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Philippe Schaeffer, Lucile Bailly, Estelle Motsch, Pierre Adam. The effects of diagenetic aromatization on the carbon and hydrogen isotopic composition of higher plant di- and triterpenoids: evidence from buried wood. Organic Geochemistry, Elsevier, 2019, 136, pp.103889. 10.1016/j.orggeochem.2019.06.010 . hal-02317649

HAL Id: hal-02317649
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Submitted on 16 Oct 2019

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1 # The effects of diagenetic aromatization on the carbon and
2 hydrogen isotopic composition of higher plant di- and
3 triterpenoids: evidence from buried wood

4

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13

14

15 **Abstract**

16 The widely distributed aromatic di- and triterpenoids from higher plants occuring in
17 sediments are formed by diagenetic microbial processes affecting their precursor plant lipids,
18 and these compounds and their stable isotopic composition ($\delta^{13}C$ and $\delta^{2}H$) have the potential
19 to be used for palaeoenvironmental and palaeoclimatic studies. In the present study, the
20 isotopic composition of di- and triterpenoids aromatized to a different extent has been
21 measured to examine the isotopic effects associated with the aromatization reaction. To
22 overcome the possibility of multiple higher plant sources as is generally the case with
23 sedimentary lipids, the $\delta^{13}C$ and $\delta^{2}H$ values of aromatized di- and triterpenoids from lipid
24 extracts recovered from conifer and angiosperm buried wood have been determined, allowing
25 an unambiguous genetic precursor/product relationship to be insured. The results show that
26 the $\delta^{13}C$ compositions of both di- and triterpenes do not seem to be significantly affected by

27 progressive aromatization, whereas the situation is more contrasted with δ^2H values. In the
28 case of diterpenoids related to abietic acid, a significant increase of the δ^2H values by up to 86
29 ‰ with ongoing aromatization was measured. This is in contrast to what is expected for
30 dehydrogenated compounds which should be globally more ^2H-depleted than their precursor
31 molecules (e.g., biosynthesized unsaturated fatty acids vs. their saturated precursors). This ^2H
32 enrichment of aromatized diterpenoids could indicate that they represent only minor residual
33 intermediates, the majority of these intermediates having been further degraded by processes
34 favoring the degradation of the ^1H-containing substrates and having a moderately pronounced
35 $^{12}C/^{13}C$ selectivity. With triterpenoids, a preservation of the δ^2H values was observed
36 whatever the nature and extent of the aromatization process considered, which may be related
37 to enzymatic reactions showing a limited carbon and hydrogen isotopic effect upon
38 aromatization.

39

40 Key words: Diterpenes, Triterpenes, Early diagenesis, Aromatization, Isotopes, Hydrogen,
41 Carbon, Buried wood.

42
43

44 1. INTRODUCTION

45 The stable carbon ($\delta^{13}C$) and hydrogen ($\delta^{2}H$) isotopic composition of *n*-alkyl lipids from
46 plants (*n*-alkanes, *n*-alkanoic acids) in sediments have been actively investigated to acquire
47 plaeoenvironmental and palaeoclimatic information on, notably, the relative contributions of
48 C_3 and C_4 plants (e.g., Huang et al., 2001; Liu et al., 2005; Tanner et al., 2007; Agrawal et al.,
49 2014; Diefendorf and Freimuth, 2017; Rao et al., 2017) or on the reconstruction of past
50 precipitation regimes or temperatures (e.g., Sauer et al., 2001; Sachse et al., 2004; Garcin et
51 al., 2012; Diefendorf and Freimuth, 2017; Freimuth et al., 2017; Rao et al., 2017). The same
52 palaeoenvironmental and palaeoclimatic information may potentially be provided by the
53 determination of the $\delta^{13}C$ and $\delta^{2}H$ compositions of di- and triterpenic biomarkers from plants
54 in sediments. However, contrary to straight-chain lipids which occur in all plant species, di-
55 and triterpenes can sometimes be associated to a specific plant familly or plant species (e.g.,
56 Jacob et al., 2008; Le Milbeau, 2010; Schnell et al., 2012, 2014). Investigation of their carbon
57 and hydrogen isotopic composition might therefore potentially provide additional and more
58 accurate information than that obtained from less or non-specific plant constituents. However,
59 terpenoids generally undergo alteration processes upon diagenesis, such as aromatization
60 which affects the structure of their genuine carbon skeleton and results in the loss of carbon
61 and hydrogen atoms (Spyckerelle et al., 1977; Laflamme and Hites, 1979; Wakeham et al;,
62 1980; Wolff et al., 1989; Stout, 1992; Simoneit et al., 1986; Le Milbeau et al., 2010;
63 Nakamura et al., 2010; Schnell et al., 2012, 2014). Since cleavage of several C-C and C-H
64 bonds might be associated to important carbon and hydrogen isotopic effects, a key question
65 arises whether the $\delta^{13}C$ and $\delta^{2}H$ values of aromatized terpenoids keep or not the original
66 isotopic composition of their biological precursor molecules. This point is of particular
67 interest for studies in which the stable isotopic signatures of lipids are used. In addition,
68 investigation of the carbon and hydrogen isotopic effects associated with aromatization might

69 also potentially give information about the mechanisms involved in these processes which
70 remain largely unknown.

71 To our knowledge, there are only two studies dealing specifically with the impact of early
72 diagenetic processes on the carbon (Freeman et al., 1994; Jacob et al., 2011) or hydrogen
73 (Jacob et al., 2011) isotopic compositions of sedimentary aromatized triterpenoids from
74 angiosperms and one dedicated to the aromatisation processes affecting diterpenoids in coals
75 and mustones (Tuo et al., 2006). Nevertheless, comparison of the $\delta^{13}C$ or $\delta^{2}H$ values of di- or
76 triterpenoids aromatized to a different extent may occasionaly be found in articles not
77 specifically dealing with the impact of aromatization processes on carbon and hydrogen
78 isotopic composition, this aspect being generally not discussed (e.g., Schouten et al., 2007;
79 Tuo et al., 2003; Nakamura et al., 2010; Suzuki et al., 2010). The main problem while
80 investigating isotopic fractionation during aromatization of terpenes resides in the fact that a
81 given aromatized terpenoid within a sediment sample might originate from mixed plant
82 sources. It results that the $\delta^{13}C$ and $\delta^{2}H$ differences between the aromatized intermediate
83 molecules within this sample might be due to fractionation effects associated with the
84 aromatisation process itself, but may also be related to source effects (i.e., contribution of
85 multiple source organisms with different isotopic compositions). To overcome this problem,
86 we have investigated the case of aromatized terpenoids within ancient buried woods samples.
87 They indeed represent ideal candidates to study the effects of early diagenetic aromatization
88 on carbon and hydrogen isotopic compositions of di- and triterpenoids since all the terpenoid
89 intermediates from a given buried wood sample must be unambiguously genetically related.
90 We report here the determination and possible significance of the variations of the $\delta^{13}C$ and
91 $\delta^{2}H$ signatures of aromatic terpenoids from buried wood samples comprising conifer wood
92 (*Pinus sylvestris*) investigated for diterpenoids, and *Quercus robur* and *Alnus sp.* wood for
93 triterpenoids.

94

95 ## 2. MATERIALS AND METHODS

96 ### 2.1. Samples

97 Three buried wood samples were collected and analysed for their terpenoid content. They
98 comprise:

99 Sample 1: Piece of trunk of *Pinus sylvestris* buried under a mudslide (age : > 4000 BP; SE
100 France).

101 Sample 2 (cf. Schnell et al., 2014): Inner part of an oak trunk (heartwood) unearthed from a
102 palaeochannel of the Rhine river (^{14}C age: ca. 3700 BP; Gerstheim, NE France).

103 Sample 3 (cf. Schnell et al., 2014; Adam et al., 2014): Piece of alder heartwood (*Alnus sp.*)
104 from a palaeochannel of the Rhine river (^{14}C age: ca. 3700 BP; Gerstheim, NE France).

105 ### 2.2. GC-MS

106 GC-MS analyses were performed on a Thermo Scientific Trace Ultra gas chromatograph
107 coupled to a Thermo Scientific TSQ Quantum mass spectrometer equipped with a Tri Plus
108 autosampler and a programmed temperature vaporizing (PTV) injector. MS conditions were:
109 source 220 °C, electron ionization (EI) at 70 eV and scanning *m/z* 50 to 700. GC conditions:
110 HP5-MS column (30 m x 0.25 mm, 0.1 µm film thickness) using He as carrier gas (constant
111 flow, 1.1 ml/min); oven program: 70 °C-200 °C (10 °C/min), then to 300 °C (held 40 min) at
112 4 °C/min).

113 ### 2.3. GC-irmMS

114 GC-irmMS measurements were carried out on a Delta V Plus mass spectrometer (Thermo
115 Scientific) coupled to a GC Trace GC Ultra gas chromatograph equipped with a Triplus

116 autosampler, an on-column injector, and an Agilent HP5-MS column (30 m x 0.25 mm i.d. x
117 0.1 μm film thickness), and connected to a ConFlow IV interface system and a GC Isolink II
118 conversion unit, comprising a combustion oven at 1000 °C for $\delta^{13}C$ measurements (resp. a
119 pyrolysis oven at 1420°C for $\delta^{2}H$ measurements). The temperature program was: 80 °C – 310
120 °C (4°C/min) – isothermal at 310 °C (40 min). Each analysis was repeated 3 x. Before and
121 after each triplicate, the carbon (resp. hydrogen) isotopic composition of a certified *n*-alkane
122 mixture (Type A5; Arndt Schimmelmann, Biogeochemical Laboratories, Indiana University,
123 USA) was measured and used for calibration. The stability of the measurements was checked
124 using pulses of reference CO_2 (resp. H_2) prior (5 pulses) and after (3 pulses) each run. H_3^+
125 factor was determined every day. The data were analyzed using Isodat 3.0 software. For
126 correction of the $\delta^{13}C$ and $\delta^{2}H$ values of derivatized lipids, see section 2.7.

127 ## 2.4. Extraction and fractionation of lipids from the pine wood sample

128 The buried pine tree wood sample was extracted with CH_2Cl_2/CH_3OH (1:1 v/v) according to
129 the procedure described by Schnell et al. (2014). 975 mg of total lipid extract (TLE) were
130 obtained from 5.52 g of wood, an aliquot of which (240 mg) being derivatized (1.
131 Ac_2O/Pyridine 1:1 v/v; 2. esterification with a solution of diazomethane in Et_2O, cf. Schnell et
132 al., 2014). The derivatized TLE was fractionated on a silica gel column yielding three
133 fractions eluted respectively with CH_2Cl_2/EtOAc (8:2 v/v) (F1, 168 mg; 2 x dead volume -D_v-
134); CH_2Cl_2/AcOEt (F2, 20.5 mg; 2 x D_v), and CH_2Cl_2/CH_3OH (1:1 v/v) (F3; 22.5 mg; 2 x D_v).
135 Fraction F1 was analysed by GC-MS.

136 Besides, the remaining underivatized extract (ca. 730 mg) was fractioned on a silica gel
137 column (D_v: 33 ml) to isolate diterpenoid hydrocarbon fractions for determination of their
138 carbon and hydrogen isotopic compositions. Eight fractions were recovered eluting,
139 respectively with cyclohexane (F’1 to F’5, 25 ml each), cyclohexane/CH_2Cl_2 (1:1 v/v, F’6 and

140 F'7, 25 and 30 ml respectively) and CH_2Cl_2/CH_3OH (1:1 v/v) (F'8; 50 ml). Fractions F'1-F'5
141 were analysed by GC-MS and GC-irmMS. Fraction F'8 was mainly composed of
142 tetrahydroabietic acid **1** and was further purified on a silica gel column using CH_2Cl_2/EtOAc
143 (95:5; v/v) as eluent, yielding almost pure tetrahydroabietic acid **1**. The latter was silylated
144 (section 2.7) prior analysis by GC-MS and GC-irmMS.

145 2.5. Fractionation of lipids from the oak wood sample

146 The sample from buried oak wood was extracted as described by Schnell et al. (2014). An
147 aliquot of the crude extract (40 mg) was fractionated on a silica gel column using CH_2Cl_2 as
148 eluent yielding, respectively, fraction F1 (11 mg; 3 D_v) and a more polar fraction F2 (5 mg; 3
149 D_v).

150 2.6. Fractionation of lipids from the alder wood sample

151 A moderately polar fraction (18 mg) obtained from a buried alder wood sample following a
152 procedure involving extraction, derivatization and elution with CH_2Cl_2/EtOAc (8:2 v/v) on a
153 silica gel column (cf. Adam et al.,2014) was further fractionated on a silica gel column using
154 successively cyclohexane/CH_2Cl_2 (8:2; v/v) and EtOAc/CH_2Cl_2 (8:2; v/v) as eluents, yielding,
155 respectively, an apolar fraction F1 (1.8 mg) containing the aromatic hydrocarbons which was
156 further analyzed by GC-MS and GC-irmMS and a more polar fraction F2 (16 mg) not further
157 investigated.

158 2.7. Derivatization

159 Fractions containing alcohols, phenols or carboxylic acids were derivatized prior GC-MS and
160 GC-irmMS analysis using pyridine (40 µl) and *bis*-(*N,O*-trimethylsilyl)trifluoroacetamide
161 (BSTFA; 150 µl, 70 °C, 2 h) having a known carbon and hydrogen isotopic composition of
162 the trimethylsilyl group ($\delta^{13}C$: -41.14 ‰; $\delta^{2}H$: -154.09 ‰). The solvent and excess reagent

163 were removed under a stream of N_2. δ^2H and $\delta^{13}C$ values of derivatized biomarkers were
164 corrected according to Rieley (1994) for the added trimethylsilyl group.

165 3. RESULTS AND INTERPRETATIONS

166 3.1. Diagenetic aromatization of diterpenoids

167 GC-MS analysis of the organic extract of the sample of buried *Pinus sylvestris* (Figure 1)
168 revealed the presence of diagenetic transformation products of diterpenoid acids from the
169 abietane and pimarane series such as tetrahydroabietic acid **1**, monounsaturated derivatives of
170 abietic and pimaric acids (**2**, **3**) and dehydroabietic acid **4**. The former was the predominant
171 constituent of the fraction and is considered as a typical microbial reduction product formed
172 under anaerobic conditions (Reunanen et al., 1990; Bailly et al., 2016). Beside diterpenic
173 acids, various saturated (fichtelite **5**), monounsaturated (**6**), and aromatic hydrocarbons such
174 as 18-norabietatriene **7**, tetrahydroretene **8** and retene **9** were detected. Such diterpenic
175 structures are frequently encountered in sediments with a significant contribution of biomass
176 originating from conifers (e.g., Wakeham et al., 1980; Simoneit et al., 1986; Hautevelle et al.,
177 2006; Tuo et al., 2006; Nakamura et al., 2010; Suzuki et al., 2010). The aromatic compounds
178 **7-9** are postulated to be the result of microbial aromatization processes operative under anoxic
179 conditions (Tavendale et al., 1997a,b; Martin et al., 1999).

180 For the investigation of the isotopic effects associated with aromatization processes, we have
181 measured the carbon and hydrogen isotopic composition of the aromatic compounds **7**, **8**, and
182 **9** after chromatographic separation of the total hydrocarbons from the TLE (see below). For
183 comparison, the δ^2H and $\delta^{13}C$ values of partly reduced compounds such as tetrahydroabietic
184 acid **1**, one monounsaturated tricyclic hydrocarbon **6** and fichtelite **5** have been measured.
185 This allowed us to evaluate specifically the influence of decarboxylation on the carbon
186 isotopic composition of diterpenes (comparison between **1** and **5**). Because of partial

187 coelutions in GC, determination of the δ^2H and δ^{13}C values of the various diterpenoids
188 discussed above could not be performed directly on the lipid fraction shown in Figure 1.
189 Therefore, further liquid chromatography fractionation was carried out, leading to several sub-
190 fractions enriched in the diterpenoids of interest and allowing the δ^2H and δ^{13}C compositions
191 of compounds **1** and **5-9** to be determined.

192 The results show that the carbon isotopic composition of the diterpenoids is retained,
193 independently of the diagenetic alteration process considered (decarboxylation, reduction, and
194 aromatization) (Figure 2). On the contrary, the δ^2H signatures seem to be strongly affected by
195 these processes, which apparently result in a progressive increase of the δD values with
196 ongoing aromatization (from -238 ‰ for 18-norabietatriene **7** to -152 ‰ for retene **9**). A
197 similar observation was made by Tuo et al. (2006) in the case of diterpenoids occurring in a
198 series of mudstone samples from the Liaohe Basin (China). Thus, the δ^2H values determined
199 for dehydroabietane **10** by Tuo et al. (2006) were generally lower than those measured for the
200 related diaromatic diterpenoid simonellite **11** within the same samples, except in one case.

201 Hydrogenation processes affecting abietic acid and leading to the formation of
202 tetrahydroabietic acid **1**, hydrocarbon **6** and fichtelite **5** in our wood sample (Figure 2) are
203 likely induced by microorganisms, which might explain the slightly lower δ^2H values (ca. 25
204 ‰ difference) between the hydrogenated compounds **1** and **5** and their unsaturated
205 counterpart (**6**). The D-depletion of the hydrogenated compounds can be explained by the fact
206 that the biological reactions involved in the reduction processes indeed favor the
207 incorporation of ^{2}H-depleted hydrogen. Such a situation has been observed, for instance, by
208 Chikaraishi et al. (2009) for the biosynthesis of phytol from geranyl geraniol. By contrast, the
209 progressive increase of the δ^2H values observed during aromatization of 18-norabietatriene **7**

210 (-238 ‰) to retene **9** (-152 ‰) is more difficult to interpret and will be discussed in detail in
211 section 3.3.1.

212 3.2. Diagenetic aromatization of triterpenoids

213 3.2.1. *Quercus robur*

214 The triterpenoids recovered from the inner part of buried trunk of *Q. robur* unearthed from a
215 palaeochannel of the Rhine (NE, Gerstheim; France) have been investigated. A detailed
216 molecular analysis of the sample can be found in Schnell et al. (2014). The predominant
217 aromatized triterpenoids identified (**12-17**) bear an oxygen functionality at C-2 (Le Milbeau et
218 al., 2010; Schnell et al., 2012, 2014). These compounds have inherited this uncommon
219 structural feature from the predominant biological triterpenoid precursors **18** and **19**
220 functionalized at both C-3 and C-2 which occur in fresh *Q. robur* wood. They result from
221 aromatization processes starting in ring *D* from the triterpenoid skeletons and progressing
222 towards ring *A* (Figure 3). Two chromatographic fractions, one enriched in the two
223 monoaromatic ketones **14-15** and tetraaromatic phenols **16-17** (Figure 4a) and the other
224 dominated by the alcohols **12** and **13** (Figure 4b) could be separated from the lipid extract of
225 the oak wood sample obtained according to the procedure described in Schnell et al. (2014).
226 These compounds correspond to two triplets of unambiguously genetically related
227 triterpenoids (Figure 3) which are ideal candidates for the evaluation of the effects of
228 aromatization processes on both carbon and hydrogen isotopic compositions. The $\delta^{13}C$ and
229 $\delta^{2}H$ values of triterpenoids **12-17** are given in Figure 3. Surprizingly, despite the loss of up to
230 six methyl groups from the precursor molecule **19** to its tetraaromatic phenol counterpart **17**,
231 neither the carbon, nor the hydrogen isotopic compositions of the different intermediates seem
232 to be significantly affected by these aromatization processes progressing from ring *D* to ring
233 *A*.

234 *3.2.2. Alnus sp.*

235 For comparison, the impact of aromatization processes on the carbon and hydrogen isotopic
236 compositions during aromatization of C-3-oxygenated triterpenes (as opposed C2,C3-
237 difunctionalized precursor molecules) following the "classical" pathway starting from ring *A*
238 and progressing towards ring *D* (e.g., Wolff et al., 1989; Stout, 1992) has been investigated.
239 For that purpose, the carbon and hydrogen isotopic composition of aromatic triterpenoid
240 hydrocarbons related to lupanol **20** and β-amyrin **21** occurring in the organic extract of *Alnus*
241 *sp.* wood found at the same site as the oak wood sample have been determined. A detailed
242 analysis of the lipid extract of this sample is presented in Schnell et al. (2014) and Adam et al.
243 (2014). The partial gas chromatogram of the aromatic hydrocarbon fraction focusing on the
244 pentacyclic triterpenoids is presented in Figure 5. They comprise mono- (**22**) (Wolff et al.,
245 1989), tri- (**23**, **24**) and tetraaromatic (**25**) triterpenoids (Spyckerelle et al., 1977; Laflamme
246 and Hites, 1979; Wakeham et al., 1980; Chaffee & Fuchs, 1988), all being closely genetically
247 related (Figure 6). Comparison of the δ^2H and $\delta^{13}C$ values of the triterpenes from both
248 oleanane and lupane series showed that the hydrogen and carbon isotopic are not significantly
249 affected by the degree of aromatization, as was observed with the triterpenoids from the oak
250 sample (see above). Freeman et al. (1994) obtained similar results for carbon isotopes in the
251 case of aromatized triterpenoids occurring in Eocene oil shale samples (Messel formation,
252 Germany). In this case, however, the genetic relationship between the various aromatized
253 higher plant triterpenoids from the same series could not be guaranteed since the triterpenes
254 likely originated from multiple higher plant sources. Regarding $^2H/^1H$ isotopes, there is only
255 one study which is devoted to the comparison of the δ^2H composition of triterpenoids
256 aromatized to a different extent in sediments (Jacob et al., 2011). In this study, important
257 variations of up to 100 ‰ could be observed between potential triterpenoid precursors and
258 related aromatized compounds, a difference which we did not observe in our study. However,

259 as for the study of Freeman et al. (1994), the authors were dealing with triterpenoids extracted
260 from sediments, and for which multiple plant sources with different hydrogen isotopic
261 compositions can be expected.

262 3.3. Evolution of isotopic compositions upon aromatization

263 Early diagenetic aromatization processes observed in buried wood are triggered by anaerobic
264 microbial processes (e.g., Tavendale et al., 1997a,b; Harder and Foß, 1999; Martin et al.,
265 1999) as demonstrated by incubation experiments involving either diterpenic acids
266 (Tavendale et al., 1997a,b) or triterpenoids (e.g., Trendel, 1985; Lohmann, 1988; Lohmann et
267 al., 1990). Therefore, since the formation of aromatized di- and triterpenoids is biologically-
268 mediated, one could envisage that the carbon and hydrogen isotopic shifts induced by these
269 processes have some analogies with those from enzymatic reactions involved in the
270 dehydrogenation of fatty acids via desaturases (e.g., Behrouzian et al., 2001; Buist, 2004;
271 Buist and Behrouzian, 1998; Savile et al., 2001; Shanklin et al., 2009) or in the aromatization
272 of androgen steroids via aromatases (e.g., Simpson et al., 1994; Akhtar et al., 2011). Indeed,
273 similarly to the aromatization processes leading from sterols to aromatic steroid hormones
274 such as estradiol, the aromatization of diterpenoids and triterpenoids involves the loss of
275 angular methyl groups. Like for the aromatization of the ring *A* of androgen steroids,
276 terpenoid aromatization might proceed via the functionalization of the angular methyl groups.
277 Hydrogen isotopic effects related to the dehydrogenation reactions or loss of methyl groups
278 catalyzed by the enzymes involved have often been investigated in the frame of studies aimed
279 at elucidating their mechanisms, but the carbon isotope effects associated with these reactions
280 are generally not documented. Thus, for instance, in the case of fatty acid desaturases, the
281 hydrogen kinetic isotope effect has been shown to be strongly dependent on the class of
282 enzymes involved. This effect can be important in the case of membrane-bound desaturases
283 (e.g., Savile et al., 2001; Buist and Behrouzian, 1998), but is sometimes negligible with

284 soluble plant desaturases (e.g., Behrouzian et al., 2001; Buist, 2004). It should however be
285 noted that the enzymes involved in these biosynthetic pathways bear iron at their active site
286 and are O_2-dependent (Shanklin et al., 2009; Akhtar et al., 2011), being able to activate C-H
287 bonds aerobically. Therefore, since the early diagenetic aromatization of di- and triterpenes
288 apparently only occur under anaerobic conditions according to incubation experiments
289 (Trendel, 1985; Lohmann, 1988; Lohmann et al., 1990; Tavendale et al., 1997a,b; Harder and
290 Foß, 1999; Martin et al., 1999), it is likely that the enzymatic processes initiating anaerobic
291 aromatization are significantly different and comprise C-H bond cleavage in the absence of
292 oxygen following different pathway(s). These pathways possibly involve the formation of a
293 radical by abstraction of an hydrogen atom as has been described for the enzymes responsible
294 for the biodegradation of hydrocarbons under strictly anaerobic conditions (e.g., Spormann
295 and Widdel, 2000; Buckel and Golding, 2006; Heider, 2007; Booker, 2009). Nevertheless,
296 regardless of the enzymatic pathway(s) involved (aerobic vs. anaerobic), the isotopic
297 selectivity in the initiation step of the aromatization processes can probably be expected to be
298 similar in aerobic and anaerobic processes and to favor, globally, light isotopes (i.e., ^{1}H and
299 ^{12}C vs. ^{2}H and ^{13}C).

300 3.3.1. Hydrogen isotopic composition

301 In the case of fatty acids biosynthesized by some marine macroalgae, it has been observed by
302 Chikaraishi et al. (2004) that the $\delta^{2}H$ values of saturated fatty acids are significantly increased
303 compared to their unsaturated metabolites, which is probably related to the important
304 hydrogen isotopic effect associated to dehydrogenation reactions induced by membrane-
305 bound desaturases (e.g., Savile et al., 2001; Buist and Behrouzian, 1998). In the case of
306 sequential dehydrogenation processes such as those leading from saturated fatty acids to
307 mono- di-, tri and tetra-unsaturated fatty acid analogues, the evolution of the $\delta^{2}H$ values of the
308 intermediates was more difficult to predict. According to Chikaraishi et al. (2004), it appears

309 that these processes depend on the relative fluxes into the various unsaturated products and on
310 the conversion rates, since each dehydrogenation step affecting an intermediate is supposed to
311 result in a ^{2}H-enrichment of this intermediate relative to the product formed. Similar
312 observations have been made when the hydrogen isotopic composition of alkenones was
313 compared to that of their related dehydrogenated products (D'Andrea et al., 2007; Schwab
314 and Sachs, 2009). It should, however, be noted that Zhang and Sachs (2007) observed a
315 different trend for the biosynthesis of C_{18} unsaturated fatty acids from stearic acid by the
316 Chlorophyceae *Eudorina unicocca* and *Volvox aureus*. With these organisms, the δ^{2}H values
317 measured for the C_{18} unsaturated fatty acids were similar or slightly higher than those
318 determined for stearic acid. These authors explained this observation by the fact that the
319 desaturases involved in the biosynthesis of unsaturated fatty acids in these organisms belong
320 to the so called "soluble" desaturases. As mentioned above, the action of these enzymes was
321 indeed shown to induce only very limited isotope effects (e.g., Behrouzian et al., 2001; Buist,
322 2004). In our case, the absence of a significant evolution of the δ^{2}H value of the triterpenoids,
323 whatever the series considered (i.e., lupane vs. oleanane), the aromatization process (i.e., from
324 ring *A* to ring *D* or ring *D* to ring *A*; Figure 3 and Figure 6, respectively), and aromatization
325 progress (i.e., from mono- to tetraaromatic derivatives), might thus be explained by the fact
326 that the ^{1}H/^{2}H isotopic effect related to the bacterial enzymes involved in these aromatization
327 processes operating under oxygen-depleted conditions is limited, similarly to the "soluble"
328 desaturases reported from plants.

329 In the case of the aromatized diterpenoids from *Pinus silvestris*, the significant increase of the
330 δ^{2}H values observed with increasing aromatization is more difficult to explain in this context.
331 One would indeed expect the aromatized diterpenes to have lower δ^{2}H values or, as observed
332 in the case of the triterpenoids, almost similar δ^{2}H values than their precursor molecules. The
333 present observation can only be explained if the aromatized diterpenoids identified

334 correspond to minor residual intermediates which have survived overall degradation processes
335 that led to further degradation products not detected in our study or to complete
336 remineralization. Such degradation processes would indeed most likely strongly favor the
337 degradation of ^{1}H-enriched substrates, resulting in an increase of the δ^{2}H values of the
338 residual aromatic diterpenes. A similar δ^{2}H trend has been reported in the case of the
339 anaerobic biodegradation of aromatic hydrocarbons such as toluene by denitrifying, sulfate-
340 reducing and fermenting bacteria using the succinate pathway (e.g., Morash et al., 2001;
341 Meckenstock et al., 2004; Vogt et al., 2008). In this connection, it should be noted that the
342 δ^{2}H values determined by Tuo et al. (2006) for dehydroabietane **10** in a series of mudstone
343 samples from the Liaohe Basin were generally lower (except in one case) than those measured
344 for the related diaromatic diterpenoid simonellite **11** within the same samples. This overall
345 trend was thus close to that observed in our sample. Tuo et al. (2006) nevertheless proposed
346 another explanation for this trend, arguing that dehydrogenation processes most likely lead to
347 a ^{2}H-enrichment of the products relative to the substrate, since hydrogen is preferentially
348 removed during such processes. This is, however, not the case as illustrated by the evolution
349 of the δ^{2}H values of unsaturated lipids formed by dehydrogenation processes induced by
350 desaturases as compared to that of their saturated precursors (e.g., Chikaraishi et al., 2004;
351 D'Andrea et al., 2007; Schwab and Sachs, 2009). When a dehydrogenation reaction occurs,
352 the increase of the δ^{2}H value of the remaining substrate relative to that of the unsaturated
353 product formed is due to the fact that the hydrogen isotopic effect mainly affects the hydrogen
354 atom at the C-H position involved in the in the first C-H cleavage reaction (e.g. Buist and
355 Behrouzian, 1996, 1998), this hydrogen atom involved in the desaturation being finally lost.
356 The δ^{2}H value of the hydrogen atoms from the unsaturated product (or aromatized compound,
357 in our case) formed should thus be almost identical to that of the substrate at the beginning of
358 the reaction, whereas that of the remaining unreacted substrate should progressively increase

359 (Figure 7). In the case of cascade reactions as observed for the diagenetic di- and
360 triterpenoids, it is of importance to note that each intermediate also corresponds to the
361 substrate of the next reaction, the same type of ^{2}H isotope fractionation applying hence at each
362 step. Furthermore, at each step, the extent of ^{2}H isotopic fractionation will depend on the yield
363 of the reaction, the δ^{2}H composition of the substrate increasing with increasing yield relative
364 to the product(s) formed. Therefore, given these considerations, it appears that the
365 interpretation of the ^{1}H/^{2}H signatures of biomarkers formed by diagenetic processes are
366 extremely difficult to interpret in the case of palaeoenvironmental studies (e.g., past climatic
367 changes, palaeohydrology).

368 3.3.2. Stable carbon isotopic composition

369 The results show that the carbon isotopic compositions of both di- and triterpenes do not seem
370 to be significantly affected by progressive aromatization, regardless of the nature of the
371 substrate (di- vs. triterpenes), of the process involved (triterpene aromatization progressing
372 from ring *A* to ring *D* or from ring *D* to ring *A*) and of the number of carbon atom(s) lost
373 during the process (with up to 6 methyl groups lost in the case of tetraaromatic triterpenoids).
374 This suggests that the kinetics of the enzymatic processes for aromatization are not sensitive
375 to carbon isotopic effects. In the same way as that proposed to explain the evolution of the
376 δ^{2}H signatures of terpenoids in relation with intense alteration processes (see above), one
377 might envisage that these degradation processes might potentially also lead to an increase of
378 the δ^{13}C values of the residual, undegraded aromatic terpenes. However, such a trend has not
379 been observed, and might be due to the fact that anaerobic biodegradation processes affecting
380 aromatic hydrocarbons can be significantly more sensitive to hydrogen isotopes than to
381 carbon isotopes, at least in some cases, as demonstrated for the anaerobic biodegradation of
382 toluene (e.g., Morash et al., 2001; Meckenstock et al., 2004; Vogt et al., 2008) or with
383 diterpenes (the present study).

384

385 ## 4. CONCLUSIONS

386 The stable carbon and hydrogen isotopic composition of aromatic di- and triterpenoid
387 biomarkers from conifer and angiosperm buried wood has been investigated to determine the
388 extent of the isotopic fractionation involved in these diagenetic aromatization processes. Our
389 results indicate that the stable carbon isotopic composition is not significantly affected by
390 aromatization regardless of the substrate (di- vs. triterpenoids), of the processes involved
391 (aromatization progressing from ring *A* to ring *D* or from ring *D* to ring *A*) and of the number
392 of carbon atom(s) lost during the process. This absence of changes in the terpenoid $\delta^{13}C$
393 signatures may be attributed to the limited sensitivity of the enzymes involved in the
394 biological aromatization processes towards carbon isotopic effects, and indicates that $\delta^{13}C$
395 signatures of aromatic higher plant lipids may thus be used with confidence to trace potential
396 biological sources (e.g., C3 vs. C4 plants).

397 In the case of the hydrogen isotopic composition of diagenetic terpenoids, our observations
398 are more contrasted, with the diterpenoid δ^2H signatures showing a strong increase of the δ^2H
399 values with ongoing aromatization, and triterpenoid δ^2H composition remaining almost
400 constant whatever the nature and extent of the aromatization process involved. In the case of
401 diterpenoids, the trend observed could be attributed to the fact that the different aromatic
402 diterpenoids analyzed represent extremely minor intermediates which have survived to further
403 degradation/mineralization which affected the majority of the diterpenoid pool. In contrast,
404 the constancy of the triterpenoid δ^2H signature suggests that $^1H/^2H$ isotopic effects related to
405 the bacterial enzymes involved in these aromatization processes operating under oxygen-
406 depleted conditions is limited, similarly to what is reported for the "soluble" desaturases from
407 plants. The high variability of the $^1H/^2H$ values measured for aromatic terpenoids suggests

408 that the interpretation of the $^1H/^2H$ signatures of biomarkers formed by such diagenetic
409 processes may be extremely difficult to interpret in connection with palaeoenvironmental
410 studies (e.g., climatic changes, palaeohydrology).

411

412 ACKNOWLEDGMENTS

413 D. Schwartz (Université de Strasbourg) is thanked for the gift of the wood sample of *Pinus*
414 *sylvestris*, and N. Schneider (Institut National de Recherches Archéologiques Preventives) for
415 the oak and alder wood samples. LB thanks the French "Ministère de l'Enseignement
416 Supérieur et de la Recherche" for a doctoral fellowship. The "Centre National de la Recherche
417 Scientifique" and "Université de Strasbourg" are thanked for financial support.

418

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591

592 FIGURE CAPTIONS

593 FIGURE 1 Partial gas chromatogram (GC-MS) of the apolar fraction F1 (cf. section 2.4) from
594 the TLE recovered from buried pine wood (SE France). Carboxylic acids were analysed as
595 methyl esters.

596 FIGURE 2 Scheme showing the genetic relationship between abietic acid and its related
597 diagenetic diterpenoids occurring in buried pine wood. Measured δ^2H and $\delta^{13}C$ values are
598 indicated under the corresponding structure. In the case of tetrahydroabietic acid **1**, the
599 isotopic values were measured on the related trimethylsilyl derivative and were corrected for
600 the added derivatization group introduced (cf. section 2.7). **a**: aromatization; **b**:
601 hydrogenation; **c**: decarboxylation.

602 FIGURE 3 Scheme showing the genetic relationship between aromatic C-2 oxygenated
603 triterpenoids **12**-**17** occurring in buried oak wood and bartogenic **18** and 23-
604 hydroxybartogenic **19** acids (adapted from Schnell et al., 2012 and 2014). Measured δD and
605 $\delta^{13}C$ values are indicated under the corresponding structure. Alcohols **12-13** and phenols **16-**
606 **17** were analysed as trimethylsilyl derivatives and the δ^2H and $\delta^{13}C$ values were corrected for
607 the derivatization group introduced (cf. § 2.7).

608 FIGURE 4 Partial gas chromatograms (GC-MS) of fraction (a) F1; (b) F2 (cf. section 2.5)
609 isolated from the TLE from a buried oak (*Q. robur*) trunk (heartwood) sample collected in a
610 palaeochannel from the Rhine river (Gerstheim, NE France). Alcohols and phenols were
611 analysed as trimethylsilyl derivatives.

612 FIGURE 5 Partial gas chromatograms (GC-MS) of the apolar fraction F1 (cf. section 2.6)
613 isolated from the lipid extract from a buried alder heartwood (*Alnus sp.*) sample collected in a
614 palaeochannel from the Rhine river (Gerstheim, NE France).

615 FIGURE 6 Scheme showing the genetic relationship between aromatic triterpenoids **22**-**25**
616 occurring in buried alder wood found in Gerstheim (NE France). Measured δ^2H and $\delta^{13}C$
617 values are indicated under the corresponding structures.

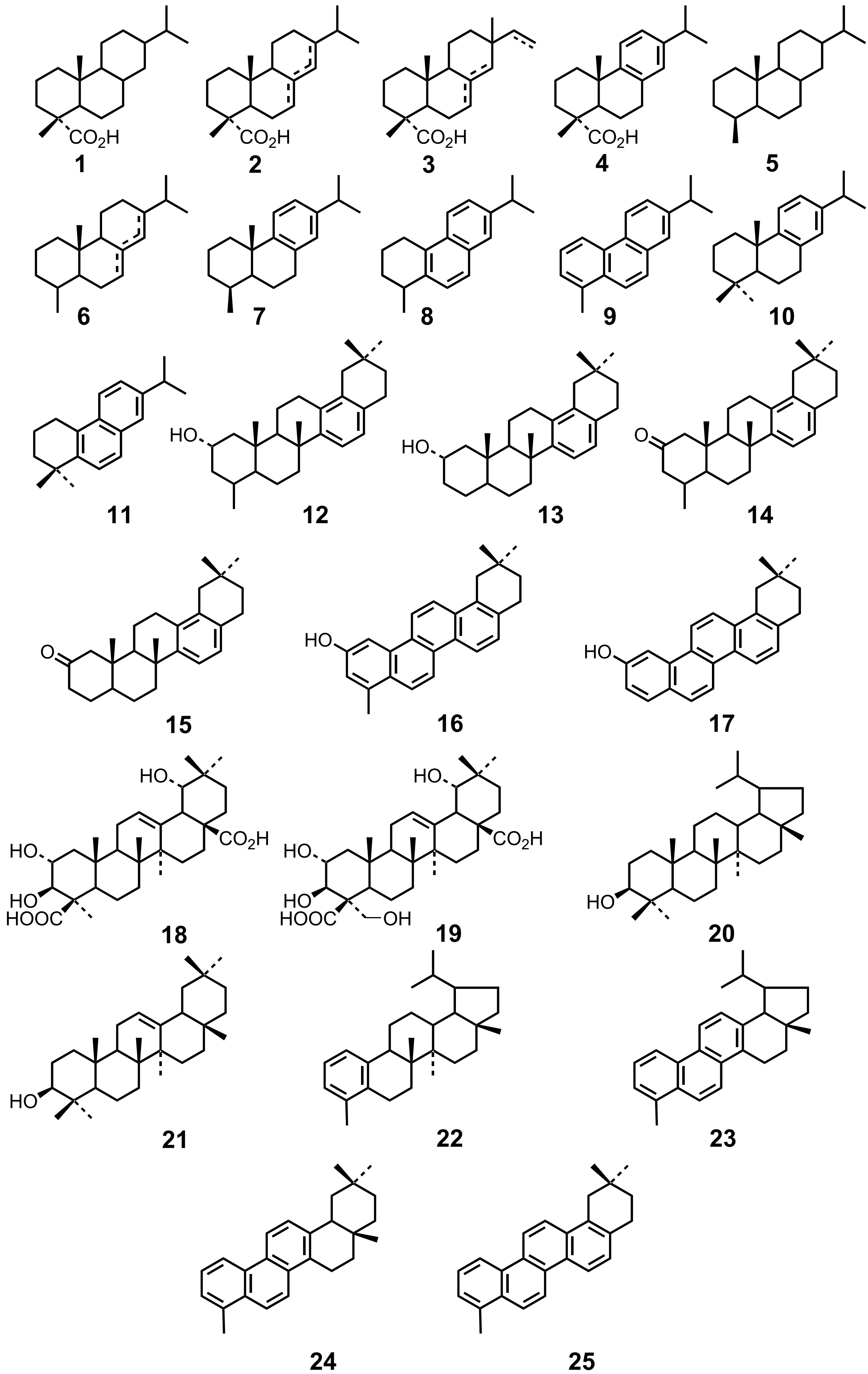
618 FIGURE 7 Scheme summarizing the evolution of the hydrogen isotopic composition of the
619 monounsaturated transformation product and the residual substrate during early diagenetic

620 dehydrogenations. Biologically triggered dehydrogenation reactions are generally sensitive to
621 the isotopic composition of the hydrogen atom ($H_{1a\ biol}$) involved in the first C-H cleavage
622 reaction and not to that involving the second hydrogen atom ($H_{2a\ biol}$) removed during the
623 dehydrogenation step (e.g., Buist and Behrouzian, 1996 ; 1998). The role on the isotopic
624 composition of the hydrogen atoms not involved in the reaction should be negligible
625 (secondary isotopic effects). It results that the δ^2H value of the remaining substrate should
626 increase mainly due to the progressive ^2H-enrichement of the hydrogen at the position
627 involved in the first C-H cleavage reaction whereas the δ^2H value of the monounsaturated
628 transformation product should be close to that of the original biological substrate prior
629 dehydrogenation (unless the latter serves as a substrate for further dehydrogenation).

630

631

Appendix



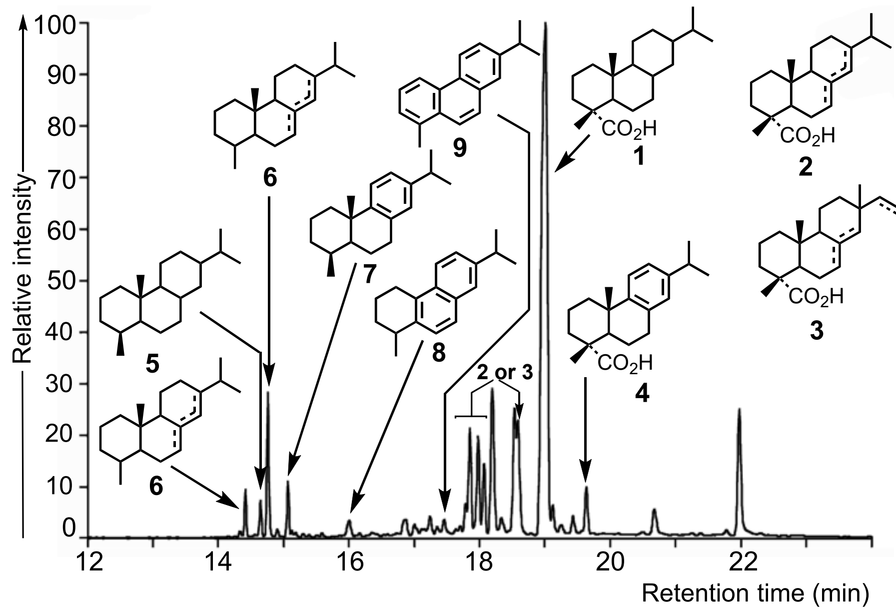


Fig. 1

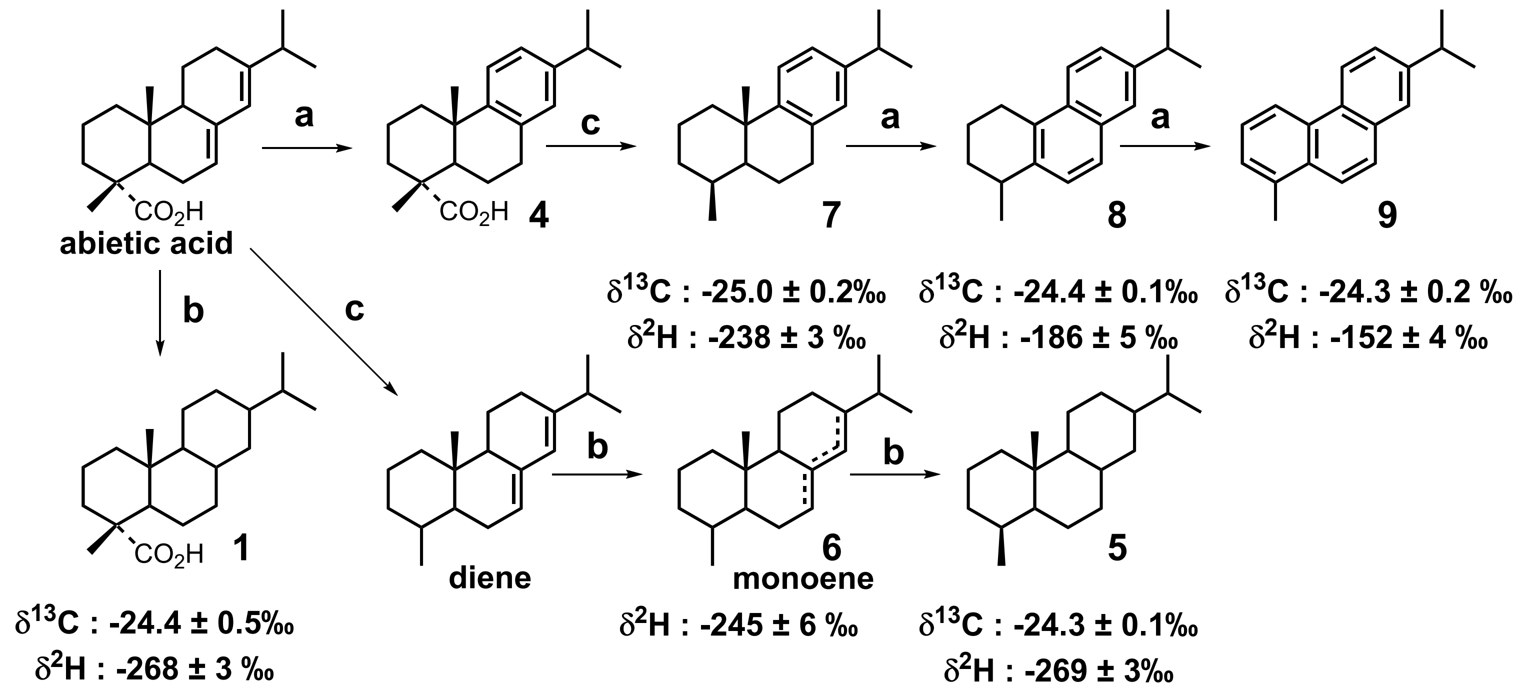


Fig. 2

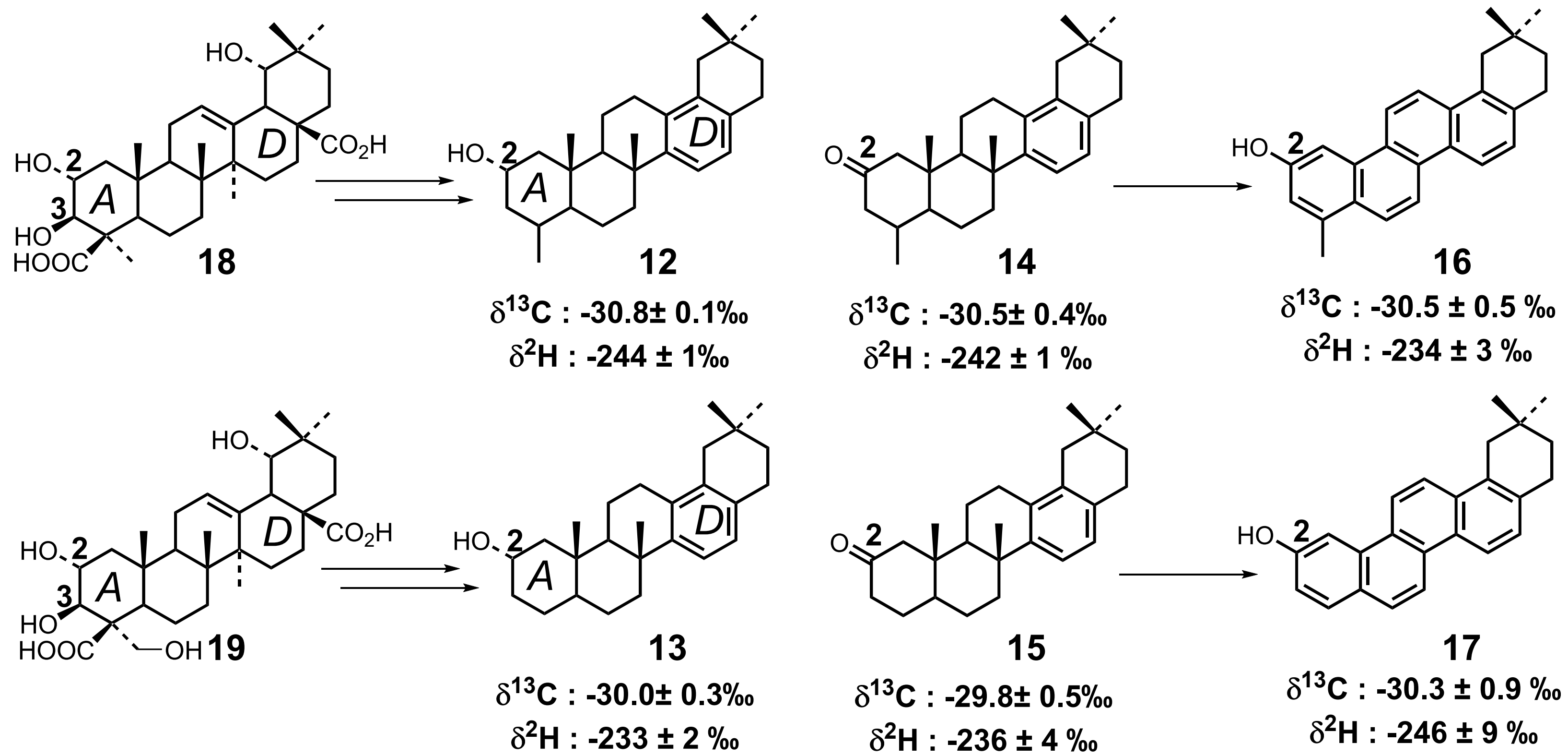


Fig. 3

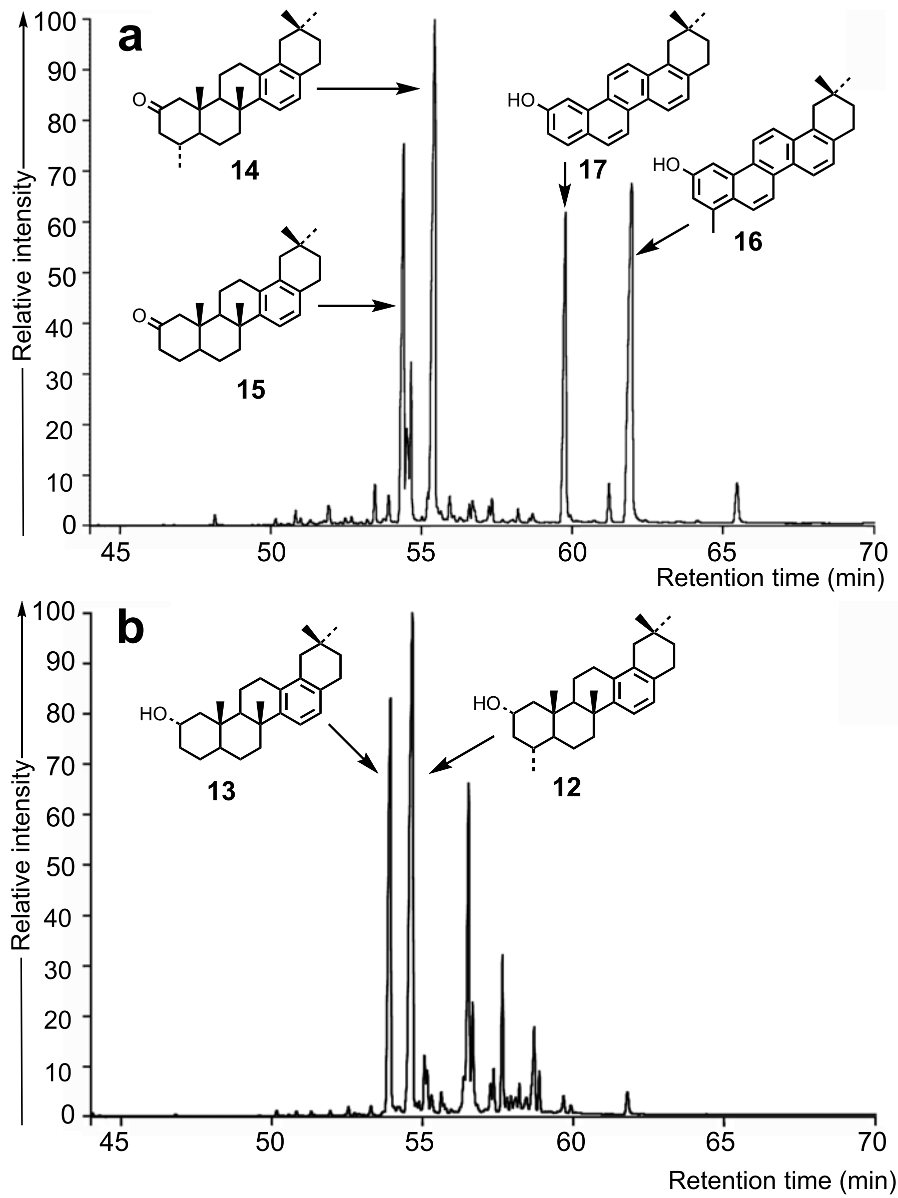


Fig. 4

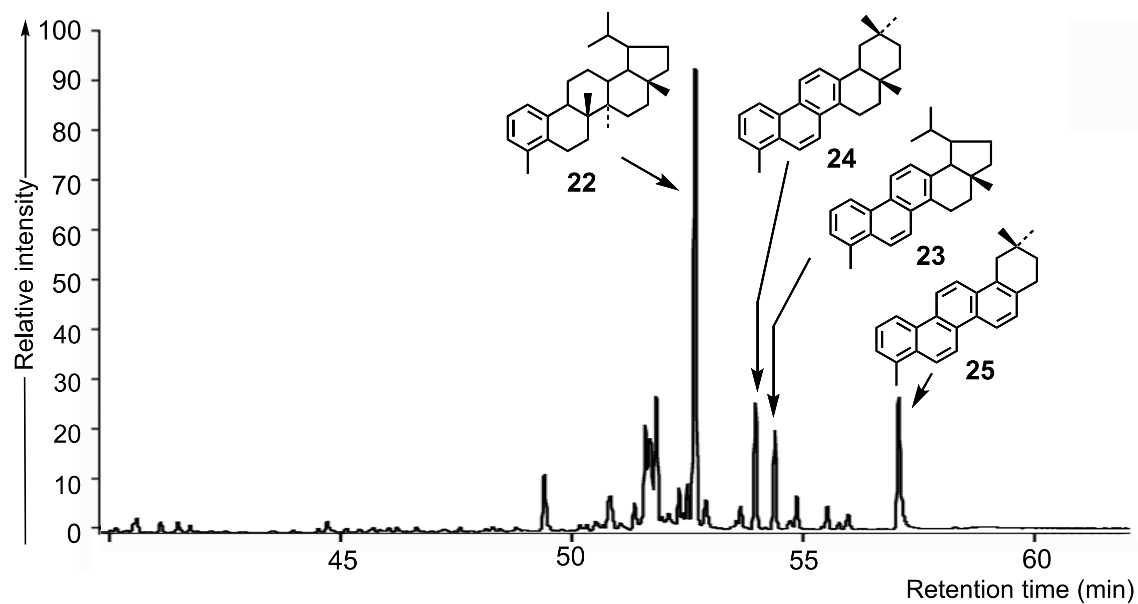


Fig. 5

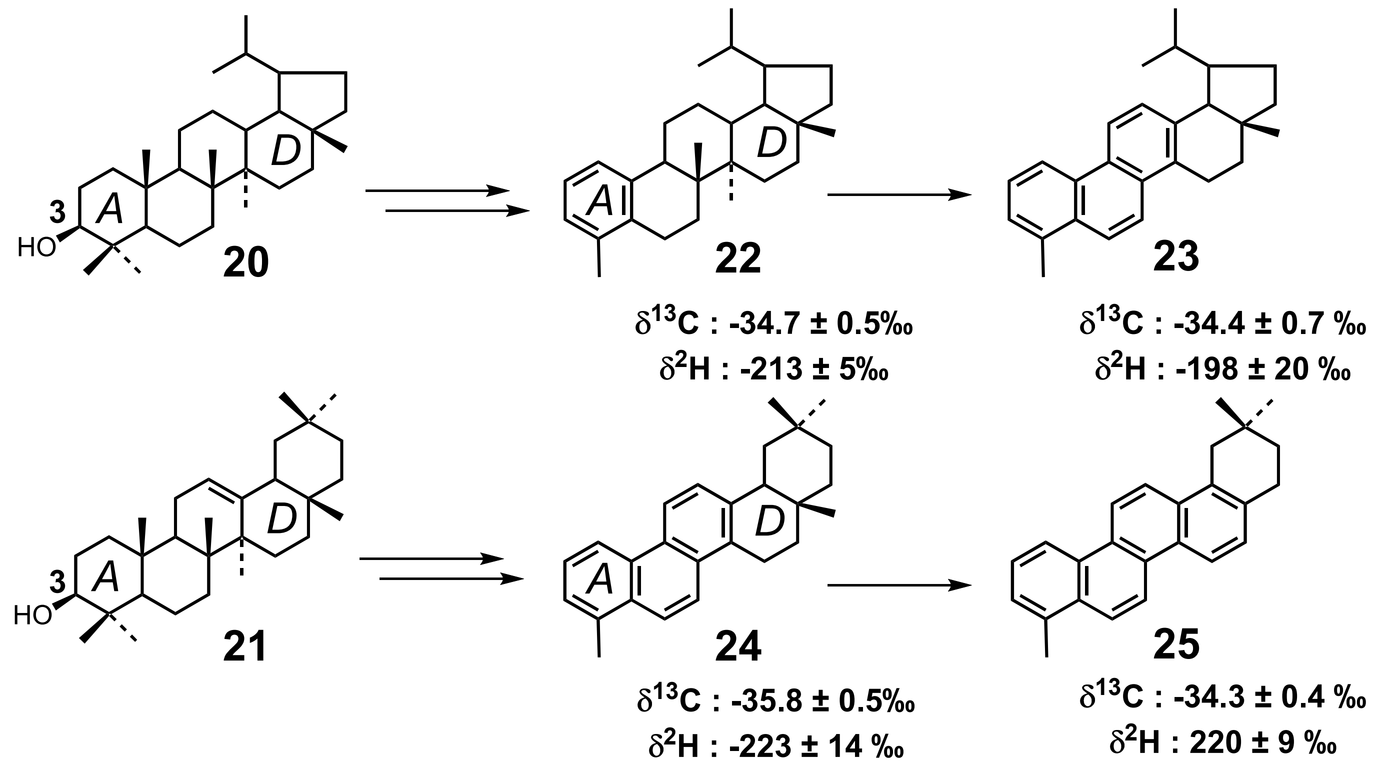


Fig. 6

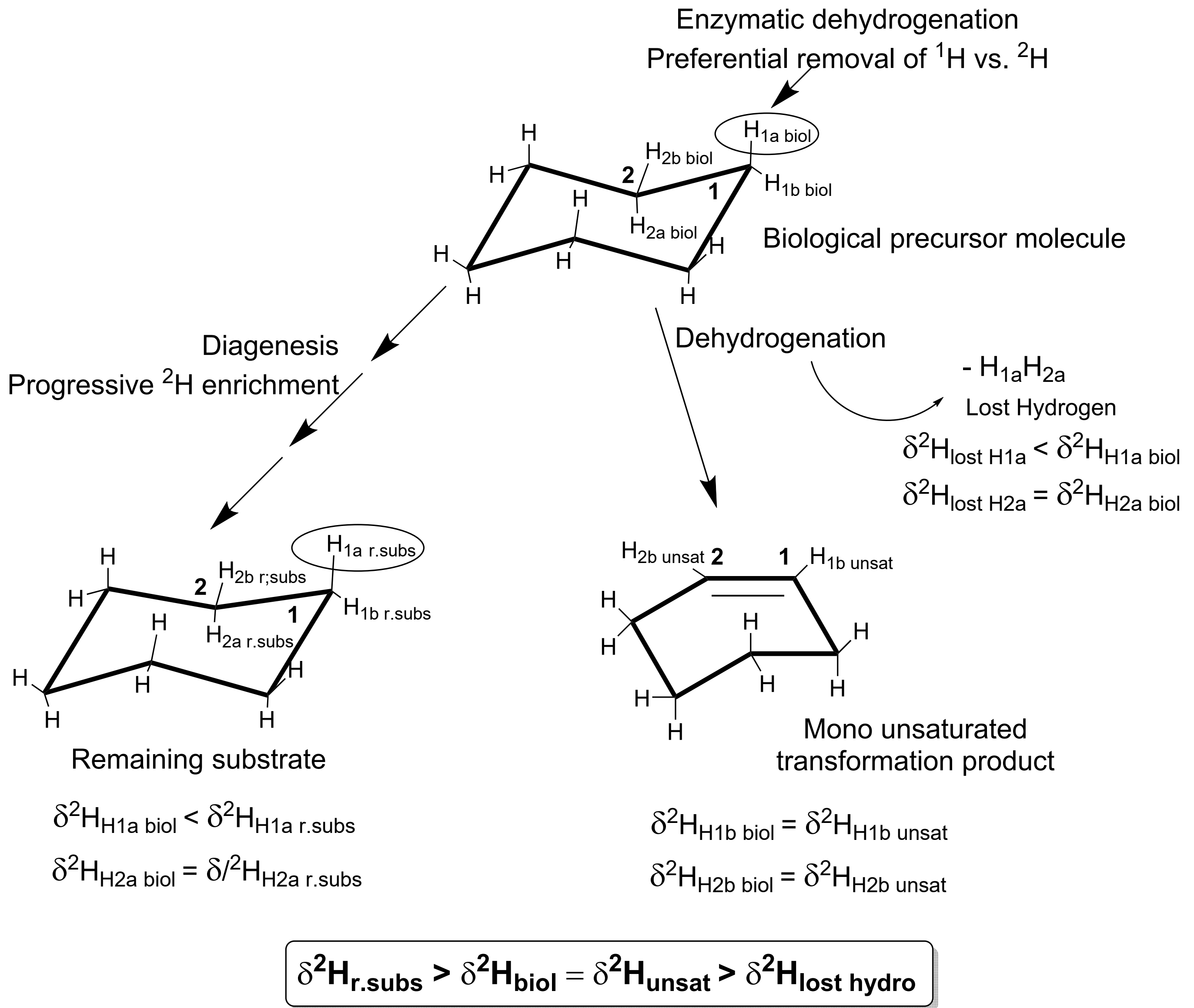


Fig. 7