillars were stored in
172 ambient room conditions whilst attempts were made to obtain funding for their study.

173 Prior to sub-sampling sediment was removed and discarded from the surface of the exposed
174 side to a depth of c. 3 cm until sediment with original appearance (e.g. colour, degree of
175 integrity) was reached. Sub-sampling of each cleaned pillar was completed within one day.
176 Cleaning and sub-sampling was undertaken using sharp single-edged razor blades that had
177 been rinsed in alcohol and treated in a furnace prior to use. A fresh razor blade was used for
178 each sub-sample. Sub-samples specifically for geochemical analysis were taken from near
179 the centre of the pillar, avoiding the sides. These samples were dried and individually
180 wrapped in aluminium foil, which had previously been treated in a furnace, then transported
181 to the University of Bristol for extraction. The current study utilised aliquots of total lipid
182 extract (TLE) originally prepared by Pancost et al. (2007) and which had been stored dry and
183 frozen (-20°C) until transported to Newcastle in 2012 for BHP analysis.

184 The Cobham Lignite sequence is underlain by a sand and mud unit, beneath which is the
185 late Paleocene Upnor Formation (shallow marine). The Early Eocene Woolwich Shell Beds
186 (marginal marine/lagoonal) overly the lignite. The Woolwich Shell Beds contain the
187 *Apectodinium* acme (Collinson et al., 2009), thus, in combination with the CIE in the upper
188 part of the lower laminated lignite (Pancost et al., 2007), demonstrate that all of the blocky
189 lignite is within the PETM and probably in the early part of the PETM (Collinson et al., 2009).

190 The site, stratigraphy, lithology, palynology, vegetation change and wetland plant
191 mesofossils have been described in detail elsewhere (Table 1; Collinson et al., 2003, 2007,
192 2009, 2013; Steart et al., 2007).

193
194 The Cobham Lignite sequence includes two thick units of lignite and two thin clay layers. A
195 basal thin pale clay layer (3 cm thick) underlies the lower laminated lignite unit (up to 55 cm
196 thick). The uppermost 25 mm of the lower laminated lignite is transitional and contains thin
197 clay laminae (upper part of Figure 3.3 in Steart et al., 2007). A thicker (up to 10 cm) pale clay
198 layer separates the laminated lignite from the overlying blocky lignite unit (up to 132 cm
199 thick). The bottom of the basal thin pale clay layer is set to 0 cm for the section (Table 1). In
200 total thirty five samples from the original set described by Pancost et al. (2007) were chosen
201 for BHP analysis. The samples ranged from the underlying sand and mud unit through the
202 laminated lignite, middle clay and blocky lignite including samples from the onset of the CIE
203 in the upper laminated lignite (Table 1).

204

205 *2.2 Extraction and derivatisation*

206 Prior to lipid extraction samples were powdered with a mortar and pestle. The powdered
207 samples were extracted by sonication with a sequence of increasingly polar solvents (four
208 times with dichloromethane (DCM), four times with DCM/methanol (1:1 v/v) and three times
209 with methanol) to produce a total lipid extract (TLE; Pancost et al., 2007).

210

211 An aliquot of each TLE was acetylated by adding acetic anhydride and pyridine (1 ml each)
212 and heating at 50 °C for 1 h then left at room temperature overnight to yield acetylated
213 BHPs. The acetic anhydride and pyridine were removed under a stream of N₂. The resulting
214 acetylated extract was dissolved in MeOH/propan-2-ol (3:2, v/v) and filtered through a 0.45
215 micron PTFE filter, blown down to dryness and redissolved in 500 µl MeOH/propan-2-ol (3:2,
216 v/v) for analysis. Sessions et al. (2013) showed using GCMS analysis that acetylation of

217 hopanoids can produce anhydroBHT if run for more than 15-30 minutes. However, similar
218 tests on a range of acetylation reagent volumes and reaction times showed no statistically
219 significant variation in concentration or production of anhydroBHT on samples analysed by
220 ion-trap LCMS (Spencer-Jones, 2016).

221

222 As no standards were added prior to splitting of the TLE and derivatisation, only relative
223 abundances within individual samples are reported below and are based on LCMS data
224 only.

225

226 *2.3 Analytical HPLC-APCI-MS*

227

228 BHPs were measured using reversed-phase HPLC-APCI-MS as described previously (e.g.
229 Cooke et al., 2008a; van Winden et al., 2012a). Compounds were separated using a Thermo
230 Finnigan Surveyor HPLC system equipped with a Phenomenex Gemini C18 5 μ m column
231 (150 mm x 3.0 mm i.d.) and a security guard column of the same material. The flow rate was
232 0.5 ml/min at 30 °C with the following gradient: 90% A and 10% B (0 min); 59% A, 1% B and
233 40% C (at 25 min); isocratic (to 40 min), returning to starting conditions over 5 min and
234 stabilizing for 15 min, with A = MeOH, B = water and C = propan-2-ol (all Fisher HPLC
235 grade). The HPLC system was connected to a Thermo Finnigan LCQ ion trap MS instrument
236 equipped with an APCI source operated in positive ion mode. Settings were: capillary
237 temperature 155 °C, APCI vaporiser temperature 400 °C, corona discharge current 8 μ A,
238 sheath gas flow 40 and auxiliary gas 10 (arbitrary units). The instrument was tuned as
239 described previously (Talbot et al., 2003b,c). Detection was achieved at an isolation width of
240 m/z 4.0 and fragmentation with normalised collisional dissociation energy of 30% and an
241 activation Q value (parameter determining the m/z range of the observed fragment ions) of
242 0.15. LCMSⁿ was carried out in data-dependent mode with three scan events: SCAN 1 – full
243 mass spectrum, range m/z 300–1300; SCAN 2: data-dependent MS² spectrum of the most

244 intense ion from SCAN 1; SCAN 3: data-dependent MS³ spectrum of the most intense ion
245 from SCAN 2.

246

247 For standard runs dynamic exclusion was turned on limiting the number of MS² scans per
248 individual parent ion to 3 before automatically switching to the next most abundant ion.

249 Where necessary, additional targeted analyses were performed to increase the number of
250 MS² scans per peak. Structures were assigned from comparison with published spectra
251 where possible (Talbot et al., 2003b,c, 2005, 2007a,b) or by comparison of their APCI
252 spectra with those of known compounds as described below.

253

254 Although full quantification was not possible, we applied the usual response factors
255 determined previously for BHPs with and without nitrogen where N-containing compounds
256 give an average relative response 1.5 times that of BHPs without a N atom (see van Winden
257 et al., 2012a for details). Furthermore, co elution of aminotriol (**If**) and anhydroaminotriol (**Ii**)
258 complicates the identification and assessment of the anhydroaminotriol peak area.

259 Anhydroaminotriol has a base peak [M+H]⁺ of m/z 654. Aminotriol has [M+H]⁺ of m/z 714 but
260 also produces some m/z 654 as well via loss of 1 acetylated functional group (CH₃COOH)
261 which is also seen in the full mass spectrum (SCAN 1). Therefore even when only aminotriol
262 is present there will still be a minor m/z 654 peak. To minimise this effect (i.e. minimise any
263 overestimation of anhydroaminotriol) the relative areas of the m/z 714 and 654 peak in all
264 samples where both are present were compared (Table 1; Column **If/Ii**). The lowest relative
265 amount of the 654 peak in any sample is equal to 17.9% of the 714 peak area. Therefore
266 17.9% of the corresponding 714 peak area was subtracted from the m/z 654 peak for each
267 individual sample. The reduced value for the m/z 654 was then used in the calculation of the
268 relative abundance of anhydroaminotriol as a proportion of total BHPs (Table 1).

269

270 *2.4 GC-MS Analysis*

271 Acetylated hopanepolyols, dissolved in DCM, were analysed by GC-MS using an Agilent
272 7890A GC split/splitless injector (300°C) linked to an Agilent 5975C MSD (electron energy
273 70 eV; filament current 220 mA; source temperature 230°C; multiplier voltage 2000V;
274 interface temperature 350°C). A 15 m DB5-HT fused silica column (0.25 mm i.d.; 0.1 mm
275 film thickness) was used with helium as the carrier gas. The oven temperature was
276 programmed from 50 to 200°C at 15°C/min (held for 1 min), from 200 to 250°C at 10 °C/min
277 (held for 1 min) and from 250 to 350°C at 5°C/min (held for 8 min; Talbot et al., 2003a).
278 Hopanoids were identified from full scan (m/z 50–750) analysis of selected samples, by
279 comparison with authentic standards and published spectra and by relative retention times.
280 Additionally, one sample (11.95 cm; Table 1) was analysed in SIM mode targeting ions m/z
281 191, 205, 391, 405, 449, 493 and 507 (see below).

282

283 **3. Results**

284

285 *3.1. Identification of polyfunctionalised hopanoids in the Cobham lignite*

286

287 3.1.1. Biohopanoids

288 Bacteriohopanetetrol (BHT; **1c**) was present in 26 of the 35 samples and was the only
289 biohopanoid observed in any of the Cobham lignite samples which did not contain an amine
290 at the C-35 position (Fig. 1a, b for example; Table 1). Most samples also contained the
291 common tetrafunctionalised aminotriol (**1f**), typically accompanied by similar relative amounts
292 of the pentafunctionalised aminotetrol (**1g**) (Fig. 2 for example; Table 1). In a smaller number
293 of samples, the hexafunctionalised aminopentol (**1h**, Fig. 2; Table 1) was also observed.
294 These compounds were identified by relative retention time (Fig. 2) and interpretation of
295 APCI mass spectra in comparison to previously published data (Talbot et al., 2003b,c).

296

297 3.1.2 AnhydroBHT and related novel hopanoids

298 The common BHP transformation product anhydroBHT (**1a**) was present in all but one
299 sample and was frequently the most abundant BHP present (Table 1). It is readily identified
300 in the diacetate form via EI mass spectrometry (peak **1a**, Figs. 1b, 3a). Although this
301 spectrum has been published previously (e.g. Bednarczyk et al., 2005), it is included to allow
302 for comparison with a group of related novel components described here for the first time
303 (Fig. 3a).

304

305 When analysed by reversed phase HPLC, diacetylated anhydroBHT (**1a**) elutes later than
306 BHT (**1c**), and has an APCI base peak ion of m/z 613 (= $[M+H]^+$; Fig. 1a). The MS² spectrum
307 (Fig. 3b; cf. Talbot et al., 2005) includes major fragments of m/z 553 and 493 indicating 2
308 neutral losses of 60 Da (i.e. the acetylated OH groups at C-33 and C-34 [CH_3COOH]) and
309 ion m/z 475 indicates loss of 18 Da (loss of the heterocyclic oxygen as H_2O). Lower intensity
310 ions indicating a 2nd pathway of initial loss of 18 Da followed by 2 losses of 60 Da are also
311 present (m/z 595, 535 and 475 respectively). There are also characteristic ions more directly
312 indicative of the hopanoid nature of the compounds at m/z 191 as seen in the EI spectrum
313 and at m/z 421 indicating neutral loss of the A+B rings from the protonated molecule. Low
314 levels of a second, earlier eluting isomer (**1a'**; possibly the α,β isomer e.g. Eickhoff et al.,
315 2014) were also present in some samples (Fig. 1a, Table 1).

316

317 In many samples, another peak (**1b**, Fig. 1a) was observed eluting just after anhydroBHT (**1a**)
318 with a base peak ion of m/z 627, potentially indicating a methylated pseudohomologue of
319 anhydroBHT. Based on the retention time this peak was anticipated to be the C-2
320 methylated homologue as the C-3 compound would elute later during both LCMS and
321 GCMS analysis (e.g. Talbot et al., 2003; Farrimond et al., 2004), however, the expected m/z
322 205 ion (indicating methylation on the A+B rings) was not present (or only at very low
323 relative abundance, see below) in the APCI MS² spectrum (Fig. 3d). An ion of m/z 191 was
324 present suggesting instead an additional CH_2 located at a position other than on the A/B
325 rings. Two ions indicating loss of 60 Da and one loss of 18 Da (m/z 567, 507 and 489

326 respectively; Fig. 3d) are also present in the spectrum of peak **Ib** (as also seen for
327 anhydroBHT; Fig. 3b) as well as the ion indicative of neutral loss of 192 (m/z 435; Fig. 3d).
328 We conclude, therefore, that this compound is related to anhydroBHT but has an additional
329 methylation, possibly in the side chain. The D/E+side-chain fragment in the EI mass
330 spectrum would therefore be an ion of m/z 405. Two peaks are observed in the EI m/z 405
331 mass chromatogram (Fig. 1b); one, (peak **Ib**) eluting directly after the main anhydroBHT
332 isomer (**Ia**) and a 2nd peak eluting later (peak **IIIb**, Fig. 1b). The EI mass spectrum of peak **Ib**
333 (Fig. 3c) is consistent with these interpretations, as it includes the ions indicative of a regular
334 hopanoid not methylated on the A/B rings (m/z 191) or indeed any part of the ring system
335 (m/z 369) as also seen for regular anhydroBHT, but also the expected M^+ and $[M-15]^+$ ions
336 of m/z 626 and 611, respectively.

337

338 The second peak in the m/z 405 mass chromatogram (**IIIb**; Fig. 1b) was more difficult to
339 characterise by EI mass spectrometry as it co-eluted with a number of other known
340 compounds including BHT (peak **Ic**, Fig. 1b) and multiple isomers of 32,35-
341 anhydrobacteriohopanepentol (anhydroPentol, **Id**; Fig 1b; Talbot et al., 2005). The retention
342 time of the later eluting m/z 405 peak relative to peak **Ib** is suggestive of an additional
343 methylation at the C-3 position (e.g. Farrimond et al., 2004), supported by the presence of
344 the m/z 205 and 383 ions in the EI mass spectrum (Fig. 3e). This peak (**IIIb**) could also be
345 observed in the APCI data as a peak in the m/z 641 ($= [M+H]^+$) mass chromatogram (Fig.
346 1a). The assignment of an A/B ring methylation is indicated by the ion m/z 205 in the MS^2
347 spectrum (Fig. 3f) and the neutral loss of 206 Da after loss of 2 acetylated hydroxyls to give
348 m/z 315. The relative retention time of peaks **Ib** and **IIIb** by RP-HPLC (Fig. 1a) also agrees
349 with previously reported separations between non-methylated and C-3 methylated
350 homologues via RP-HPLC (regular C-2 methylated structure would elute between **Ib** and
351 **IIIb**; e.g. Talbot et al., 2003b). Therefore peaks **Ib** and **IIIb** are proposed as a pair of novel
352 compounds related to anhydroBHT but with an additional methylation in the side chain and
353 differing from each other by the presence of a methyl group at C-3 in the latter compound.

354

355 In a few samples which contained peak **1b** ("side chain-methylated" anhydroBHT; see Table
356 1), a small shoulder was observed on the leading edge of this peak (peak **1a**, Fig. 1a). For
357 this shoulder, under APCI conditions, the MS² spectrum of *m/z* 627 did contain a small *m/z*
358 205 ion and it is therefore proposed to be the C-2 methylated homologue of anhydroBHT
359 (**1a**; e.g. Bednarczyk et al., 2005). However, this shoulder could not be readily distinguished
360 from the main, non-methylated compound and therefore measurements of peak **1b**
361 incorporate a minor contribution for peak **1a** which is estimated to account for less than 10%
362 of the main peak where present.

363

364 3.1.3 Novel N-containing BHPs

365 In addition to the "regular" N-containing biohopanoids (Figs. 2 and 4a-c) other N-containing
366 compounds were observed throughout the core including the recently described
367 "anhydroaminotriol" (**1i**, Figs. 2 and 4d; see Eickhoff et al., 2014). Identification of
368 anhydroaminotriol can be difficult by GCMS as the intensity of response of nitrogen
369 containing compounds via EI MS is much weaker than that of equivalent N-free compounds
370 (e.g. Eickhoff et al., 2014). Furthermore, under RP-HPLC-APCI-MS analysis,
371 anhydroaminotriol-triacetate co-elutes with regular aminotriol (peaks **1i** and **1f** respectively,
372 Fig. 2). In addition, the triacetate has a base peak ion of *m/z* 654 (= [M+H]⁺) which is
373 equivalent to the primary fragment ion of aminotriol under the same conditions (aminotriol
374 *m/z* 654 = [M+H-CH₃COOH]⁺; Fig. 4a; Talbot et al., 2003b,c). However, careful inspection of
375 the APCI data can still yield clues to the presence of this compound. Firstly, in the absence
376 of anhydroaminotriol, the intensity of the aminotriol base peak ion (MH⁺ = *m/z* 714) relative
377 to the *m/z* 654 ion (= [MH-CH₃COOH]⁺) in the full mass spectrum (Scan 1; Section 2.3) with
378 the conditions used is typically in the range 5:1. Therefore, when the observed ratio is closer
379 to 1:1 or higher as observed in several of the Cobham samples (Table 1, column *m/z*
380 654/*m/z* 714), it is clear that the transformation product is present. This assignment is also
381 supported by consideration of the MSⁿ spectra of *m/z* 714 and 654 respectively. It has

382 previously been shown that MS³ fragmentation of the *m/z* 654 ion derived from MS² of *m/z*
383 714 (aminotriol) leads to further fragmentation with ions *m/z* 594 and 534 as the major
384 products (Talbot et al., 2003c). However, in the presence of anhydroaminotriol, during direct
385 MS² fragmentation of *m/z* 654, the major ion observed is still *m/z* 654 (at 35% collision
386 energy; Eickhoff et al., 2014), with lower intensity ions resulting from losses of 60 Da
387 (acetylated OH groups at C-33 and C-34; Fig. 4d). This is a result of the stability of the
388 heterocyclic N atom during ion-trap fragmentation. Anhydroaminotriol was present in most
389 samples and was always the most abundant N-containing degradation product (Table 1).
390 Adjustment of the anhydroaminotriol peak area for relative quantification is described in
391 section 2.3.

392

393 We also identified 2 other compounds which we tentatively propose to be novel *N*-containing
394 BHP transformation products. The MS² spectrum (Fig. 4e) of peak **Ik** from the APCI *m/z* 728
395 mass chromatogram (Fig. 2) indicates a N-containing compound (on the basis of the even
396 numbered base peak ion) with a minor ion of *m/z* 191 (intensity typical of N-containing
397 BHPs; e.g. Fig. 4c). The base peak ion is equivalent to that of regular A/B ring methylated-
398 aminotriol (e.g. Talbot et al., 2008), however, if that were the case the peak should elute
399 directly after aminotriol (peak **If**, Fig. 2; Talbot et al., 2003b; 2008). The peak in question
400 actually elutes significantly earlier suggesting a more polar compound. Speculating therefore
401 that the 14 Da difference could result from the incorporation of ketone oxygen into the
402 molecule, it is reasonable to expect a loss of that oxygen atom as water (18 Da) under APCI
403 conditions. The major fragment ion in the MS² spectrum is *m/z* 710 (base peak minus 18 Da;
404 Fig. 4e) with 3 further losses of 60 Da (3 acetylated hydroxyls) and a minor ion at *m/z* 471
405 resulting from a final loss of an acetylated amine (see Fig. 4c for comparison of intensity of
406 ion indicating loss of amine in aminopentol spectrum). It is therefore proposed that peak **Ik**
407 (Fig. 2) is related to aminotriol and contains an amine, 3 hydroxyls and a ketone. Although
408 the location of the ketone cannot be constrained other than excluding the A/B rings on the
409 basis of the minor *m/z* 191 ion, there is one previous report of a highly functionalised BHP

410 containing a ketone oxygen at the C-32 position in the side chain. The composite structure
411 32-oxo-bacteriohopanetriol glucosamine (**Io**) was reported from *Zymomonas mobilis*, an
412 obligately ethanologenic species of alphaproteobacteria (Flesch and Rohmer, 1989). It is
413 therefore proposed that the most likely location for the ketone is at C-31 i.e. 31-oxo-35-
414 amino-bacteriohopane-32,33,34-triol (31-oxo-aminotriol; Fig. 4e).

415

416 There was no equivalent peak in the m/z 786 chromatogram to indicate a possible
417 hexafunctionalised compound (i.e. "30-oxo-aminotetrol"), even in the samples with the
418 highest level of aminopentol. However, a peak of m/z 670 would correspond to the
419 anticipated base peak for an aminodiol with additional ketone functionality. Peak **Ij**, the only
420 significant peak in the m/z 670 mass chromatogram (Fig. 2), was considered to be a likely
421 target. Note this retention time is too early to indicate a regular A/B ring methylated
422 compound and the retention time relative to aminotriol (**If**) is early, as seen for the proposed
423 ketone containing 31-oxo-amino-triol and aminotetrol (peaks **Ik** and **Ig** respectively, Fig. 2).
424 Interpretation of this spectrum was complicated, however, as in many cases the peak co-
425 eluted with an ion of m/z 669 meaning that the APCI spectrum contains fragments from both
426 parent ions. Fragment ions of m/z 652, 610 and 550 were observed and would correspond
427 exactly to the expected losses of the ketone oxygen as water or 2 acetylated OH
428 functionalities. In this compound (**Ij**) the intensity of the $[M+H-18]^+$ ion (m/z 652) was
429 significantly lower than for the equivalent ion (m/z 710) in the MS^2 spectrum of the proposed
430 31-oxo-aminotriol (**Ik**; Fig. 4e) whilst in the less functionalised compound, loss of the first
431 acetylated hydroxyl (m/z 619) appears to be a more favourable fragmentation. A minor ion at
432 491 indicates loss of the acetylated OH groups and amine, prior to loss of the ketone,
433 however, the hopanoid-specific ions at m/z 191 and 163 could be products of fragmentation
434 of the co-eluting m/z 669 ion (see below). Therefore the assignment of peak **Ij** (Figs. 2 and
435 4f) as a tetrafunctionalised amine and ketone-containing BHP i.e. 32-oxo-35-amino-
436 bacteriohopane-33,34-diol remains tentative but is included in further discussions below.

437

438 3.1.4 Other novel BHPs not containing a nitrogen atom

439 As mentioned above, a peak was observed in the m/z 669 APCI mass chromatogram which,
440 in samples where co-elution with peak **lj** (m/z 670) was least significant, produced an APCI
441 spectrum potentially indicating a polyfunctionalised BHP (Fig. 5a). Although a peak of m/z
442 669 would typically indicate an A/B ring methylated BHT (Talbot et al., 2003c) the early
443 retention time (i.e. earlier than BHT; **lc**, Fig. 1a) indicates a more polar component.

444 Assuming the validity of the assignment of peak **lk** (Figs. 2 and 4e) as a ketone-containing
445 pentafunctionalised aminoBHP, it is then reasonable to assume that the same should be
446 possible for BHPs which do not contain nitrogen i.e. structures related to BHT. A base peak
447 of m/z 669 and retention time earlier than that of BHT is therefore suggestive of a BHT-
448 ketone (m/z 669 = $[M+H-CH_3COOH]^+$). The APCI spectrum of peak **le** (Fig. 5a) shows three
449 losses of 60 Da (m/z 609, 549 and 489 respectively) assumed to be the normal losses in the
450 APCI spectrum of regular BHT (Talbot et al., 2003b,c). Although an ion of m/z 471 could be
451 observed (loss of ketone oxygen as water from m/z 489), this was weak; nonetheless, the
452 hopanoid nature of this peak is confirmed by the m/z 191, 163 and ions following neutral loss
453 of the A+B rings (m/z 477, 417, 357; Fig. 5a).

454

455 As this compound does not contain N, an equivalent peak was sought in the GCMS data.
456 The anticipated parent ion for this peracetylated compound would be m/z 728 under EI and,
457 assuming a side chain location for the ketone, a D/E+side chain fragment 14 Da higher than
458 that of BHT i.e. m/z 507 (relative to m/z 493, Fig 5b) is expected. A peak (m/z 507, **le**; Fig.
459 1b) was observed eluting after the main m/z 493 peak in the GCMS data (**lc**; Fig. 1b). The EI
460 mass spectrum of peak **le** (Fig. 5b) contained the typical hopanoid fragments of m/z 191 and
461 369 excluding any further modification from the ring system. The predicted D/E-side chain
462 fragment of m/z 507 was observed, however, so were two additional ions of m/z 447 and
463 387, potentially 2 losses of 60 Da from the full D/E-side chain fragment which are not
464 typically observed in other EI spectra of functionalised BHPs (e.g. Fig. 3a) but may be due to
465 the presence of the ketone. As the ring system ions restrict the location of the additional 14

466 Da to the side chain and in both the APCI and EI data, the relative retention time (compared
467 to regular BHT; peak **1c**) indicates a more polar structure than that resulting from a simple
468 methylation, we conclude that there is a ketone present in this structure, located somewhere
469 in the side chain, most likely at C-31 i.e. 31-oxo-BHT (**1e**).

470

471 The tetrafunctionalised ketone equivalent (i.e. BHT with one OH replaced by ketone) would
472 theoretically have an APCI base peak ion of m/z 611 ($[MH-CH_3COOH]^+$). Two peaks were
473 observed in this mass chromatogram in many samples, however, they also co-elute exactly
474 with peaks in the m/z 671 chromatogram (Fig. 1a). The latter ion is also previously reported
475 as the base peak ion for anhydropentol (**1d**) which has a major fragment ion of m/z 611 (Fig.
476 5c; Talbot et al., 2005) so the tetrafunctionalised ketone could not be confirmed by APCI-
477 MSⁿ. Under EI conditions, the D/E-side chain fragment of this proposed ketone would be m/z
478 449, identical to that of anhydropentol (Figs 1b; see Talbot et al., 2005) and therefore could
479 not be confirmed due to co-elution with multiple BHPs (Fig. 5d) although the anticipated $[M-$
480 $15]^+$ ion was present in low abundance (Fig. 5d). This compound is therefore excluded from
481 the following discussion of relative abundance of the various known biohopanoids and novel
482 BHPs including functionalised transformation products.

483

484 To summarise, a range of polyfunctionalised biohopanoids were identified including strong
485 evidence for methanotroph-derived BHPs (aminotetrol and aminopentol in particular but also
486 aminotriol) as well as a recently identified N-containing diagenetic product
487 (anhydroaminotriol) and a number of putative novel diagenetic products including structures
488 with an unusual side-chain methylation. A diverse range of ketone containing products,
489 termed “oxo” BHPs on the basis of previous studies (Flesch and Rohmer, 1989), both with
490 and without a nitrogen atom in the structure, are tentatively assigned here for the first time.

491

492 *3.2 BHP variations in the Cobham Lignite sequence*

493 The compounds BHT (**lc**), aminotriol (**lf**) and aminotetrol (**lg**) had very similar distributions in
494 the sequence when converted to % total BHPs, with the highest relative abundance in the
495 middle clay unit between the laminated and blocky lignite. However, these compounds were
496 also relatively abundant in the uppermost laminated lignite and the lowest blocky lignite
497 samples. Both of these lignite samples, to either side of the middle clay layer, also contained
498 clay laminae not seen in any other lignite samples (Table 1). The combined total abundance
499 of all biological BHPs relative to putative geo-BHPs shows two major and one minor peak
500 (Fig. 6c). The first major peak begins in the uppermost laminated lignite and spans the
501 middle clay layer reaching 100% biohopanoids at 65.3 cm (within the middle clay layer;
502 Table 1). The second maximum of 76% occurs near the top of the Blocky Lignite at 118.65
503 cm (Table 1; note the blocky lignite continues above the section investigated here; Collinson
504 et al., 2007; Steart et al., 2007). All biohopanoids show elevated levels in the samples from
505 115.65 and 118.65 cm except aminopentol which was only present in the lower of the two
506 samples where it is at its highest relative abundance throughout the entire sequence (4%;
507 Figs. 2 and 6, Table 1). The minor peak occurs in the single sand/mud unit sample
508 underlying the Cobham Lignite sequence (Fig. 6c). At the onset of the CIE (Fig. 6a,b; 54-56
509 cm) relative abundances of BHT, aminotriol and aminopentol fluctuate markedly (Fig. 6d, e
510 and g) whilst aminotetrol is consistently lower than in samples immediately pre and post this
511 interval (Fig. 6f). Aminopentol (**lh**), however, was not present in the middle clay whilst both
512 BHT and Aminotetrol had higher relative abundance (to total biohopanoids) in the middle
513 clay layer and the adjacent lignite with clay samples (Table 1; Figs. 6d and 6f respectively).
514

515 The most abundant geo-BHP in the majority of samples was anhydroBHT (**la**) accounting for
516 up to 100% of all hopanoids (one sample; Fig. 7d, Table 1). Most of the geohopanoids
517 followed a similar trend to anhydroBHT with high abundances relative to biohopanoids in
518 lignites both above and below the middle clay layer (Table 1). This was particularly
519 noticeable for the N-containing compounds in the laminated lignite, especially in samples
520 leading up to and within the onset of the CIE just below the middle clay layer.

521 Anhydroaminotriol (**li**) reached a maximum of 45.8 % of the total BHPs and 50% of the total
522 geohopanoids (40.65 cm, Table 1; Fig. 7g). The novel “side-chain methylated” anhydroBHTs
523 (**lb** and **IIIb**; Fig. 7e) and the proposed oxo-BHPs (**le**, **li**, **lj**; Fig. 7f, h-, Table 1) all had similar
524 profiles through the sequence with peaks coinciding with the onset of the CIE and at the end
525 or just after deposition of the middle clay layer.

526

527 **4. Discussion**

528

529 4.1 Origin of BHPs in the Cobham Lignite sequence

530 *4.1.1 Modern, Recent or Ancient deposition?*

531 Palaeoenvironmental interpretation of BHPs in the Cobham Lignite is contingent on these
532 BHPs being formed in the ancient environment rather than being derived from bacteria living
533 in the shallow subsurface at any point post deposition to date. Previous workers have shown
534 that bacteria do live in subsurface environments including in marine sediments (e.g. Parkes
535 et al., 2000; Kallmeyer et al., 2012), lignites and coalbeds (e.g. Kotelnikova, 2002; Pokorný
536 et al., 2005). Inagaki et al. (2015) found unexpectedly high levels of microbial cells in lignite
537 layers buried ~ 2 km below the ocean floor and microbial community analysis revealed
538 populations similar to those found in forest soils. Aerobic organisms can receive low levels of
539 oxygen via meteoric water in the subsurface environment (e.g. Kotelnikova, 2002) and
540 specific aerobic methanotrophic activity was recently identified in Carboniferous coals where
541 organisms affiliated with the Type I genera *Methylomicrobium* and *Methylocaldum* and the
542 Type II genera *Methylosinus* and *Methylocystis* were identified via 16S rRNA targeted gene
543 sequencing after enrichment (Stepniewska et al., 2013). All of these genera have been
544 shown to produce hopanoids (Cvejic et al., 2000; Talbot et al., 2001 and references therein;
545 Banta et al., 2015), although both of the Type I genera identified are known to make both
546 non-methylated and C-3 methylated biohopanoids (with a C-35 amine functionality), so are
547 unlikely to be significant sources in the Cobham Lignite sequence which did not contain any

548 C-3 biohopanoids or hopanes (Pancost et al., 2007) and only one minor geohopanoid BHP
549 (side chain-methylated-3-methylanhydroBHT, **IIIb**). In light of these findings it is difficult to
550 preclude a modern origin for some of the biohopanoid BHPs at Cobham.
551
552 A modern origin for the Cobham Lignite BHPs, however, is considered unlikely for several
553 reasons. Firstly, many aspects of the BHP distribution are consistent with an aerobic source
554 which may be more recent but would also be consistent with formation in an ancient mire
555 with abundant methanotrophs. Previously, carbon isotopic analysis of the hopanes in the
556 same sample extracts (Fig. 6b, 7b; Pancost et al., 2007) supported a methanotrophic
557 source. For example, within the upper part of the laminated lignite at the onset of the CIE,
558 the $\delta^{13}\text{C}$ values of the C_{29} - and C_{31} $17\beta(H),21\beta(H)$ hopanes decrease (to values as low as -
559 76‰ and -42‰, respectively), indicating the consumption of isotopically light methane
560 (Pancost et al., 2007). This change in isotope values occurred within the laminated lignite
561 (section 2.1; Fig. 6), but below the transitional zone to the middle clay where thin clay
562 laminae start to appear in the lignite. Therefore the isotopic changes are not linked to
563 changes in depositional environment (as far as those are expressed by changes in lithology)
564 apparently precluding a preservation artefact (Pancost et al., 2007). Within the BHP
565 assemblage, the co-occurrence of aminotriol (**If**) and aminotetrol (**Ig**) and with a **If/Ig** ratio
566 less than 20 (as observed in all samples here, except that at 11.95 cm; Table 1) appears to
567 be indicative of an aerobic methanotroph source. Indeed the value is more frequently below
568 10, whereas the lowest reported values for the only other known source of aminotetrol,
569 anaerobic sulfate reducing bacteria of the genus *Desulfovibrio* (which are unlikely to occur in
570 a freshwater environment) is over 20 and can be as high as 100 (Blumenberg et al., 2006,
571 2009, 2012; See also review in Talbot et al., 2014). An aerobic methanotroph source is also
572 supported by the occurrence of aminopentol (**Ih**) in 13 samples covering all parts of the
573 sequence, except the middle clay layer (and the lignite samples directly above and below the
574 clay) where aminotetrol dominated (Figs. 6f and 8, Table 1).
575

576 Secondly, the strong increase in the relative proportion of biohopanoids in the middle clay
577 layer and samples directly above and below containing clay laminae (Fig. 6c) is directly
578 related to lithology. Low permeability of the clay layer compared to lignite would likely result
579 in a much smaller “recent” population whilst the organic rich, more permeable lignite might
580 be expected to host a larger “recent” population (cf. Inagaki et al., 2015). As the relative
581 abundance of biohopanoid BHPs is in fact significantly lower in the lignite sections (Fig. 6c,
582 7c), this supports a sub-recent source for (some of) the BHPs with potentially better
583 preservation of ancient BHPs in the fine grained material.

584

585 Thirdly, although modern bacteria could be affected by variations in lithology and organic
586 matter content, they would not be expected to vary across the CIE onset, which occurs
587 within the laminated lignite lithology below the transition zone to the clay layer (Figs. 6 and 7)
588 where a distinct geohopanoid BHP signature with enhanced relative abundance of N-
589 containing transformation products is observed (54.4 to 56.6 cm; Fig. 7h). Following the
590 same argument made for the hopane isotope signature, that the lack of a change in lithology
591 at this depth (54.4 to 56.6 cm) precludes a preservation artefact (Pancost et al., 2007), then
592 an alternative explanation is required to explain the relative increase in ketone-containing
593 compounds (Fig. 7f,h). As the pathway for production of the proposed ketone-containing
594 compounds is currently unknown, they may represent either a specific transformation
595 pathway, or could be derived from as yet unknown biological sources. The only previous
596 report of a ketone containing biohopanoid was from the obligately ethanologenic bacterium
597 *Zymomonas mobilis* (alphaproteobacteria; Flesch and Rohmer, 1989). This organism was
598 cultured under microaerophilic conditions and the authors proposed that the compound 32-
599 oxo-BHT glucosamine (**1o**) could be either a precursor or catabolite of BHT (Flesch and
600 Rohmer, 1989). Gibson (2011) also tentatively identified ketone-containing
601 polyfunctionalised hopanoids (without nitrogen) in geothermal sinters that were precipitating
602 under microaerophilic conditions. We therefore tentatively propose that the oxo-amineBHPs
603 could represent production by methanotrophs under oxygen limited conditions.

604
605 Finally, no composite BHPs (structures containing a more complex moiety at the C-35
606 position such as an aminosugar; e.g. **Im**, **In**; Rohmer, 1993) were observed, even in the clay
607 layer, although these compounds would be expected in any modern or Recent terrestrial
608 setting (e.g. Cooke et al., 2008a; van Winden et al., 2012a,b; Spencer Jones et al., 2015)
609 including marine sediments containing high terrestrial fluvial input (e.g. Handley et al., 2010;
610 Wagner et al., 2014). Furthermore, adenosylhopane (**Ip**), and compounds related to this
611 structure containing an alternative terminal moiety at C-35 with or without A-ring methylation
612 (**I** or **IIq**) (e.g. Cooke et al., 2008a; Rethemeyer et al., 2010), were also absent from the
613 Cobham sequence, despite being nearly ubiquitous in terrestrial settings (see review in
614 Spencer-Jones et al., 2015). This is not surprising, if the BHPs that are present are indeed of
615 Eocene age, as adenosylhopane was found to be degraded more rapidly than all other
616 functionalised BHPs, including aminoBHPs, in a study of sediments up to 1.2 Ma from the
617 Congo deep-sea fan (ODP site 1075; Cook et al., 2008b; Handley et al., 2010).
618 Adenosylhopane was also completely removed/transformed during an artificial maturation
619 experiment on biomass of the purple non-sulfur bacterium *Rhodospseudomonas palustris*,
620 whilst some BHT (**Ic**) and aminotriol (**If**) remained after exposure to elevated pressure and
621 temperature (170°C, 120 MPa, 7 d; Eickhoff et al., 2014).

622
623 Collectively, this evidence suggests that at least some of the BHPs, and the oxo-compounds
624 in particular (Fig. 7f,h), could reflect environmental conditions associated with the original
625 deposition of the lignite. Nonetheless, a more recent source can neither be confirmed nor
626 entirely excluded and we suggest that future work conduct BHP analyses in tandem with
627 microbiological investigations (i.e. cell counts, enrichments) in an attempt to decouple
628 ancient from modern living bacterial contributions. If, however, the biohopanoid BHPs in the
629 Cobham lignite are indeed of the same age as the deposition of the lignite, this would
630 represent the oldest example of suites of solvent extractable intact biohopanoids reported to
631 date. Previously only BHT has been identified in TLE from Tanzanian sediments up 50 Ma

632 (van Dongen et al., 2006) and following chemical degradation of the Messel oil shale
633 kerogen (Eocene, 50 Ma; Mycke et al., 1987). If contemporaneous with lignite deposition
634 then this significantly extends the aminoBHP record back in time, by nearly 55 Ma,
635 highlighting their potential utility in identifying the aerobic methane oxidation process in
636 samples where other methods can be inconclusive.

637

638 4.1.2 Variability in methanotrophic sources

639 The occurrence of aminopentol (**Ih**), thought to be a marker for Type I methanotrophs in
640 terrestrial settings (see review in Talbot et al., 2014), in 13 Cobham Lignite samples is
641 consistent with a significant methanotroph input. In recent studies aminopentol accounted for
642 only a very minor proportion of the full biohopanoid BHP complement in a range of European
643 *Sphagnum* peat sections (<1%; van Winden et al., 2012a,b; Talbot and Pancost,
644 unpublished data). In contrast, the highest abundance of aminopentol relative to total
645 biohopanoid BHPs in the Cobham Lignite reaches ~20% (sample 115.65 cm; Fig. 6g) or 4%
646 relative to total BHPs (Table 1), significantly higher than that observed in any *Sphagnum*
647 sample or modern peat. Furthermore, the peak occurrence of aminopentol (115.65 cm, Fig.
648 6g) corresponds to a C₂₉ hopane carbon isotope ratio of -53.2‰ which, although less than
649 the maximum depletion during the onset of the CIE, is still significantly depleted relative to
650 pre-CIE values in the lower Laminated lignite (range -40.5 to -31.0‰; Fig 6b). Also, 3 of the
651 5 samples at the onset of the CIE where C₂₉ carbon isotope ratio reach values as low as -
652 75.7‰ also contain relatively high levels of aminopentol (up to 10% of total biohopanoids,
653 Fig. 6g). The sample at 70.3 cm, slightly above the clay layer also contains similar levels of
654 aminopentol and again corresponds to strongly depleted signature for the C₂₉ hopane of -
655 61.77‰ (Fig. 6b). Clearly there is correspondence in the Cobham Lignite between
656 aminopentol abundances (as proportions of total biohopanoids) and ¹³C-depleted isotopic
657 composition of the C₂₉ hopane. Moreover, maxima in aminopentol proportions and minima in
658 C₂₉ hopane δ¹³C values also correspond to maxima in abundances of nitrogen and ketone-
659 containing degradation products (Fig. 7h).

660

661 Aminotetrol (**Ig**) is also likely derived from methanotrophs in this terrestrial setting. It has also
662 been found only at very low levels in *Sphagnum* peat (<2% of total BHPs) such that its
663 relative abundance in the Cobham Lignite, reaching a maximum of ~65% of total
664 biohopanoid BHPs (Fig. 6f, 4.65 cm; equivalent to 14.4% of total BHPs; Table 1) is
665 remarkable, likely reflecting intense methanotrophic activity. Intriguingly and in contrast to
666 aminopentol, aminotetrol proportions do not appear to track hopane $\delta^{13}\text{C}$ values; in part, that
667 could reflect changing methanotroph communities as discussed below.

668

669 Aminotriol (**If**) and BHT (**Ic**) are much more significant components in peat (van Winden et
670 al., 2012a,b; Talbot and Pancost, unpublished data) than aminopentol and aminotetrol. They
671 are present throughout the Cobham sequence likely indicating a combination of sources
672 including Type II methanotrophs and heterotrophs (e.g. van Winden et al., 2012a). Again,
673 variations in their proportions do not correspond to those of hopane $\delta^{13}\text{C}$ values, suggesting
674 that they either do not derive from methanotrophs or derive from a different group of
675 methanotrophs, exhibiting different behaviour.

676

677 The BHP composition of both the lower laminated lignite (deposited during the latest
678 Palaeocene) and upper blocky lignite (deposited during the PETM) are broadly similar (Fig.
679 8a,d), suggesting the microbiological community was similar during both periods of
680 deposition or during later colonisation. If the former, this is unexpected given the wetter
681 conditions and presumably greater methane cycling (as inferred from hopane $\delta^{13}\text{C}$ values) in
682 the upper section, although Sherry et al. (2016) showed that there was no significant
683 changes in the overall methanotrophic community distribution in sediment slurry incubations
684 under a range of methane concentrations. Nonetheless, the blocky lignite is associated with
685 generally higher proportions of aminopentol (Fig. 6g) and that does suggest that aspects of
686 the biohopanoids BHP distribution are recording environmental change.

687

688 The most significant change in biohopanoid BHPs, both in terms of their abundance relative
689 to total BHPs and their distribution, occurs in the clay layer (and lignite with clay; Fig. 8c,
690 Table 1). Biohopanoid BHPs represented the highest proportion of total BHPs in the clay
691 layer, up to 100% at 65.3 cm (Fig. 6c; Table 1), which we attribute to enhanced preservation
692 (see above, section 4.1.1). With respect to the biohopanoid BHP distribution, the most
693 striking feature in the clay layer is the absence of aminopentol (**Ih**), particularly given the
694 high abundances of both aminotriol (**If**) and aminotetrol (**Ig**; Figs. 6e and f, 8b, Table 1). This
695 could reflect a change in the depositional environment: the clay layer has been linked to
696 changes in local hydrology leading to increased run-off and even standing water as indicated
697 by the occurrence of *Salvinia* and *Azolla* (free floating water plants) at the base of the blocky
698 lignite directly above the clay, possibly facilitated by deposition of clay reducing drainage
699 (Collinson et al., 2003, 2013; Steart et al., 2007 and references therein). This could have
700 impacted bacterial or even methanotroph communities and associated BHP signatures.

701

702 The presence of aminotetrol and absence of aminopentol has been linked to Type II
703 methanotroph sources (e.g. Talbot et al., 2001; Birgel et al., 2011), although some Type I
704 methanotrophs also do not produce aminopentol (Talbot et al., 2001; Jahnke et al., 1995;
705 Coolen et al., 2008; Banta et al., 2015). Nonetheless, it is possible that the different
706 distributions in the clay layer reflect different environmental conditions and a methanotroph
707 community dominated by Type II organisms. These are traditionally believed to be common
708 in terrestrial settings (soils, peats e.g. Hanson and Hanson, 1996), although this is certainly
709 not always the case. For example, Gray et al. (2014) found that Type I methanotrophs
710 dominated over Type II signals in soil cores from 13 sites located along the southern and
711 northern margins of Kongsfjorden in Northwestern Spitsbergen. Overall, it remains unclear
712 what dictates the predominance of different types of methanotrophs, and therefore, it is
713 difficult to explain a change in assemblages in the Cobham Lignite.

714 4.2 Known and novel polyfunctionalised hopanoid transformation products in the Cobham

715 Lignite sequence

716

717 4.2.1. AnhydroBHT and related structures

718 The most abundant degradation product, occurring in all but one sample (65.3 cm; Table 1)
719 in the Cobham sequence was anhydroBHT (**Ia**). This is expected as the compound has been
720 shown to be produced from BHT (**Ic**) and composite structures such as BHT cyclitol ether
721 (**Im**) under a range of conditions (Schaeffer et al., 2008, 2010; Eickhoff et al., 2014). It has
722 been reported in a wide range of samples as old as Jurassic in age (Bednarczyk et al., 2005)
723 and was by far the most abundant BHP in a ~50 Ma marine sediment sample from Tanzania
724 (van Dongen et al., 2006). Watson (2002), also showed a correlation between loss of BHT
725 and increase in anhydroBHT in sediments from the Benguela upwelling system down to over
726 4 Ma, as did Blumenberg et al. (2013) in younger sediments from the Baltic Sea. Whilst it is
727 yet to be conclusively demonstrated that adenosylhopane (**Ip**; or related compounds, **Iq**)
728 could also be a precursor for this transformation product, a number of studies have
729 speculated that it may be possible via reductive removal of the terminal adenine (Costantino
730 et al., 2001; Cooke et al., 2008b; Eickhoff et al., 2014). The high abundance of anhydroBHT
731 in these samples, therefore, likely indicates an amalgamated signature from a range of
732 precursor biohopanoids.

733 By careful inspection of the APCI and EI data we have identified 2 novel structures related to
734 anhydroBHT (**Ia**) but containing an unusual methylation in the side chain (Figs. 1 and 3). The
735 location of the unusual side chain methylation could not be conclusively determined based
736 on the available data and insufficient material is available to attempt isolation and NMR
737 analysis. Previously, however, Simonin et al. (1994) identified biohopanoids with methylation
738 at the C-31 position of the side chain of hopanoids from the acetic acid bacterium
739 *Acetobacter europaeus* (Phylum Alphaproteobacteria, also known as *Gluconacetobacter*
740 *europaeus* or *Komagataeibacter europaeus*). The side chain methylated compounds in that
741 study were also methylated at the C-3 position as in compound **IIIb** reported here (Fig. 3).

742 More recently, Nytoft (2011) reported C-31 methylated hopanes, although not additionally
743 methylated at the C-3 position, in coals and crude oils sourced from a range of locations
744 worldwide and linked these compounds with oxic depositional environments. The possible
745 occurrence of C-3 methylation in one of these structures (**IIIb**), the only proposed C-3
746 methylated compound in the entire data set, does agree with the potential acetic acid
747 bacterium source (Simonin et al., 1994); therefore the tentative structures are illustrated with
748 the methylation at C-31 (**Ib** and **IIIb**; Fig. 3 and Appendix). An alternative known site for
749 methylation at C-12 as reported for a BHT in the sponge *Placortis simplex* (Costantino et al.,
750 2000) could be conclusively ruled out for structure **Ib** due to the presence of the m/z 369 ion
751 in the EI spectrum (Figs. 3c). No C-3 methylated precursor biohopanoids were observed in
752 the Cobham sequence; however, given the low relative intensity of peak **IIIb** (Fig. 1; Table
753 1), this is not surprising, especially when considering that reports of other C-3 methylated
754 biohopanoids in younger samples are scarce (e.g. Talbot et al., 2003a; Talbot and
755 Farrimond, 2007; Blumenberg et al., 2007; Gibson et al., 2008; Zhu et al., 2011).

756

757 We are not aware of prior reports of polyfunctionalised biohopanoids with this unusual side-
758 chain methylation in environmental samples. The relative retention time of **Ib** to anhydroBHT
759 (**Ia**; Fig. 1) indicates that it would elute in approximately the same position as the regular C-2
760 methylated homologue (**IIa**; Fig.1). However, in various settings an apparently methylated
761 BHT eluting at the expected C-2 methyl position (i.e. directly after BHT by RP-HPLC) has
762 been observed in sediment extracts but where the MS² spectrum contains a dominant m/z
763 191 and only a minor m/z 205 ion (if present at all; Talbot et al., unpublished data).

764

765 There was no evidence of C-2 methylated biohopanoid precursors in any of the samples,
766 and only trace levels of a C-2 methylated anhydroBHT (**IIa**) could be identified in a few
767 samples indicating that some C-2 methylated precursor was occasionally present. Absence
768 of C-2 methylated structures is relatively unusual for terrestrial settings, as they are
769 frequently found, for example, in soils (e.g. Cooke et al., 2008a; Xu et al., 2009; Rethemeyer

770 et al., 2010; Kim et al., 2011; Zhu et al., 2011), lake sediments (Talbot et al., 2003a; Talbot
771 and Farrimond, 2007; Coolen et al., 2008) and tropical wetlands (Wagner et al., 2014;
772 Spencer-Jones et al., 2015). As the co-eluting peak **lb** was in all cases significantly more
773 intense than the shoulder **lla** (e.g. Fig. 1a), the compounds could not be individually
774 integrated and are combined in Table 1. Furthermore, peak **lla** could not be conclusively
775 identified by GCMS analysis.

776

777 4.2.2 *N*-containing transformation products.

778

779 Crucially, this study has revealed the presence of both previously identified and novel
780 transformation products of *N*-containing BHPs, providing new tools for examining methane
781 cycling in ancient settings. These observations are consistent with recent experimental work.
782 Eickhoff et al. (2014) reported the generation of a pair of novel *N*-containing transformation
783 products during simulated diagenetic degradation of a culture of the purple non-sulfur
784 bacterium *Rhodopseudomonas palustris*. These structures, tentatively identified as isomers
785 of anhydroaminotriol (**li**; Fig. 4d), were major components of the products after artificial
786 maturation. Their finding is significant as it represents the first polyfunctionalised *N*-
787 containing hopanoid degradation product indicating a possible distinct transformation
788 pathway for hopanoids containing a terminal amine (Eickhoff et al., 2014). The observation
789 of the later eluting and therefore presumed $\beta\beta$ isomer here in the Cobham Lignite represents
790 the first report of this compound from environmental samples. Moreover, other *N*-containing
791 products are tentatively proposed here (oxo-aminotriol [**lk**] and oxo-aminodiol [**lj**]; Figs 2 and
792 4, Table 1). Anhydroaminotriol was by far the most abundant compound of this type during
793 the onset of the CIE (Fig. 8c; Table 1), although its peak occurrence was in the sample from
794 40.65 cm suggesting that nitrogen containing precursors were particularly abundant at the
795 time of deposition of this sample. However, bulk and compound specific isotope values do
796 not indicate an enhancement in methane cycling at this point (Fig. 7; Pancost et al., 2007)
797 and therefore suggest heterotrophic sources for the precursor aminotriol.

798

799 **5. Conclusions**

800 This study presents the first identification of polyfunctionalised bio- and geohopanoids in the
801 well preserved, immature Cobham Lignite sequence from within part of the PETM interval
802 (~56 Ma) and below. Up to four different biohopanoids including BHT, aminotriol, aminotetrol
803 and aminopentol were observed in samples throughout the sequence. Although the age of
804 the BHPs cannot be constrained at this time, several lines of evidence point to a sub-Recent,
805 predominantly methanotrophic source for the BHPs. These include prior reports of
806 isotopically light hopanes (up to -74‰) indicative of enhanced methanotrophy at the onset of
807 the negative CIE, the absence of the composite biohopanoids or adenosylhopane and
808 related structures, and peaks in novel, potentially ketone containing, geohopanoid BHP
809 transformation products corresponding to the onset of the CIE.

810 The comparable averaged BHP distribution in the lower laminated lignite (deposited during
811 the latest Paleocene) and the blocky lignite (deposited during the PETM) suggest similar
812 dominant members of the microbiological community were active during both periods This is
813 unexpected given the wetter conditions and the presence of generally more ¹³C-depleted
814 hopanes in the blocky lignite although there are subtle differences in the intermittent
815 occurrence of aminopentol which corresponds with depleted C₂₉ hopane δ¹³C values.

816 Further experimental work (e.g. quantitative BHP analysis; laboratory studies of the impact
817 of different environmental conditions [temperature, methane concentration etc.] on BHP
818 distributions) and study of other lignites including cell counts, formed under both changing
819 and constant climatic conditions, is needed to interpret the palaeoenvironmental implications
820 of the methanotroph communities and their varied biomarkers.

821

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1109 **Figure legends**

1110 **Figure 1.** (a) Partial APCI mass chromatograms showing base peak ions of individual BHPs
1111 which do not contain a nitrogen atom identified via LCMSⁿ. (b) Partial EI mass
1112 chromatograms including m/z 191 and 205 fragments (A/B rings) and characteristic D/E
1113 +side chain fragments for BHPs identified via GCMS. See appendix for structures. Data from
1114 sample depth 11.95 cm (laminated lignite; Table 1).

1115 **Figure 2.** Partial APCI mass chromatograms showing base peak ions of individual BHPs
1116 identified via LCMSⁿ which contain a nitrogen atom. See appendix for structures. Data from
1117 sample depth 115.65 cm (blocky lignite; Table 1.)

1118 **Figure 3.** EI and APCI MS² mass spectra (respectively) of peracetylated derivatives of (a
1119 and b) AnhydroBHT, (c and d) proposed “side chain methylated” AnhydroBHT and (e and f)
1120 proposed “side chain methylated” 3-methyl-AnhydroBHT (note this compound co elutes with
1121 BHT and anhydroBHpentol by GCMS as indicated by the D/E+side chain fragments m/z 493
1122 and 449 respectively; underlined ions in panel e are consistent with the proposed structure).
1123 Position of “side-chain methylation” at C-31 in structures shown in parts c-f is tentative (cf.
1124 Simonin et al., 1994; Nytoft, 2011). For identification of peaks see Figure 1. Key: Ac = -
1125 COCH₃; # indicates peak related to aminotriol (**If**; Figure 2). Spectra taken from sample
1126 depth 11.95 cm, (laminated lignite, Table 1).

1127 **Figure 4.** APCI MS² spectra of peracetylated derivatives of (a) Aminotriol [**If**], (b) Aminotetrol
1128 [**Ig**], (c) Aminopentol [**Ih**], (d) anhydroaminotriol (**li**; cf. Eickhoff et al., 2014) (e) proposed 31-
1129 oxo-35-aminobacteriohopane-32,33,34-triol [**Ik**] and (f) proposed 32-oxo-35-
1130 aminobacteriohopane-33,34 diol [**Ij**]. For identification and relative retention times of peaks
1131 see Figure 2. Key: Ac = -COCH₃; * indicates loss of 42 Da i.e. partial loss of acetylated OH
1132 group as ketene COCH₂; (Talbot et al., 2003b); # indicates ions arising from fragmentation of
1133 co-eluting parent ion m/z 669, Fig. 1). Data from sample depth 115.65 cm (blocky lignite;
1134 Table 1.)

1135 **Figure 5.** APCI MS² and EI mass spectra (respectively) of peracetylated derivatives of (a)
1136 and b) proposed 31-oxo-bacteriohopane-32,33,34,35-tetrol [**le**], (c) APCI MS² spectrum of
1137 AnhydroBHpentol (isomer i; cf. Talbot et al., 2005) and (d) EI mass spectrum of
1138 AnhydroBHpentol (co-eluting with BHT [**lc**] and “side chain methylated” 3-methyl-
1139 anhydroBHT [**lllb**]). For identification of peaks see Figure 1. Data from sample depth 11.95
1140 cm (laminated lignite; Table 1).

1141 **Figure 6.** Plots of (a) $\delta^{13}\text{C}$ values of bulk OM, (b) $\delta^{13}\text{C}$ values of C₂₉ (circles) and C₃₁
1142 (diamonds) hopanes, (c) total biohopanoids as a proportion of total BHPs and (d-h)
1143 individual polyfunctionalised biohopanoids as a proportion of total biohopanoids in the
1144 Cobham lignite sequence. Open circles indicate 5 samples from within the onset of the CIE
1145 (54.4 – 56.6 cm), black circles indicate all other samples. Shaded grey bars indicate clay
1146 horizons including lignite with clay laminae in the middle section (Table 1). All isotope data
1147 from Pancost et al. (2007) and lithology after Collinson et al. (2003) and Steart et al. (2007).

1148 **Figure 7.** Plots of (a) $\delta^{13}\text{C}$ values of bulk OM, (b) $\delta^{13}\text{C}$ values of C₂₉ (circles) and C₃₁
1149 (diamonds) hopanes, (c) relative abundance of total geohopanoids as % of total BHPs and
1150 (d-h) selected known and novel polyfunctionalised hopanoid transformation products as a
1151 proportion of total geohopanoids in the Cobham Lignite sequence. Open circles indicate 5
1152 samples from within the onset of the CIE (54.4 – 56.6 cm), black circles indicate all other
1153 samples. Shaded grey bars indicate clay horizons including lignite with clay laminae in the
1154 middle section (Table 1). All isotope data from Pancost et al. (2007) and lithology from
1155 Collinson et al. (2003) and Steart et al. (2007).

1156

1157 **Figure 8.** Average relative abundance of all BHPs in Cobham lignite sequence (a) blocky
1158 lignite [67.5 – 135 cm], (b) clay layer plus uppermost laminated lignite and lowest blocky
1159 lignite [57.2-66.8 cm], (c) Onset of carbon isotope excursion [CIE] in laminated lignite [54.45
1160 – 56.6 cm] and (d) lower laminated lignite and sample from sand/mud unit [-1.5 – 54.15 cm].

1161 White bars indicate compounds containing a nitrogen atom, black bars compounds not
1162 containing nitrogen. See Appendix for structures.

1163

1164

1165 **Table 1.** Sample depth in sequence, lithology, bulk carbon isotope ratio values (‰) and relative abundance (%) of individual BHPs
 1166 from LCMS analysis and biomarker ratios in the Cobham Lignite.

1167

Level (cm)	Lithology ^a	$\delta^{13}\text{C}$ TOC ^b	Biohopanoids				Polyfunctionalised geohopanoids/Novel products									Ratios	
			lc	lf	lg	lh	la	la'	lb	lllb	ld	le	li	lj	lk	654/714 ^e	lf/li
135	Blocky Lignite	nm ^c	- ^d	2.8	-	-	60.1	-	11.5	-	4.5	1.9	13.5	5.7	-	5.0	
131.4	Blocky Lignite	-26.82	-	-	-	-	100	-	-	-	-	-	-	-	-		
118.65	Blocky Lignite	-26.93	31.4	23.8	21.1	-	16.3	-	1.8	-	-	-	5.6	-	-	0.4	1.1
115.65	Blocky Lignite	nm	4.1	8.3	4.3	4	41.9	2.3	3.9	0.5	5.2	5.5	8.7	5.3	6	1.2	1.9
102.4	Blocky Lignite	-27.33	1.6	3.6		-	62		7.7	1.3	5	2.7	8.8	4	3.3	2.6	
85.9	Blocky Lignite	-26.14	1.9	3.1	0.3	0.5	60	2.7	8.8	1.8	7.5	3.1	5.8	2.2	2.3	2.0	10.3
75.2	Blocky Lignite	-26.77	1.5	5	1.5	0.7	51.8	-	3.1		2.6	6	14.3	6.3	7.2	3.1	3.3
73.95	Blocky Lignite	-26.82	1.8	5.8	0.4	0	46.2	-	6.2	1.4	5.1	5	17.2	7.5	3.4	3.2	14.5
70.3	Blocky Lignite	nm	1.3	7	2.8	1.5	45.8	-	5.4		1	5.2	14.4	6.1	9.5	2.2	2.5
69.75	Blocky Lignite	nm		6.8	2.1	-	58	-	9.7			3.5	15.9	1.8	2.2	2.5	3.2
69.35	Blocky Lignite	-26.81	1.2	1.2	0.4	0.2	61.3	3.9	9.8	1.7	5.8	6.6	4.5	2.2	1.2	3.9	3.0
68.85	Blocky Lignite	nm	0.7	1.6	0.7	0.2	62.2	4.1	9.6	2.1	5.6	4.4	4.3	1.5	3	2.9	2.3
68.4	Blocky Lignite	nm	1.6	2.1	1	0.2	64.8	4	9.5	2.1	5.2	3.9	4	1.2	0.4	2.1	2.1
67.5	Blocky Lignite	nm	2.6	11.3	13.4	0.6	39.4	4.4	8.9	2.3	4.6	3.4	6.4	1.5	1.2	0.7	0.8
66.8	Blocky Lignite with clay layers	-24.17	7.5	22.6	48.2	-	14.4	-	4.1	0.5	0.7	0.9	1.1	-	-	0.2	0.5
65.3	Clay	-24.83	42.6	29.6	27.8	-	-	-	-	-	-	-	-	-	-	0.2	1.1
58.75	Clay	-25.45	29.1	28.4	33.1	-	6	-	-	-	-	-	3.4	-	-	0.3	0.9
57.2	Laminated Lignite with clay layers	-25.75	14.6	43.1	35.9	-	5.1	-	-	-	-	-	1.3	-	-	0.2	1.2
56.6	Laminated Lignite	-26.97	2	5.2	1.6	0.7	50.3	-	7.6	0.8	6.5	3.7	11.9	5.3	4.4	2.5	3.3
55.9	Laminated Lignite	-26.72	1.2	5.4	0.9	0.8	44.5	-	6	0.7	5.2	3.8	12.6	8	10.9	2.5	6.0
55.3	Laminated Lignite	-27.10	-	5.2	-	-	39.9	-	-	-	-	7.8	29.7	8.6	8.8	5.9	
54.85	Laminated Lignite	-27.16	-	4	0.9	0.8	44.7	-	3.6	-	3	5	15.8	9.5	10.5	4.1	4.4

54.45	Laminated Lignite	-27.01	-	2.5		-	42.4	-	-	-	-	-	26	17.9	11.2	10.7	
54.15	Laminated Lignite	-26.01	-	11.7	5.3	-	70.8	-	-	-	-	-	12.2	-	-	1.2	2.2
43.3	Laminated Lignite	-25.51	1.4	14.2	4.8	-	43.7	-	6.8	0.6	1.7	2.1	20.4	3.1	1.2	1.6	3.0
42.4	Laminated Lignite	-25.20	32.9	-	-	-	50	-	-	-	-	-	17.1	-	-		
41.5	Laminated Lignite	-26.01	1.2	9.3	1.5	-	52.7	-	4.9	1.5	1.6	1.6	19.8	3.8	2.1	2.3	6.2
40.65	Laminated Lignite	-25.28	-	8.3	-	-	45.9	-	-	-	-	-	45.8	-	-	5.7	
39.8	Laminated Lignite	-26.10	1.5	10.1	0.9	-	53.5	-	6.1		5.7	1.5	17.6	2.4	0.7	1.9	11.2
36.1	Laminated Lignite	-25.21	-	7.3	0.6	-	44.4	-	8.3	1.4	5.1	1.9	22.5	5.9	2.6	3.3	12.2
23.05	Laminated Lignite	-25.62	2.9	4.1	0.5	0.6	66.5	2.6	8.6	1.1		2.9	7.3	2	0.9	2.0	8.2
16.5	Laminated Lignite	-25.83	2.1	4.2	0.5	0.2	59.9	2.3	10.6	1.3	4.4	4.4	7.5	2.1	0.5	2.0	8.4
11.95	Laminated Lignite	-26.41	1.8	2.5	0.1	-	60.3	3.5	12.6	1.9	4.6	3.5	7.6	1.4	0.2	3.2	25.0
4.65	Laminated Lignite	-24.77	4.5	1.8	14.3	-	56.5	3.3	9.7	1.2	4.2	3	0.7	0.8	-	0.6	0.1
-1.5	Sand/mud	-25.62	9.6	7.3	20.4	-	45.5	-	10	3.5	2.7	-	1	-	-	0.3	0.4

1168

1169

1170 ^a Detailed description of the lithologies can be found in Collinson et al. (2003) and Steart et al. (2007)

1171 ^b Values from Pancost et al. (2007)

1172 ^c nm =not measured

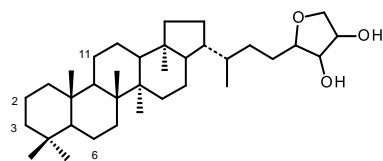
1173 ^d - indicates compound below detection limit.

1174 ^e ratio calculated from raw peak area before adjustment of the anhydroaminotriol value (see section 2.3).

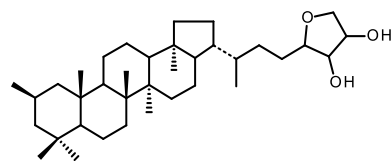
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1176 **Appendix 1.**

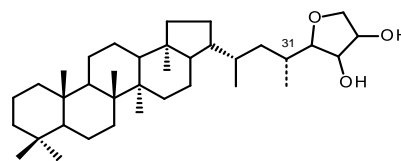
1177 Structures referred to throughout the text and common abbreviated names.



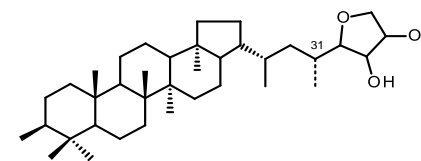
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AnhydroBHT



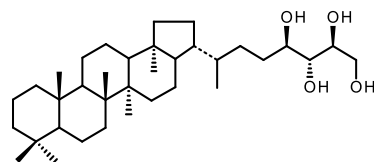
IIa
2-methyl-AnhydroBHT



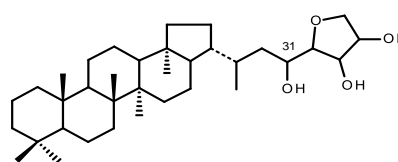
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31-methyl-AnhydroBHT^a



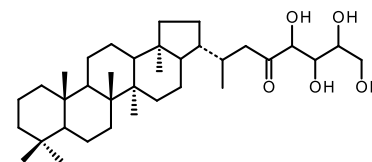
IIIb
3,31-methyl-AnhydroBHT^a



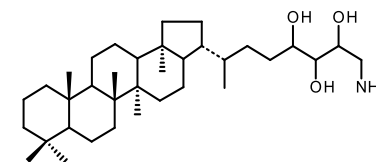
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Bacteriohopanetetrol (BHT)



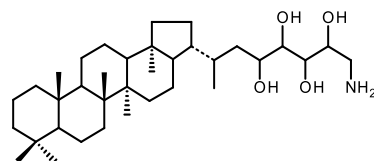
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AnhydroBHpentol



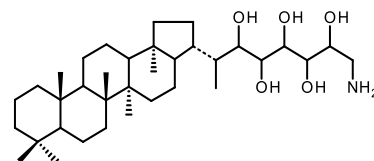
Ie
31-oxo-BHT^b



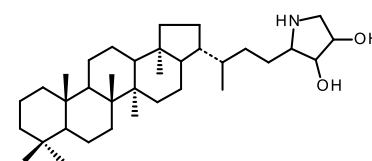
If
Aminotriol



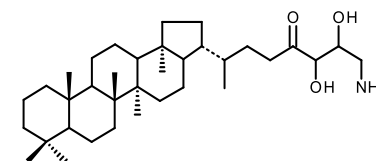
Ig
Aminotetrol



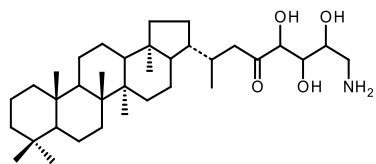
Ih
Aminopentol



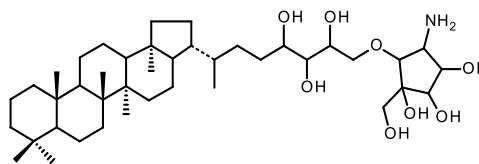
Ii
Anhydroaminotriol



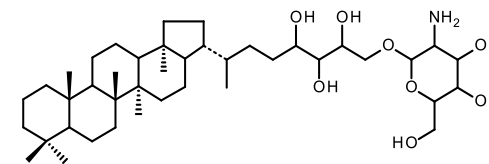
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32-oxo-35-Aminodiol^b



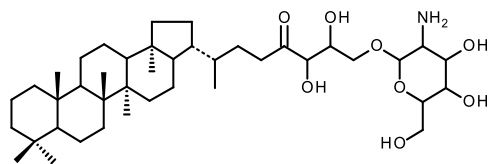
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31-oxo-35-Aminotriol^b



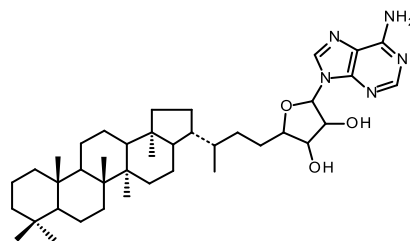
lm
BHT cyclitol ether



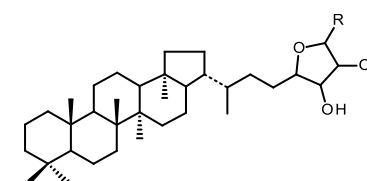
ln
BHT glucosamine



lo
32-oxo-bateriohopanetriol glucosamine



lp
Adenosylhopane



lq
Adenosylhopane-type^c
(R = unknown)

1178

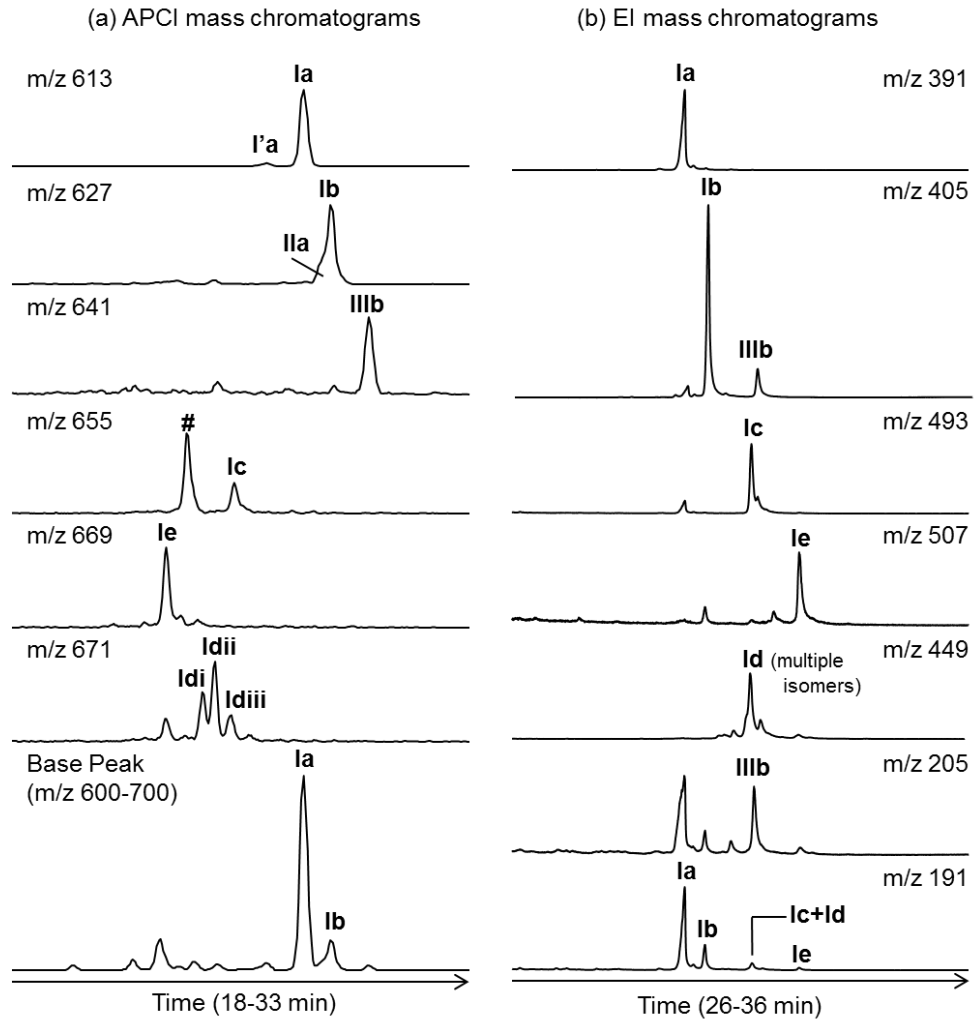
1179 ^a position of methylation at C-31 is tentative but limited to the side chain based on EI spectra (Fig. 3) and indicated at C-31 based
1180 on previous studies (Simonin et al., 1994; Nytoft, 2011)

1181 ^b location of ketone is tentative but is limited to the side chain in **le** based on EI spectrum (Fig. 5)

1182 ^c terminal group structure (R) unknown but can be differentiated from **lp** based on APCI base peak ion and MS² spectrum (Cooke
1183 et al., 2008a; Rethemeyer et al., 2010).

1184

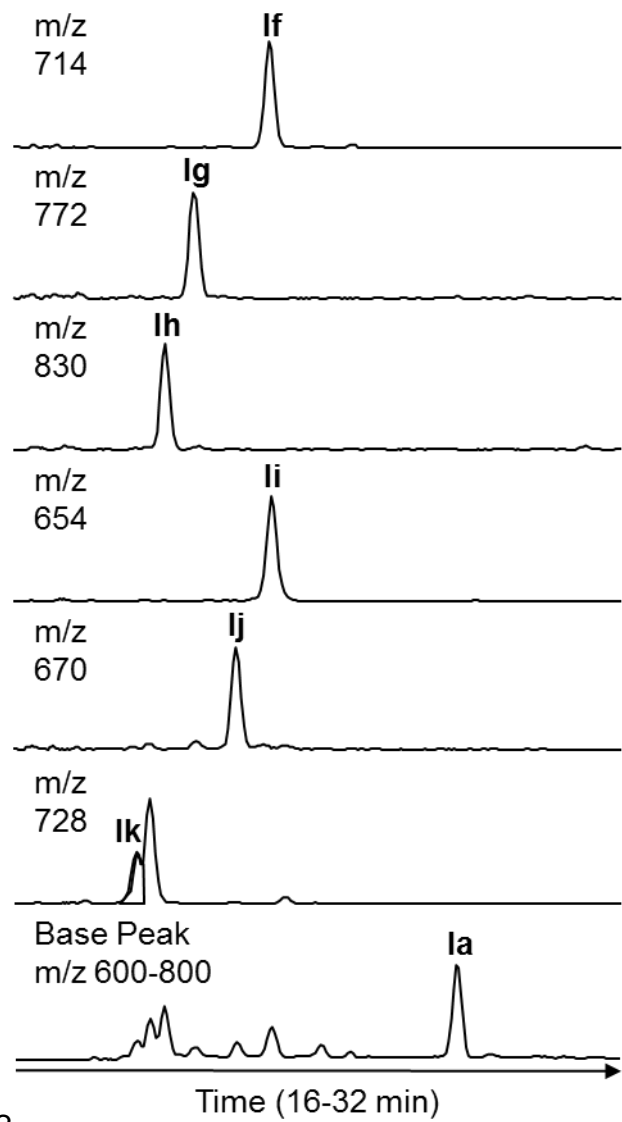
1185



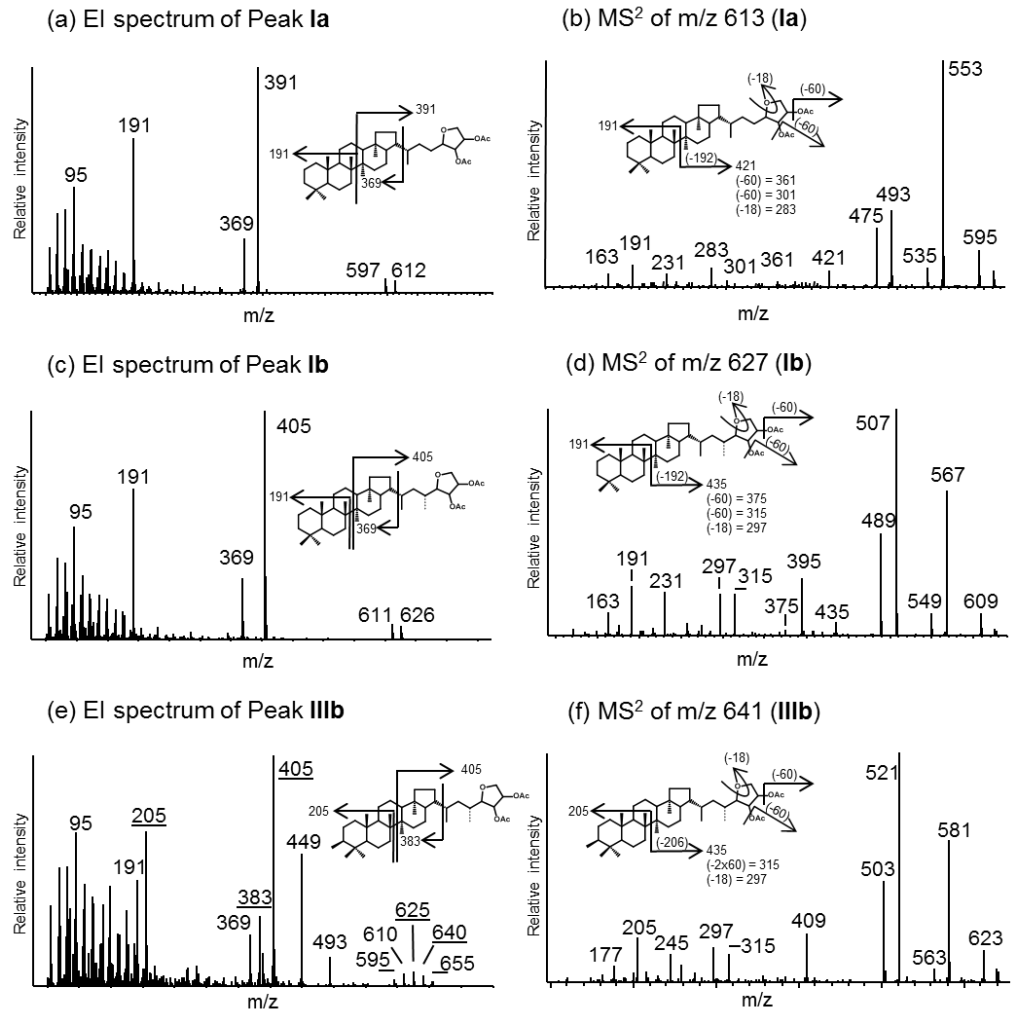
1186

1187 Figure 1

1188



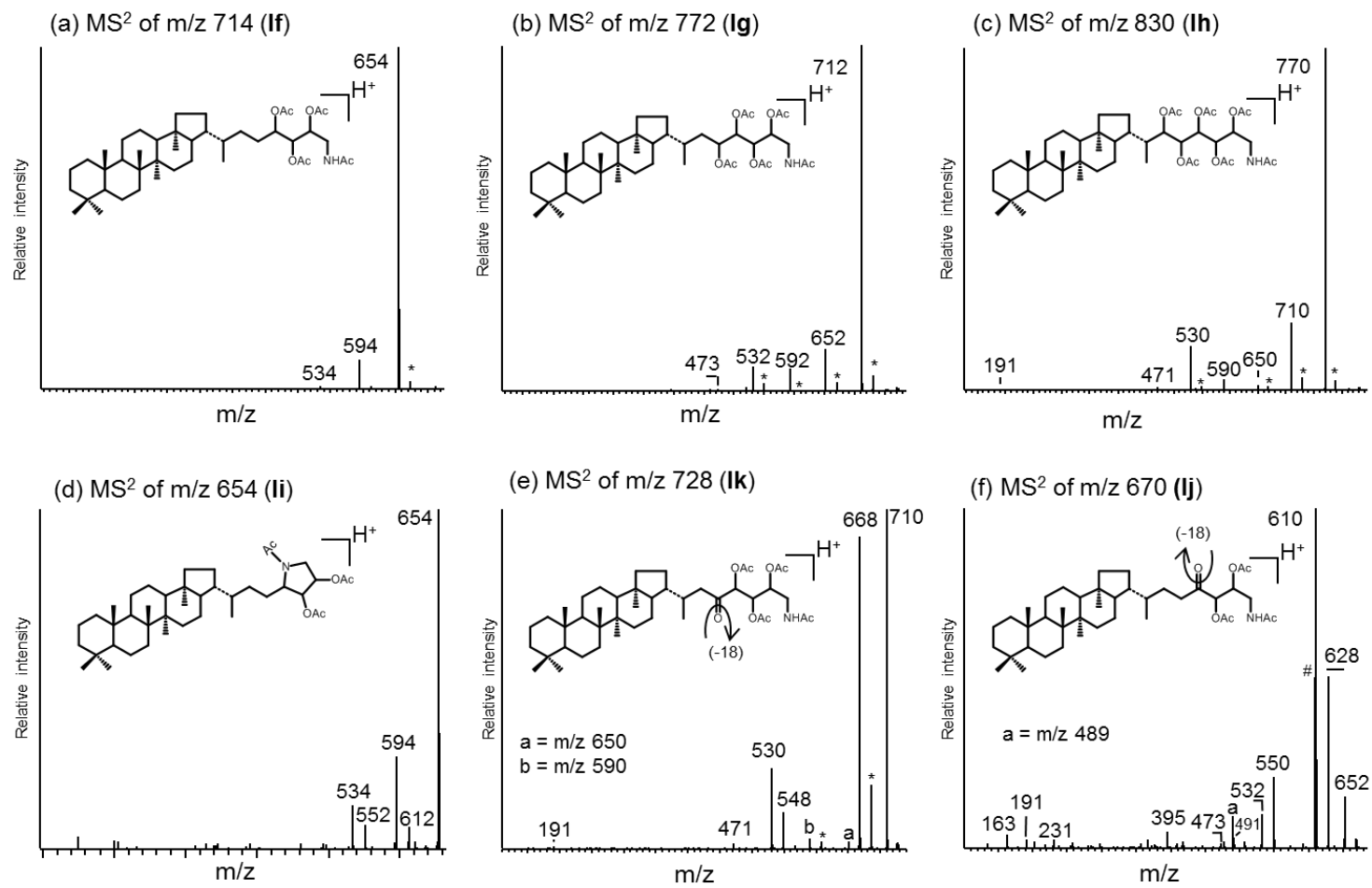
1189 Figure 2



1190

1191 Figure 3.

1192



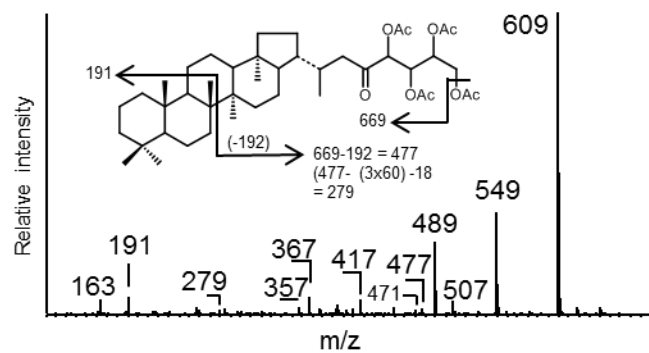
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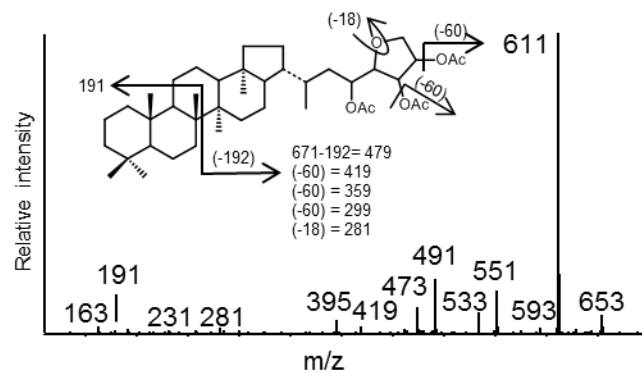
1195 Figure 4.

1196

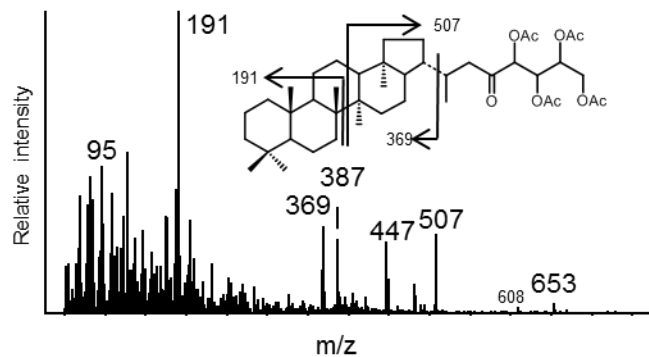
(a) MS² of m/z 669 (peak **le**)



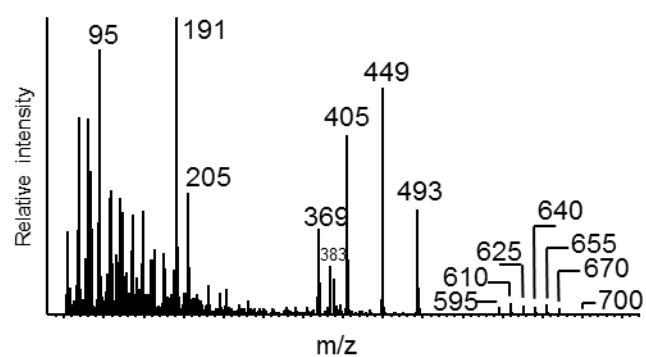
(c) MS² of m/z 671 (peak **Idi**)



(b) EI MS of peak **le**



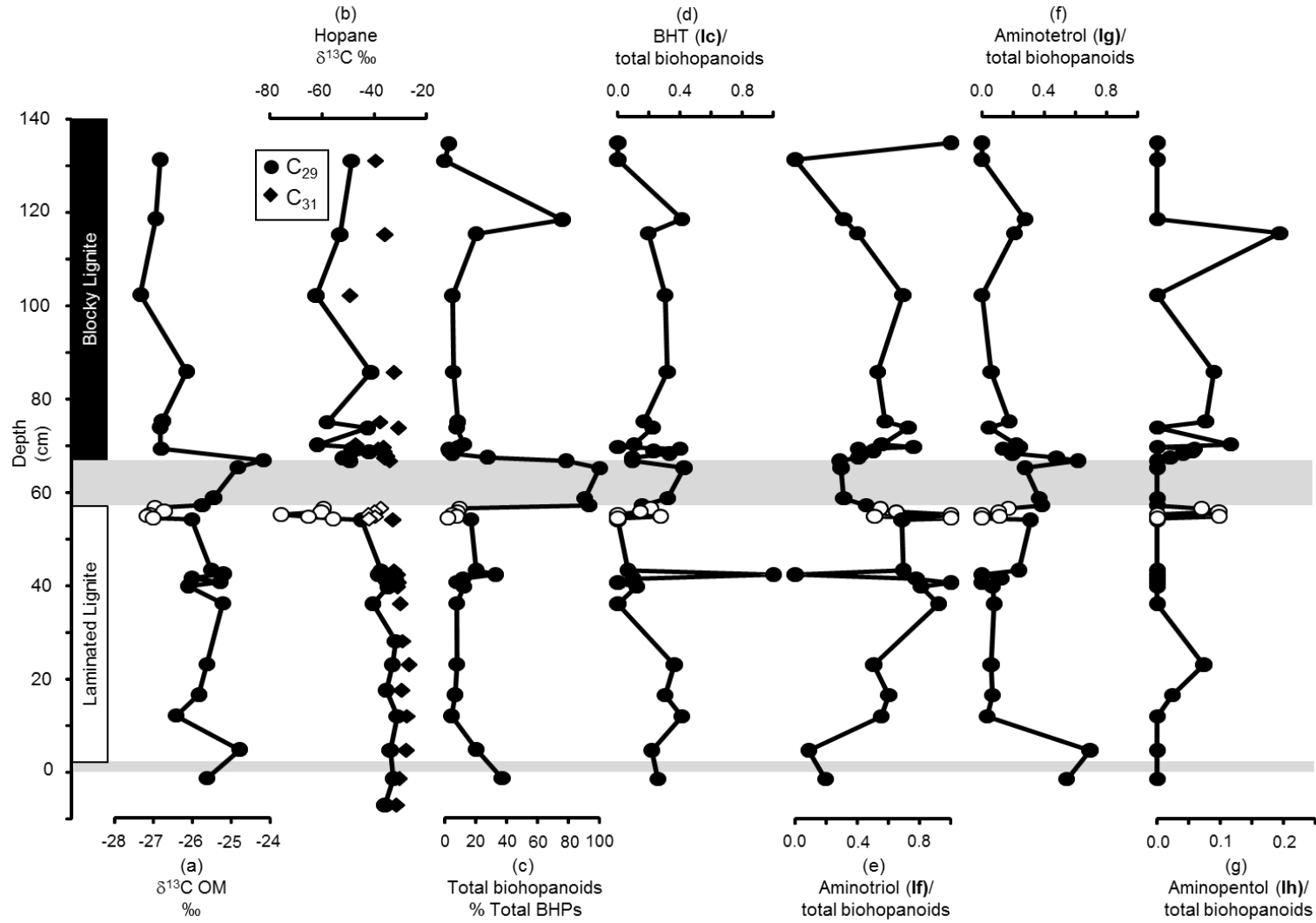
(d) EI MS of peak (**Ic+Id+IIIb**)



1197

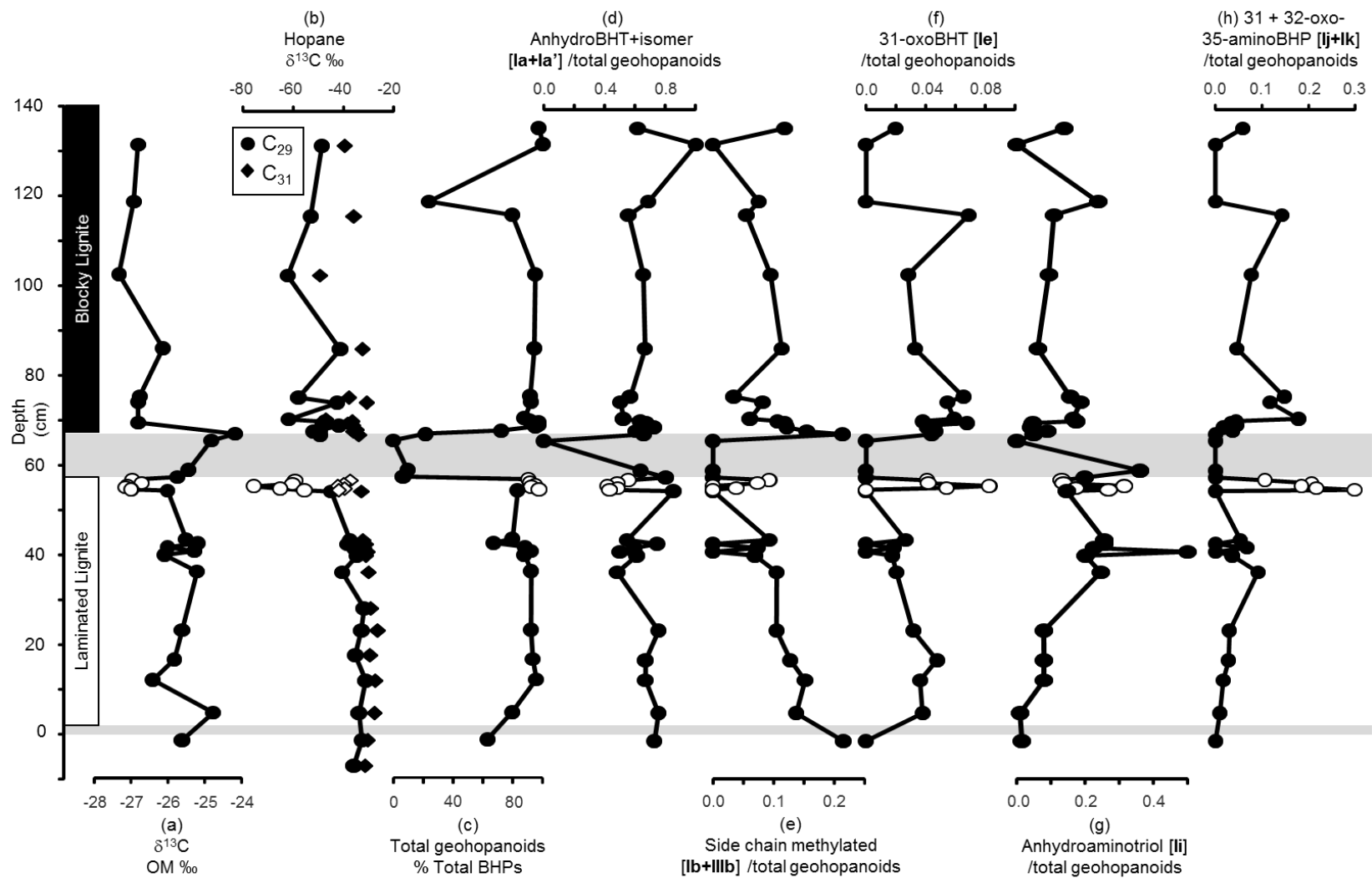
1198 Figure 5.

1199



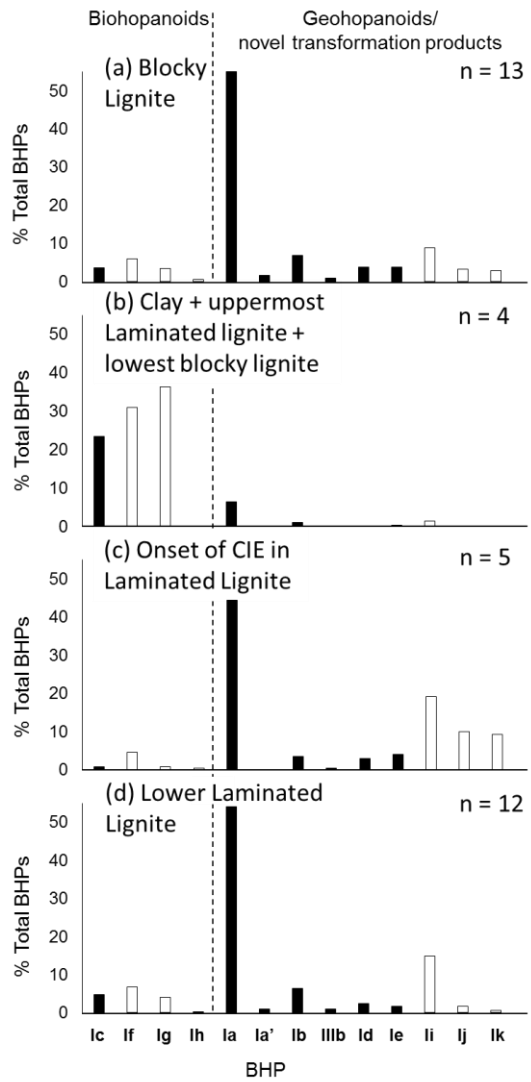
1200

1201 Figure 6.



1202

1203 Figure 7.



1204

1205 Figure 8.