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3 **BETULIN-RELATED ESTERS FROM BIRCH BARK TAR:**

4 **IDENTIFICATION, ORIGIN AND ARCHAEOLOGICAL**

5 **SIGNIFICANCE**

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21

22 **Abstract**

23 Birch bark tar, an organic material frequently encountered during
24 archaeological excavations, has been identified from its lipid composition on
25 the cracks of a ceramic dated to the late Neolithic. Lipids of this black
26 substance were dominated by a characteristic triterpenoid assemblage of
27 lupane-related triterpenoids from birch bark together with their thermal
28 degradation products formed during preparation of the tar. Among the
29 latter, four main series of unusual triterpenoid esters have been detected
30 and were postulated to correspond to esters of Δ^2-betulin and Δ^2-
31 dihydrobetulin based on their mass spectra and hydrolysis experiments.
32 Their conclusive identification has been achieved by synthesis of reference
33 compounds. These compounds most likely originate from the esterification
34 between triterpenoid alcohols related to betulin and fatty acids from suberin
35 formed upon heating of birch bark tar. They could be considered as markers
36 of intense heating during birch bark tar preparation using the "single
37 pot" procedure.

38

39 Key words: Archaeology, Birch bark tar, Lupane-related triterpenoids,
40 Triterpenoid esters, Neolithic.

41

42 Declarations of interest: none

43 **1. Introduction**

44 Natural plant resins and resinous materials have played an important role
45 in the daily life since ancient times, explaining their frequent occurrence at

46 archaeological sites. Among them, birch bark tar is a vegetal substance
47 produced by dry distillation of birch bark (Aveling and Heron, 1998; Urem-
48 Kotsou et al., 2002; Rageot et al., 2016; Courel et al., 2018). This sticky and
49 hydrophobic material has been used since the Paleolithic (Koller et al., 2001;
50 Grünberg, 2002) for various purposes, like the hafting of lithic tools or
51 jewellery (Sauter et al., 2000; Bosquet et al., 2001; Koller et al., 2001; Courel
52 et al., 2018), the repairing or caulking of ceramics (Charters et al., 1993;
53 Connan et al., 2000; Bosquet et al., 2001; Rageot et al., 2016; Reunanen et
54 al., 1993; Urem-Kotsou et al., 2002), the decorating of ceramic vessels (Vogt,
55 1949) and has even been used as chewing-gum (Aveling and Heron, 1999;
56 Karg et al., 2014). Birch bark tar has a typical molecular composition,
57 making it easy to identify by detection of predominant lupane-related
58 triterpenoids using gas chromatography coupled to mass spectrometry - GC-
59 MS - (Hayek et al., 1989, 1990; Reunanen et al., 1996; Aveling and Heron,
60 1998; Schnell et al., 2014).
61 In the frame of a study dedicated to the use of pottery during the Neolithic,
62 we have investigated an organic black substance having most likely served
63 to repair different ceramics (Fig. 1) found at the site of La Rouvière (Rogues,
64 Gard, Occitanie, Southern France) and dated to the late Neolithic (ca. 3000
65 BCE). GC-MS analysis of this substance led to its identification as birch
66 bark tar. Along with typical triterpenoid markers from the lupane series,
67 four series of uncommon compounds eluted late on the gas chromatogram
68 were detected. Based on MS interpretations, they were postulated to

correspond to triterpenes ester-linked to monocarboxylic or dicarboxylic acids. One of this compound series was previously reported to occur in birch bark tar samples from Roman archaeological sites in Great Britain, but the identification, based on MS interpretations and hydrolysis experiments, remained tentative (Dudd and Evershed, 1999). We report here the conclusive identification of the four series of compounds by synthesis of one homologue of each series. Their origin as well as their significance with regard to the mode of preparation of the organic material are discussed.

2. The archaeological site of La Rouvière

The site of La Rouvière corresponds to a settlement unearthed in the city of Rogues (Southern France). It has been discovered in 1989 by J. Halgand and colleagues (members of the « Groupe de Recherches et d'Explorations Souterraines du Vigan ») and excavated by P. Galant from 1989 to 2004 (Galant et al., 2000). The archaeological site is composed of a sinkhole (discovered in 1989) and an outdoor establishment (discovered in 1999) linked by a narrow corridor (Fig. 1a). The site was used to collect and store water. Two main phases of occupation both dated to the late Neolithic (ca. 3000 BCE) were clearly distinguished, with the latest ending with a fire event. More than 40 jars related to the last occupation stage were discovered inside the establishment. One of them, named I15, was found in the corridor at the entrance of the sinkhole and was probably used for water storage (Galant and Halgand, 2004; Fig. 1a). In this jar, black organic residues were

91 discovered along the edges of ancient cracks on the inner surface, suggesting
92 that this material was used to repair/waterproof the ceramic. The
93 archaeological sample investigated corresponds to a black organic residue
94 collected on the jar I15 (Fig. 1b).

95 3. Experimental

96 3.1. Extraction

97 The archaeological sample was extracted by sonication (20 min) using a
98 mixture of CH_2Cl_2/MeOH (1:1 v/v) followed by filtration of the supernatant
99 through celite and removal of the solvent under reduced pressure. 25.0 mg of
100 organic extract were obtained from 204.7 mg of starting material.

101 3.2. Fractionation of the solvent extract

102 3.2.1. Protocol A

103 An aliquot of the extract was acetylated (Ac_2O, Pyridine, 2 h, 60 °C) and, after
104 removal of the solvents and excess reagents under a stream of Ar, esterified
105 with a solution of diazomethane in diethyl ether. The derivatized extract was
106 fractionated by liquid chromatography (LC) on silica gel into an apolar
107 fraction ($F_{A.1}$) eluted with CH_2Cl_2/EtOAc (8:2, v/v; 3 dead volumes - D_{vol} -) and
108 a more polar fraction ($F_{A.2}$) eluted with CH_2Cl_2/MeOH (1:1, v/v; 2 D_{vol}). $F_{A.1}$
109 was analyzed by GC-MS.

110 3.2.2. Protocol B

111 An aliquot of the extract was fractionated by liquid chromatography (LC) on
112 silica gel without derivatization. A first fraction ($F_{B.1}$) containing the fatty
113 acyl esters from series **E1** and **E2** was recovered by elution with CH_2Cl_2 (1.4
114 D_{vol}). A second fraction ($F_{B.2}$), eluted with a mixture of CH_2Cl_2/MeOH (1:1 v/v;
115 2 D_{vol}), was treated with a solution of diazomethane in diethyl ether. The
116 resulting methylated fraction $F_{B.2}$ was re-fractionated into three fractions.
117 The first fraction ($F_{B.2.1}$ eluted with CH_2Cl_2 (1.25 D_{vol}) was shown to contain
118 mainly methylated fatty acids, and the second fraction ($F_{B.2.2}$), also eluted
119 with CH_2Cl_2 (2 D_{vol}), comprised dicarboxylic esters from series **E3** and **E4**. The
120 last eluted one ($F_{B.2.3}$), recovered using a mixture of CH_2Cl_2/MeOH (1:1 v/v; 2
121 D_{vol}), corresponded to more polar material not further investigated.

122 **3.3. GC-MS**

123 GC-MS analyses were carried out using a Thermo Trace gas chromatograph
124 (Thermo Scientific) coupled to a Thermo Scientific TSQ Quantum mass
125 spectrometer equipped with an autosampler Tri Plus and a programmed
126 temperature vaporizing (PTV) injector. The temperature of the source was set
127 at 220 °C. The mass spectrometer was operating in the electron impact (EI)
128 mode at 70 eV and scanning m/z 50 to 700 or 50 to 900. Gas chromatographic
129 separations were performed on a HP5-MS column (30 m x 0.25 mm ; 0.25 μm
130 film thickness) using He as carrier gas.

131 Two oven temperature programs were used:

132 **Program 1 :** 70 °C (1 min), 70 °C-200 °C (10 °C/min), 200 °C-320 °C (4 °C/min),
133 isothermal at 320 °C for 40 min.

134 **Program 2 :** 70 °C (1 min), 70 °C-320 °C (10 °C/min), isothermal at 320 °C
135 for 80 min.

136 3.4. NMR

137 The NMR spectra were recorded on a Bruker Avance I 500 MHz spectrometer.
138 The chemical shifts are reported in ppm relative to tetramethylsilane with
139 the solvent used as internal standard ($CDCl_3$: δ^1H 7.26 ppm ; $\delta^{13}C$ 77.16 ppm).

140 3.5. LiAlH4 hydrogenolysis of ester bonds

141 Fraction $F_{B.1}$ (cf. § 3.2.2.) was submitted to hydrogenolysis using $LiAlH_4$. A
142 small amount of $LiAlH_4$ (in powder) prewashed with distilled cyclohexane was
143 put under stirring in a vial containing the fraction $F_{B.1}$ dissolved in THF (1 h,
144 room temperature). The mixture was transferred dropwise into a round-
145 bottom flask containing MeOH in order to remove the excess of reagent. After
146 removal of the solvents under reduced pressure, the crude mixture was
147 transferred into a separatory funnel and extracted using EtOAc after addition
148 of distilled water. The solvent extract was filtered on a small silica gel column
149 and acetylated (Ac_2O/Pyr, 1:1 v/v, 2 h, 60 °C) before analysis using GC-MS.

150 3.6. Synthesis of reference compounds

151 Synthesis of the reference compounds was based on the articles from Sun et
152 al. (1998a, 1998b).

153 3.6.1. Dehydration of betulin derivatives (Fig. 2a)

154 Diethylazodicarboxylate (0.15 mL, 4 eq) was added dropwise to a solution
155 containing 100 mg of betulin **T1** (respectively 100 mg of dihydrobetulin **T9**)

156 in THF (3-4 mL), triphenylphosphine (237 mg, 4 eq) and 3,3-
157 dimethylglutarimide (129 mg, 4 eq) at 0 °C under Ar atmosphere. The mixture
158 was left for 3 h at room temperature. After removal of the solvent under
159 reduced pressure, the crude residue was fractionated by silica gel
160 chromatography with a mixture of EtOAc/CH_2Cl_2 (5:95 v/v) to obtain 68 mg of
161 pure Δ^2-betulin **T5** (respectively 46 mg of Δ^2-dihydrobetulin **T7**) with a yield
162 of 71% (respectively 48%).
163 *Δ^2-betulin **T5*** (Fig. 3a)*:* GC-MS (acetate derivative of **T5**) (EI, 70 eV) *m/z* (rel.
164 intensity) 466 ($M^{+\cdot}$, 10%), 406 (8), 393 (20), 229 (12), 216 (21), 203 (40), 189
165 (100), 187 (55), 173 (27), 159 (32), 147 (35), 133 (42), 119 (56), 107 (48), 93 (40).
166 ^1H NMR (500 MHz; $CDCl_3$): 0.86 (3H, s), 0.87 (3H, s), 0.94 (3H, s), 0.99 (3H,
167 s), 1.05 (3H, s), 1.69 (3H, s, H-30), 2.40 (1H, td, J = 5.5; 11.0 Hz, H-19), 3.34
168 (1H, d, J = 10.5 Hz, H-28), 3.82 (1H, d, J = 10.5 Hz, H-28), 4.59 (1H, s, H-29),
169 4.69 (1H, s, H-29), 5.35 (1H, dd, J = 2.0; 10.0 Hz, H-3), 5.40 (1H, ddd, J = 1.0;
170 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.9, 15.8, 16.5, 19.2, 19.6,
171 21.4, 22.7, 25.5, 27.2, 29.3, 29.9, 31.9, 33.5, 34.1, 34.8, 36.5, 37.6, 41.1, 41.4,
172 42.9, 47.9, 48.0, 48.9, 49.2, 52.2, 60.7, 109.8, 121.7, 138.1, 150.7.
173 *Δ^2-dihydrobetulin **T7*** (Fig. 3b)*:* GC-MS (acetate derivative of **T7**) (EI, 70 eV)
174 *m/z* (rel. intensity) 468 ($M^{+\cdot}$, 9%), 408 (3), 395 (54), 229 (10), 217 (13), 204 (26),
175 191(41), 189 (100), 177 (36), 161 (22), 147 (27), 135 (47), 122 (62), 119 (47),
176 107 (43), 95 (35). ^1H NMR (500 MHz; $CDCl_3$): 0.77 (3H, d, J = 7.0 Hz, H-29 or
177 H-30), 0.84 (3H, d, J = 7.0 Hz, H-29 or H-30), 0.87 (3H, s), 0.88 (3H, s), 0.95
178 (3H, s), 0.97 (3H, s), 1.06 (3H, s), 3.31 (1H, d, J = 11.0 Hz, H-28), 3.79 (1H, d,

179 *J*= 11.0 Hz, H-28), 5.36 (1H, dd, *J* = 2.5; 10.0 Hz, H-3), 5.40 (1H, ddd, *J* = 1.5;
180 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.8, 15.1, 15.8, 16.5, 19.6,
181 21.4, 21.9, 22.8, 23.1, 27.1, 27.1, 29.4, 29.6, 31.9, 33.6, 34.2, 34.8, 36.5, 37.1,
182 41.2, 41.4, 43.0, 44.7, 48.1, 48.2, 48.9, 52.2, 60.8, 121.7, 138.1.

183 **3.6.2. Esterification of betulin derivatives**

184 Synthesis of **E1a** and **E2a** (Fig. 2b)
185 A solution containing Δ^2-betulin **T5** (14.7 mg) (respectively Δ^2-
186 dihydrobetulin **T7**, 17.9 mg), nonanoyl chloride (50 µL, ~7 eq), N-methyl
187 imidazole (N-Me Im, one drop) and pyridine (0.6 mL) was placed in a vial at
188 90 °C for 4 h. After transfer of the mixture into a separatory funnel and
189 addition of an aqueous solution of $CuSO_4$ (3 mL, 9/1 wt:wt), the organic
190 layer was recovered using CH_2Cl_2. The organic phase was washed with
191 distilled water, and the solvent removed under reduced pressure. The crude
192 residue was fractionated by silica gel chromatography with toluene to obtain
193 4.3 mg of pure **E1a** with a yield of 22% (resp. 4.4 mg of E2a; 19%, yield).
194 *Compound **E1a*** (Fig. 3c)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 564 ($M^{+\cdot}$,
195 6%), 406 (18), 393 (19), 229 (14), 216 (22), 203 (46), 189 (100), 187 (64), 175
196 (25), 173 (26), 159 (34), 147 (34) 135 (34), 133 (40), 121 (44), 119 (48), 107
197 (45), 95 (37), 81 (27). ^{1}H NMR (500 MHz; $CDCl_3$): 0.94 (3H, s), 0.99 (3H, s),
198 1.06 (3H, s), 1.69 (3H, s, H-30), 2.32 (2H, t, *J* = 7.5 Hz, H-2'), 2.46 (1H, td, *J*
199 = 6.0; 11.5 Hz, H-19), 3.86 (1H, d, *J* = 11.0 Hz, H-28), 4.26 (1H, d, *J* = 11.0
200 Hz, H-28), 4.59 (1H, s, H-29), 4.69 (1H, s, H-29), 5.35 (1H, dd, *J* = 2.0; 10.0
201 Hz, H-3), 5.40 (1H, ddd, *J* = 1.0; 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz;

202 $CHCl_3$): 14.2, 14.9, 15.9, 16.5, 19.3, 19.6, 21.3, 22.8, 22.8, 25.3, 25.5, 27.2,
203 29.3, 29.4, 29.4, 29.8, 29.9, 31.9, 32.0, 33.5, 34.7, 34.8, 34.8, 36.5, 37.8, 41.1,
204 41.4, 42.9, 46.6, 47.9, 48.9, 49.2, 52.3, 62.7, 109.9, 121.7, 138.1, 150.4, 174.5.
205 *Compound **E2a*** (Fig. 3d)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 566 ($M^{+\cdot}$,
206 5%), 408 (8), 395 (47), 229 (16), 217 (14), 204 (39), 191 (43), 189 (100), 177
207 (37), 161 (23), 159 (24), 149 (28), 147 (30), 135 (50), 122 (51), 107 (42), 95
208 (42), 81 (30). ^{1}H NMR (500 MHz; $CDCl_3$): 0.77 (3H, d, J = 6.5 Hz, H-29 or H-
209 30), 0.84 (3H, d, J = 7.0 Hz, H-29 or H-30), 0.87 (3H, s), 0.88 (3H, s), 1.07
210 (3H,s), 2.31 (2H, t, J = 8.0 Hz, H-2'), 3.83 (1H, d, J = 11.0 Hz, H-28), 4.26
211 (1H, d, J = 11.0 Hz, H-28), 5.36 (1H, dd, J = 2.0; 10.0 Hz, H-3), 5.41 (1H,
212 ddd, J= 1.0; 6.0; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.2, 14.8, 15.1,
213 15.9, 16.5, 19.6, 21.3, 21.8, 22.8, 22.8, 23.1, 25.3, 27.1, 27.1, 29.3, 29.4, 29.4,
214 29.6, 30.1, 31.9, 32.0, 33.6, 34.7, 34.8, 34.9, 36.5, 37.4, 41.1, 41.4, 43.0, 44.7,
215 46.7, 48.3, 48.9, 52.2, 62.7, 121.7, 138.1, 174.5.

216 Synthesis of **E3a** and **E4a** (Fig. 2b)
217 A solution containing 12.9 mg of Δ^2-betulin **T5** (resp. 13 mg of Δ^2-
218 dihydrobetulin **T7**), methyl 8-chloro-8-oxooctanoate (50 µL, 11 eq), N-methyl
219 imidazole (N-Me Im) (one drop) and pyridine (0.6 mL, was placed in a vial at
220 90 °C for 4 h. To remove pyridine and N-Me Im, the mixture was transferred
221 into a separatory funnel, shaken with 3 mL of an aqueous solution of $CuSO_4$
222 (9/1 wt:wt) and extracted 2x with CH_2Cl_2 and 2x with EtOAc. The combined
223 organic extracts were evaporated under reduced pressure, dissolved in
224 EtOAc and washed with distilled water. The crude residue obtained after

225 removal of the solvent under reduced pressure was fractionated by silica gel
226 chromatography with a mixture of EtOAc/CH_2Cl_2 (5:95 v/v) to obtain 8.9 mg
227 (49 % yield) of pure **E3a** (respectively 10.1 mg of **E4a**, 55 % yield).
228 *Compound **E3a*** (Fig. 3e)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 594 ($M^{+\cdot}$,
229 2%), 406 (31), 391 (13), 363 (7), 229 (15), 215 (23), 203 (41), 202 (41), 189
230 (100), 187 (62), 173 (28), 159 (32), 147 (36), 133 (41), 119 (51), 107 (47), 95
231 (39), 81 (26). ^{1}H NMR (500 MHz; $CDCl_3$): 0.86 (3H, s), 0.87 (3H, s), 0.94 (3H,
232 s), 0.98 (3H, s), 1.06 (3H, s), 1.68 (3H, s, H-30), 2.30 (2H, t, *J* = 7.5 Hz, H-2'
233 or H-7'), 2.32 (2H, t, *J* = 7.5 Hz, H-2' or H-7'), 2.45 (1H, td, J = 5.5; 11.5 Hz,
234 H-19), 3.66 (3H, s, OCH_3), 3.86 (1H, d, *J* = 11.5 Hz, H-28), 4.27 (1H, d, *J* =
235 11.0 Hz, H-28), 4.59 (1H, s, H-29), 4.69 (1H, s, H-29), 5.35 (1H, dd, *J* = 2.0;
236 10.0 Hz, H-3), 5.39 (1H, ddd, *J* = 1.0; 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz;
237 $CHCl_3$): 14.9, 15.9, 16.5, 19.3, 19.6, 21.3, 22.8, 24.9, 25.0, 25.5, 27.2, 28.9,
238 28.9, 29.8, 29.9, 31.9, 33.5, 34.1, 34.5, 34.7, 34.8, 36.5, 37.8, 41.1, 41.4, 42.9,
239 46.6, 47.9, 48.9, 49.2, 51.6, 52.3, 62.7, 110.0, 121.7, 138.1, 150.4, 174.3,
240 174.3.
241 *Compound **E4a*** (Fig. 3f)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 596 ($M^{+\cdot}$,
242 2%), 408 (15), 395 (16), 365 (8), 326 (7), 229 (13), 217 (11), 204 (39), 191 (38),
243 189 (100), 177 (27), 159 (22), 149 (24), 147 (25), 135 (43), 119 (40), 107 (37),
244 95 (38), ^{1}H NMR (500 MHz; $CDCl_3$): 0.77 (3H, d, *J* = 7.0 Hz, H-29 or H-30),
245 0.84 (3H, d, *J* = 7.0 Hz, H-29 or H-30), 0.87 (3H, s), 0.88 (3H, s), 0.94 (3H, s),
246 0.96 (3H, s), 1.07 (3H, s), 2.30 (2H, t, *J* = 7.5 Hz, H-2' or H-7'), 2.31 (2H, t, *J*
247 = 7.5 Hz, H-2' or H-7'), 3.66 (3H, s, OCH_3), 3.83 (1H, d, *J* = 11.0 Hz, H-28),

248 4.26 (1H, d, J = 11.0 Hz, H-28), 5.36 (1H, dd, J = 2.0; 10.0 Hz, H-3), 5.40
249 (1H, ddd, J = 1.0; 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.8, 15.1,
250 15.9, 16.5, 19.6, 21.3, 21.8, 22.8, 23.1, 24.9, 25.0, 27.1, 27.1, 28.9, 28.9, 29.6,
251 30.0, 31.9, 33.5, 34.1, 34.5, 34.8, 34.9, 36.5, 37.4, 41.1, 41.4, 43.0, 44.7, 46.7,
252 48.3, 48.9, 51.6, 52.2, 62.8, 121.7, 138.1, 174.3, 174.3.

253 4. Results and discussion

254 4.1. Triterpenoids as markers of birch bark tar

255 The gas chromatogram of fraction $F_{A.1}$ (cf. § 3.2.1.) isolated from the
256 archaeological sample is shown in Fig. 4. Its lipid distribution was
257 dominated by triterpenoids from the lupane series, indicating a major
258 contribution from a vegetal source derived from angiosperms (Fig. 4). The
259 triterpenoids notably comprised betulin **T1**, lupeol **T2** and betulone **T3**
260 which are native triterpenoids occurring in birch bark (Hayek et al., 1989,
261 1990; Schnell et al., 2014). However, these compounds represent generally
262 by far the predominant triterpenoids in the case of fresh (or even weathered)
263 birch bark (Hayek et al., 1990; Aveling and Heron, 1998; Schnell et al.,
264 2014; Courel et al., 2018), whereas they appear only as minor constituents
265 in the archaeological sample. In the latter case, the distribution is
266 dominated by compounds **T4**, **T5**, **T10**, **T7** and **T11**, which all belong to the
267 series of Δ^2-betulin-related triterpenoids. These compounds, together with
268 the allobetulane derivatives **T10**-**T13**, are known from the literature to be

269 biomarkers of birch bark tar (Bosquet et al., 2001; Modugno et al., 2006;

270 Regert et al., 2006; Courel et al., 2018; Rageot et al., 2019).

271 Δ^2-triterpenoids are formed by elimination of the oxygenated function at C-3

272 of lupeol **T2** and betulin **T1**, this reaction being generally induced by a

273 thermal treatment as is the case for the preparation of birch bark tar

274 (Courel et al., 2018; Rageot et al., 2019). Similarly, it was shown that the

275 same thermal treatment leads to the formation of allobetulane derivatives

276 **(T10-T13)** which results from an acid-catalyzed intramolecular

277 rearrangement of the ring *E* of betulin and by-products (Davy et al., 1951a,

278 1951b; Green et al., 2007; Salvador et al., 2009). In some cases, both

279 reactions may co-occur, resulting in the formation of Δ^2-allobetulin

280 derivatives such as **T10** and **T11**.

281 Similar observations regarding birch bark tar composition, in which

282 alteration products dominate over genuine ones, are reported in the

283 literature in the case of archaeological samples (e.g., Urem-Kotsou et al.,

284 2002; Regert et al., 2003), and were interpreted as being the result of an

285 intense heating of the material upon tar preparation. Such seems to be the

286 case with our sample as well which was likely submitted to an abnormally

287 high thermal stress, resulting in the almost complete transformation of the

288 genuine triterpenoids (Fig. 4). In addition to these triterpenoids closely

289 related to birch bark tar, four unusual late-eluted compound series were

290 detected (named "series **E1-E4**"; Fig. 4), which present fragmentation

291 patterns in MS (Fig. 3c-3f) very similar to those of Δ^2-betulin **T5** (Fig. 3a)

292 and Δ^2-dihydrobetulin **T7** (Fig. 3b) but with quite higher molecular masses
293 in the range 494-748 Da. In order to have a more detailed look to these
294 different compounds, a fractionation procedure of the lipid extract was
295 developed and led to obtaining chromatographic fractions considerably
296 enriched in triterpenoids from series **E1**-**E4** (cf. § 3.2.2).

297 **4.2. Late-eluted triterpenoid esters from series E1-E4**

298 **4.2.1. Identification of triterpenoid esters from series E1-E2**

299 Detailed investigation of fraction $F_{B.1}$ (cf. § 3.2.2.) revealed the occurrence of
300 compounds belonging to the series **E1** and **E2** (Fig. 5). Homologues from
301 series **E1** have a mass fragmentation pattern very similar to that of Δ^2
302 betulin **T5** (Fig. 3a and 3c) and a molecular weight of $494 + n \times 14$ (n = 0-
303 18), while those from series **E2** have mass spectra close to that of Δ^2
304 dihydrobetulin **T7** (Fig. 3b and 3d) and with a molecular weight shifted
305 upwards by 2 mass units compared to series **E1** (i.e., M^+ of $496 + n \times 14$, n =
306 0-18). According to the literature, the generic structure of compounds from
307 series **E1** has been previously proposed to correspond to Δ^2 betulin **T5**
308 esterified at C-28 with saturated monocarboxylic acids of different chain
309 lengths (Dudd and Evershed, 1999). By analogy, it can be proposed that
310 compounds from the series **E2** have the same generic structure, but without
311 the $\Delta^{20(29)}$ unsaturation. For both series, the fatty acyl moiety is ranging
312 from C_4 to at least C_{22} (Fig. 5). However, since the original structural
313 identification of compounds from series **E1** was based on MS interpretations
314 and hydrolysis experiments (Dudd and Evershed, 1999), thus remaining

315 tentative, we have carried out the synthesis of one reference compound from
316 each series **E1** and **E2** (Fig. 2 and § 3.6.) for firm identification. Briefly, the
317 hydroxy group at C-3 from betulin **T1** (resp. dihydrobetulin **T9**) was
318 selectively dehydrated following the method reported by Sun et al. (1998b),
319 leading to the formation of Δ^2-betulin **T5** (resp. Δ^2-dihydrobetulin **T7**). The
320 remaining hydroxy group at C-28 from **T5** (resp. **T7**) was then esterified
321 with nonanoyl chloride to yield the C_9 ester of Δ^2-betulin **E1a** (resp. **E2a**).
322 Since the mass spectra and retention times in GC of the synthetic and
323 naturally-occurring compound **E1a** (resp. **E2a**) were identical, it can be
324 considered that both compound series have been successfully identified.

325 **4.2.2. Identification of the triterpenoid esters from series E3-E4**

326 The fraction $F_{B.2.2}$ recovered after purification of the solvent extract (cf. §
327 3.2.2.) revealed the occurrence of late eluting compounds from series **E3** and
328 **E4** (Fig. 6). Like compounds from series **E1**, the mass spectra of the
329 homologues from series **E3** (Fig. 3e) showed close similarities with those of
330 Δ^2-betulin **T5** (Fig. 3a) but with molecular ions at $538 + n \times 14$ (n = 0-6).
331 Similarly, the mass spectra of compounds from series **E4** (Fig. 3f) closely
332 resemble that of Δ^2-dihydrobetulin **T7** (Fig. 3b), with a molecular weight of
333 $540 + n \times 14$ (n = 0-6). Based on these data, it was proposed that compounds
334 from series **E3** (resp. **E4**) could correspond to dicarboxylic acids esterified at
335 the C-28 hydroxy group of Δ^2-betulin **T5** (resp. Δ^2-dihydrobetulin **T7**). For
336 both series, the dicarboxylic acid moieties comprised C_4-C_{10} homologues with
337 a predominance of the C_8 and C_9 homologues. In order to confirm these

338 hypotheses, the synthesis of one homologue of each series was performed
339 following the same synthetic scheme as for compounds **E1a** and **E2a**, except
340 for the acylating agent which was methyl-8-chloro-8-oxooctanoate (Fig. 2b).
341 Mass spectra and retention times in GC of the synthetic references and of
342 the archaeological sample were in good agreement, confirming our
343 structural hypotheses.

344 **4.2.3. Mode of formation of the triterpenoid esters E1-E4**

345 As proposed by Dudd and Evershed (1999), the esters from series **E1** and **E2**
346 may originate from esterification reactions involving compounds **T5** and **T7**
347 and monocarboxylic acids during the heating of birch bark or birch bark tar
348 with fat in a process aimed at producing birch bark tar with modified
349 properties. However, at least in our case, we propose that the
350 monocarboxylic acids may rather originate from the thermal degradation of
351 suberin, a biopolymer of birch bark (Fig. 7), and not from the input of fat.
352 Our hypothesis is notably based on the presence in the lipid extract of free
353 saturated fatty acids dominated by the C_{16}, C_{18} and C_{22} homologues,
354 together with that of C_{20}-C_{22} α,ω-hydroxyacids and C_{16}-C_{22} α,ω-diacids (Fig.
355 8a). Such a distribution closely resembles that of bound fatty acids released
356 from birch bark suberin (Holloway, 1972; Ekman, 1983; Ferreira et al.,
357 2013) and from birch bark tar (Charters et al., 1993; Reunanen et al., 1993,
358 1996; Courel, 2016; Rageot et al., 2019). Furthermore, fraction $F_{B.1}$
359 containing the triterpenoid esters from series **E1** and **E2** was treated with
360 $LiAlH_4$, and the distribution of the resulting alcohols released by

361 hydrogenolysis of the acyl chains (Fig. 8c) was compared to that of the free
362 fatty acids from the same sample (Fig. 8b). It appeared from this experiment
363 that the free fatty acids and the alcohols shared the same type of
364 predominance, with $C_{16} > C_{18} > C_{22} > C_{20} = C_{21}$, confirming an origin from
365 fatty acids from suberin for the esterifying moieties.
366 The esters **E3** and **E4** might correspond to the oxidation products of
367 triterpenoids **T5** and **T7** originally esterified with mid-chain unsaturated
368 fatty acid such as those occurring in birch bark suberin (Ekman, 1983;
369 Ferreira et al., 2013) and which have been oxidized during ageing of the
370 material or during tar preparation (Fig. 7). In this respect, it is worth noting
371 that oleic and linoleic acids (both bearing a Δ^9 double bond) are predominant
372 among the unsaturated acids present in suberin. Interestingly, it has been
373 shown that oleic acid can form C_2-C_{12} dicarboxylic acids during
374 photochemical degradation (Rontani, 1998; Tedetti et al., 2007), with azelaic
375 acid (C_9) predominating (Passi et al., 1993; Tedetti et al., 2007), as is the
376 case with the distribution of compounds from series **E3** and **E4** dominated
377 by homologues esterified with a C_9 diacid moiety.

378 **4.3. Mode of preparation of the archaeological birch bark tar**

379 The presence of lupane-related biomarkers and of their thermal degradation
380 products, like Δ^2-betulin derivatives, in the archaeological sample collected
381 from the ceramic I15, clearly indicates that the organic residue corresponds
382 to birch bark tar. As it was found on ancient cracks, it is very likely that it
383 has been used to repair the ceramic (Charters et al., 1993; Connan et al.,

384 2000; Bosquet et al., 2001; Rageot et al., 2016; Urem-Kotsou et al., 2002).
385 However, compared to the distributions generally reported in the literature
386 (Aveling and Heron, 1998, 1999; Courel et al., 2018; Koller et al., 2001), the
387 triterpenoid distribution was unusually dominated by thermal degradation
388 products, whereas genuine compounds from bark, like betulin **T1**, were
389 present in very low abundance. Such a situation has been seldom reported
390 (Urem-Kotsou et al., 2002; Regert et al., 2003), and was interpreted as being
391 the result of a drastic heating during tar preparation. In this respect, birch
392 bark tar making procedures used in the past are still little documented, but
393 thanks to archaeological findings and experimental archaeological research
394 on tar-making, two main procedures known as autothermic and allothermic
395 procedures (Rageot et al., 2019) have been identified. For the former, the
396 raw material is exposed directly to the heat source (Kurt et al., 2008),
397 whereas an indirect heat transfer by a conductor is required for the latter
398 (Rageot et al., 2019). However, according to Rageot et al. (2019), it seems
399 that the autothermic process has been used mainly for preparing conifer tar
400 and not birch bark tar since birch bark is easily flammable. Within the
401 allothermic systems, two main ways of tar production are described: one
402 without separation, and the other with separation, this technique being
403 named “per descensum” (Rageot et al., 2019). In the first case, the tar
404 remains in the reaction chamber with the bark until the end of the process.
405 In the second case, the fresh tar formed gives drops that fall into a second
406 receptacle isolated from the fire. The containers used for tar making could

407 have been ceramics leading to the so called “single-pot” and “double-pot”
408 systems or, for aceramic societies, could be made of clay, sand, ash, turf
409 (Kozowyk et al., 2017; Schenck and Groom, 2018; Rageot et al., 2019). To
410 our knowledge, the oldest allothermic ceramic system known for tar making
411 is dated to the final Bronze Age and was a double-pot system (Dal Ri and
412 Tecchiati, 2003). Ceramic systems and especially the “double-pot” are more
413 frequently encountered in Roman times and in the Middle Age in Europe
414 (Balsan, 1951; Connan et al., 2002; Regert et al., 2003; Trintignac, 2003;
415 Burri, 2009, 2010; Burri et al., 2018). According to the distribution of the
416 triterpenoids, and particularly given the almost absence of genuine
417 triterpenoids from birch bark, it is likely that the archaeological birch bark
418 tar investigated in the present study was prepared using a system without
419 separation. With this method, the produced tar is staying with the bark in
420 the reaction chamber until the end of the heating phase, thus explaining the
421 presence of thermal degradation products in high proportions and the low
422 amounts of betulin **T1** in the sample. This hypothesis is in agreement with
423 the findings of Rageot et al. (2019) who provided new molecular data on tar
424 production by single or double-pot processes and showed that the birch bark
425 tars with low amount of betulin were exclusively produced using the “single-
426 pot” procedure. However, caution is needed since the use of ceramics for tar
427 production is unclear during the Neolithic time (Pietrzak, 2012).

428 **4.4. Triterpenoid esters as new biomarkers of birch bark tar**

429 Dudd and Evershed (1999) originally reported on the presence of fatty acyl
430 esters of betulin-related triterpenoids from archaeological samples. These
431 samples, found in Roman archaeological sites in Great Britain, were
432 interpreted as being the result of the condensation between birch bark tar
433 triterpenoids and fatty acids from animal fat upon strong heating. Since
434 such compounds were never previously reported in birch bark tar, they were
435 considered by the authors as being molecular indicators of a composite
436 material made of birch bark tar and fat. Based on the present work, it seems
437 that esters of triterpenoids can (also) be formed during the preparation of
438 birch bark tar by esterification reactions between birch triterpenoids and
439 thermal degradation products of suberin (i.e., fatty acids, α,ω-dicarboxylic
440 acids) during heating, without the presence of an additional ingredient (i.e.,
441 fat; Fig. 7). In this respect, it is interesting to note that recently, Urem-
442 Kotsou et al. (2018) reported the occurrence of the same fatty acyl
443 triterpenoids than those from the study of Dudd and Evershed (1999), which
444 were interpreted as the result of a mixture between birch bark tar and fat.
445 However, these authors also reported, in the same samples, on the presence
446 of free fatty acids, as well as long and short chain dicarboxylic acids with
447 distributions typical of thermal degradation products of suberin, which
448 could indicate that triterpenoid esters occurring in these samples may also
449 have been directly formed from birch bark during tar preparation. In this
450 case, triterpenoid esters **E1**-**E4** should be considered as molecular indicators
451 of strong thermal processes during birch bark tar preparation, such as those

452 expected to occur in the “single-pot” procedure. The
453 condensation/esterification reactions between the triterpenoid alcohols and
454 the compounds bearing a carboxylic acid functionality may have been
455 favored by an acid catalysis, possibly provided by phenols formed during the
456 pyrolytic degradation of lignin (Faix et al., 1990; Reunanen et al., 1996;
457 Dudd and Evershed, 1999; Regert et al., 2006; Colombini et al., 2009; Orsini
458 et al., 2015). Further chemical investigations of archaeological samples and
459 birch bark tar production experiments should be undertaken to further
460 clarify under which conditions esters of triterpenoids may be formed, the
461 latters representing potentially new molecular tools allowing different
462 modes of preparation of birch bark tar to be distinguished.

463 **5. Conclusion**

464 The set of lipids identified in an organic material found on an ancient crack
465 on a jar from the late Neolithic led to its identification as birch bark tar that
466 has been used to repair the ceramic. Further chemical investigation of the
467 sample allowed identification of four unusual series of triterpenoid esters by
468 synthesis of reference compounds, three of them being reported here for the
469 first time. The investigation of these compounds allowed a new
470 interpretation of their mode of formation to be proposed. We suggest that
471 these markers represent the esterification products between triterpenoid
472 alcohols related to betulin and carboxylic acids from suberin formed upon
473 heating of birch bark tar. They could be indicators of a high level of heating

474 during birch bark tar preparation using the “single pot“ procedure rather
475 than biomarkers resulting from the mixture between birch bark tar and
476 animal fat, as envisaged previously (cf. Dudd and Evershed, 1999; Urem-
477 Kotsou et al., 2018).

478

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661

662 **Figure Captions**

663 **Fig. 1.** Map of the site of La Rouvière **(a)**, Jar I15 discovered at La Rouvière
664 and location of the black organic residues **(b)**. The star indicates sampling
665 location for molecular studies. Drawings by P. Debel.

666

667 **Fig. 2.** Synthesis of **(a)** Δ^2-belulin **T5** and Δ^2-dihydrobetulin **T7**; **(b)**
668 triterpenoid esters **E1a**-**E4a**.

669

670 **Fig. 3.** Mass spectra (EI, 70 eV) of Δ^2-betulin **T5 (a)**, Δ^2-dihydrobetulin **T7**
671 **(b)**, and triterpenoid esters **E1a**-**E4a (c-f)**. The hydroxy group of **T5** and **T7**
672 is analyzed as an acetate derivative and the carboxylic acid group from **E3a**
673 and **E4a** as a methyl ester derivative.

674

675 **Fig. 4.** Gas chromatogram of fraction $F_{A.1}$. Bold numbers refer to structures
676 shown in Appendix. Alcohols are analyzed as acetates and carboxylic acids
677 as methyl esters. Fx: fatty acid, x: number of carbon atoms of the
678 hydrocarbon skeleton.

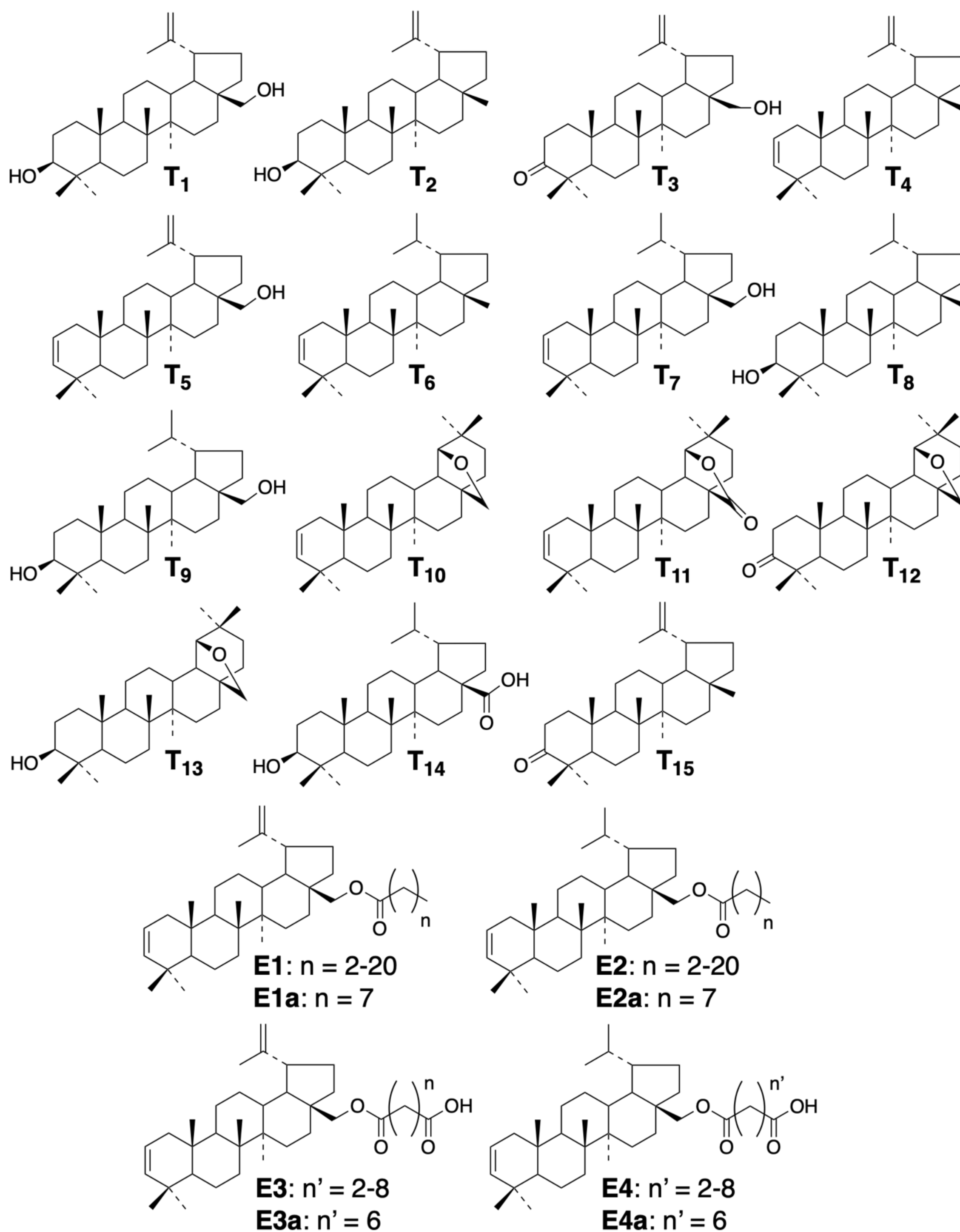
679

680 **Fig. 5.** Partial gas chromatogram (RIC) showing the distribution of the
681 triterpenoid fatty acyl esters from series **E1** and **E2** present in fraction $F_{B.1}$.

682

683 **Fig. 6.** Partial gas chromatogram (RIC) showing the distribution of the
684 triterpenoid dicarboxylic esters from series **E3** and **E4** present in fraction
685 $F_{B.2.2}$. Carboxylic acids are analyzed as methyl esters.
686
687 **Fig. 7.** Hypothetical pathway leading to the formation of the triterpenoid
688 esters from series **E1**-**E4**.
689
690 **Fig. 8. (a)** Partial gas chromatogram (RIC) showing the aliphatic
691 compounds from fraction $F_{A.1}$. Fx: Monocarboxylic fatty acid, Dx:
692 dicarboxylic acid, Wx: ω-hydroxyacid. x: number of carbon atoms. **(b)** Partial
693 mass chromatogram *m/z* 74 showing the distribution of the fatty acids from
694 fraction $F_{A.1}$. **(c)** Partial gas chromatogram (RIC) showing the distribution of
695 the fatty alcohols released by hydrogenolysis using $LiAlH_4$ of the
696 triterpenoid esters **E1**-**E2** present in fraction $F_{B.1}$. Ax: Alcohol. x: number of
697 carbon atoms. Alcohols are analyzed as acetates.
698
699

Appendix: structures cited in the text



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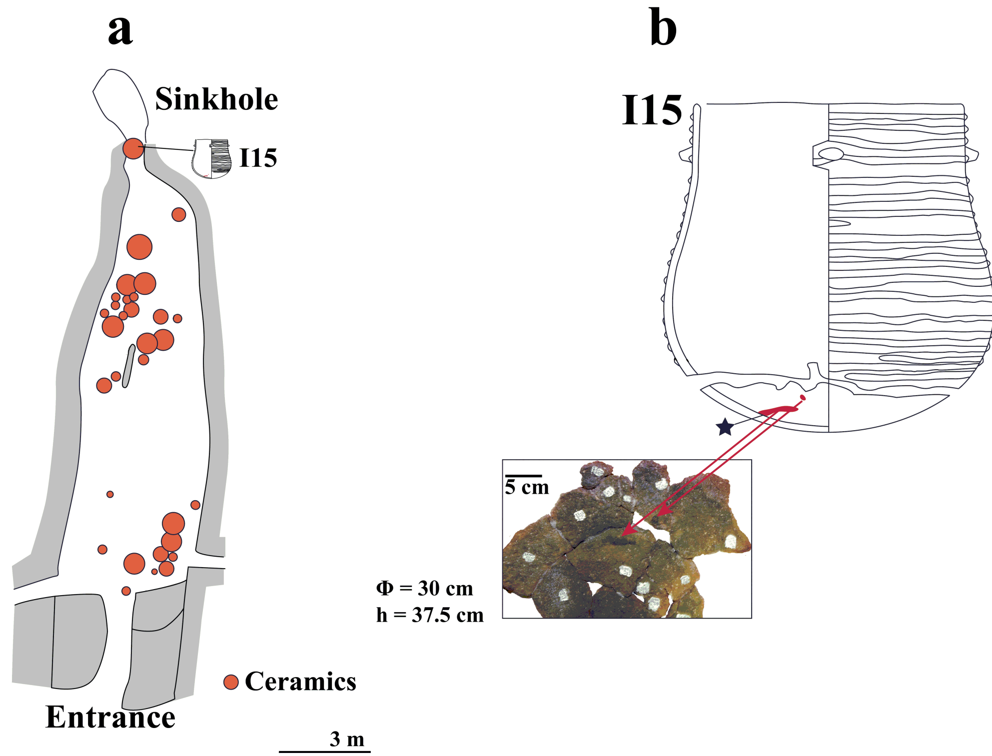

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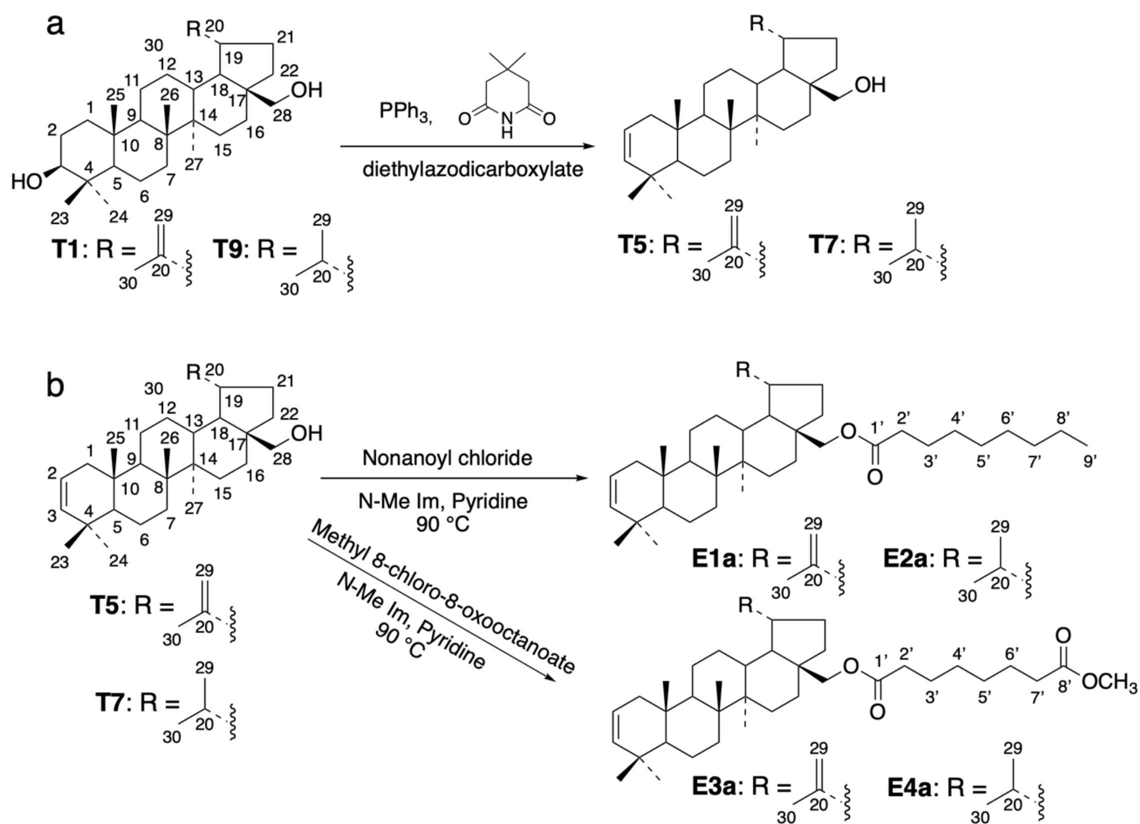

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711 Figure 2

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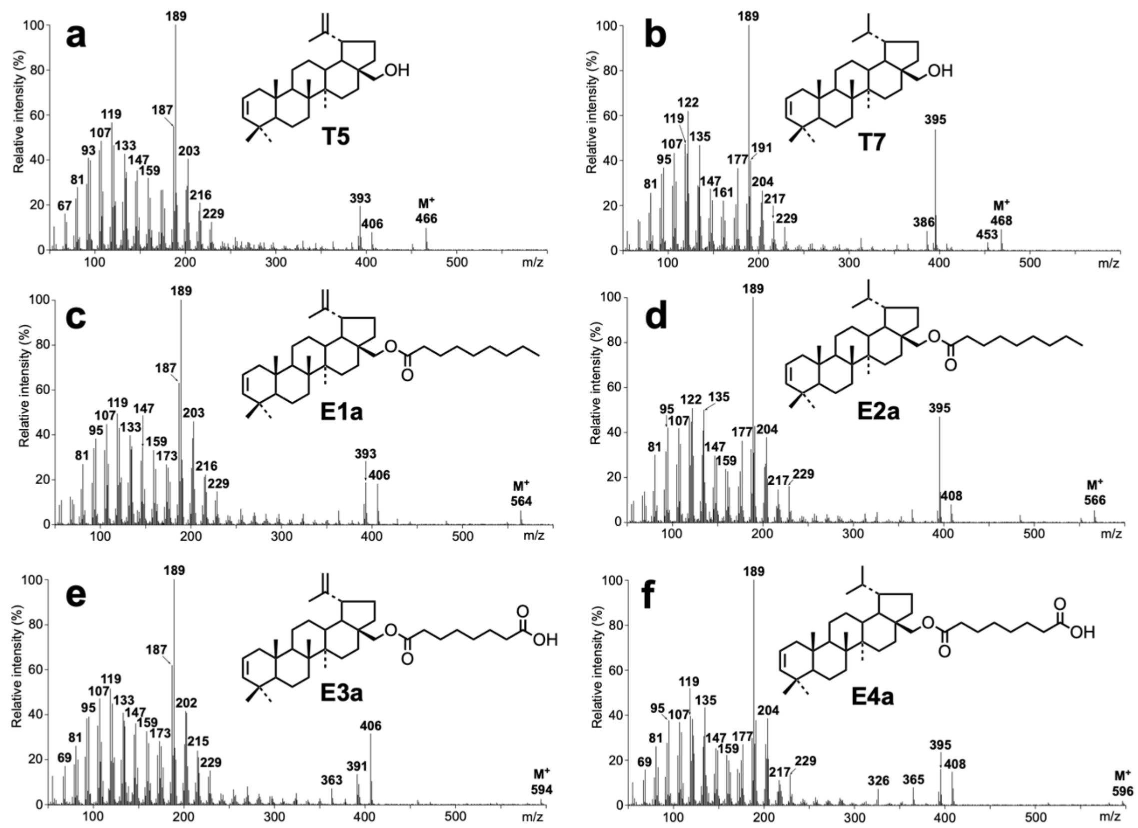

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717 Figure 3

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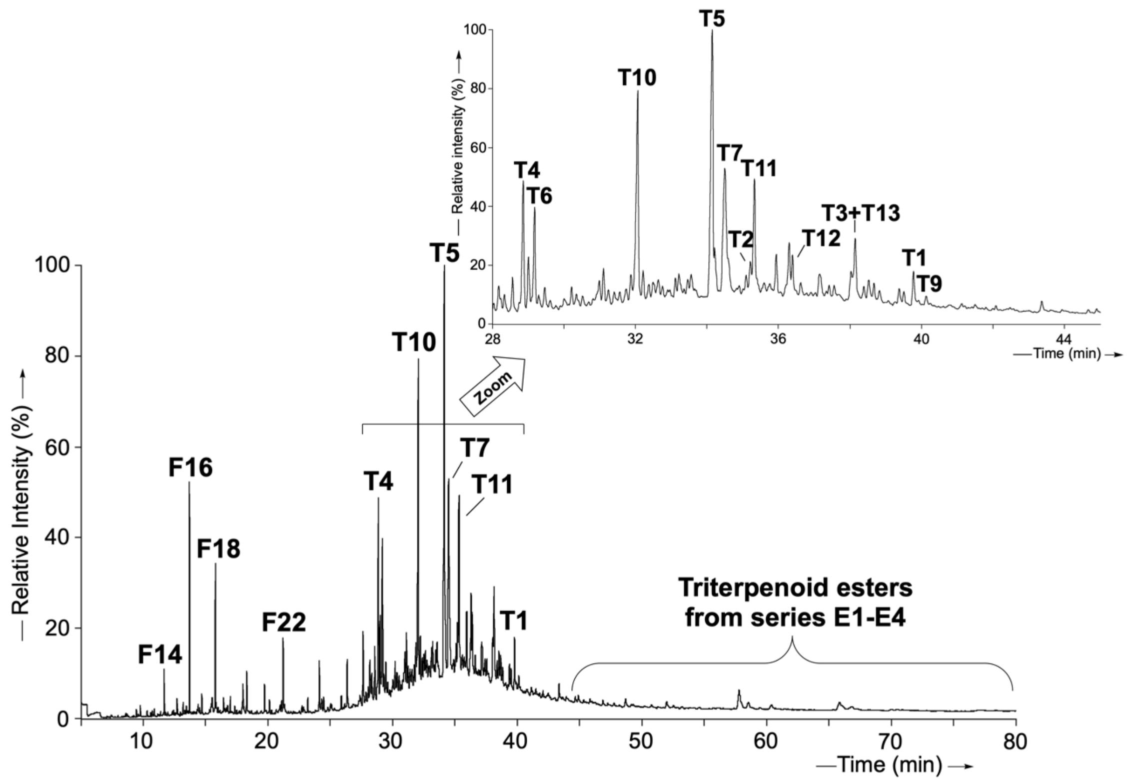

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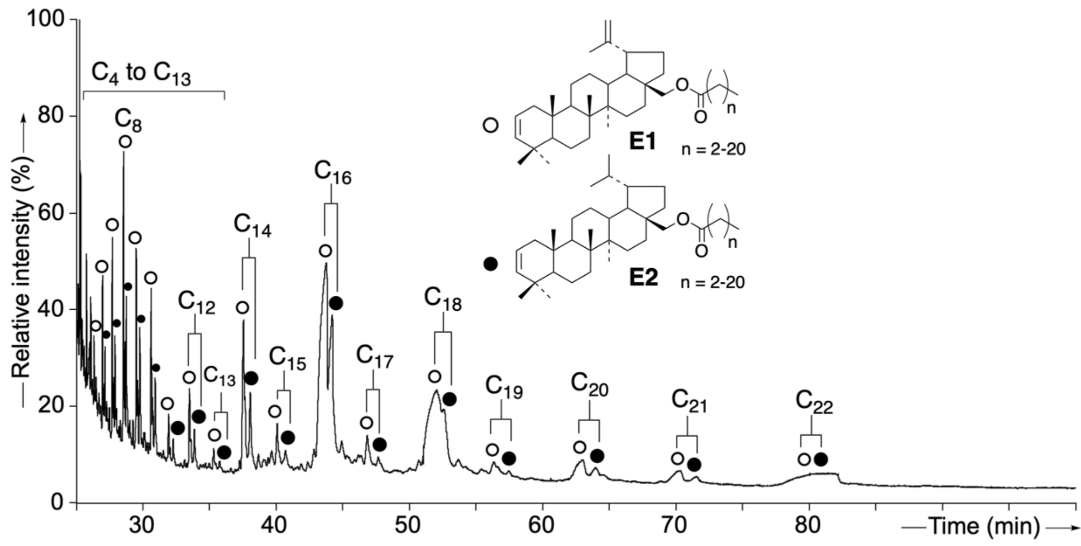

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728 Figure 5

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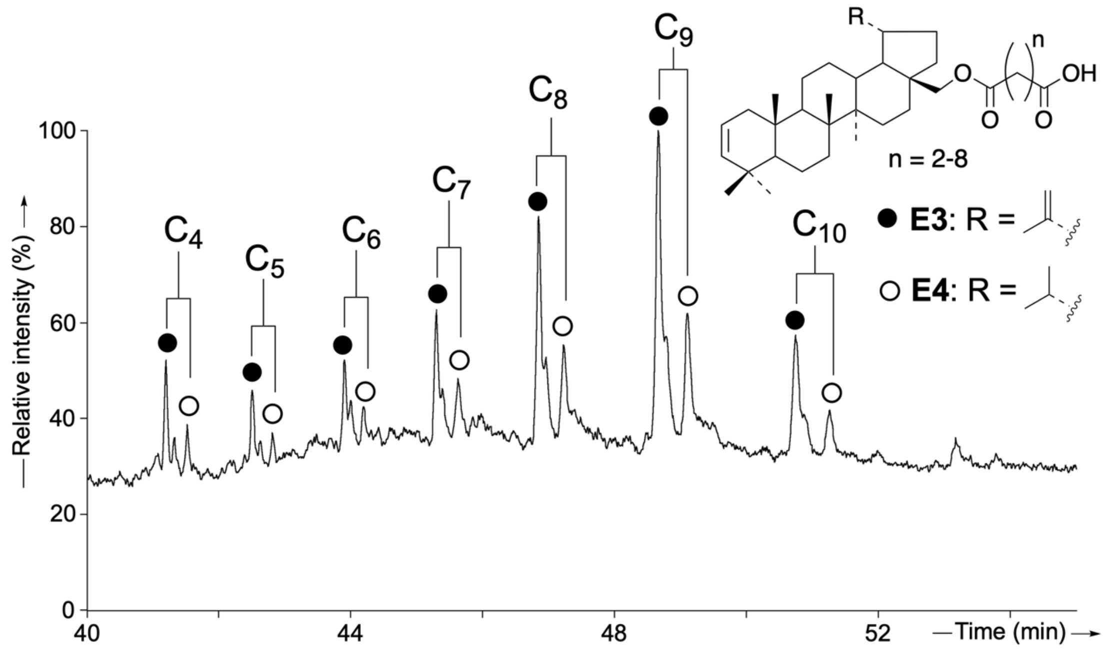

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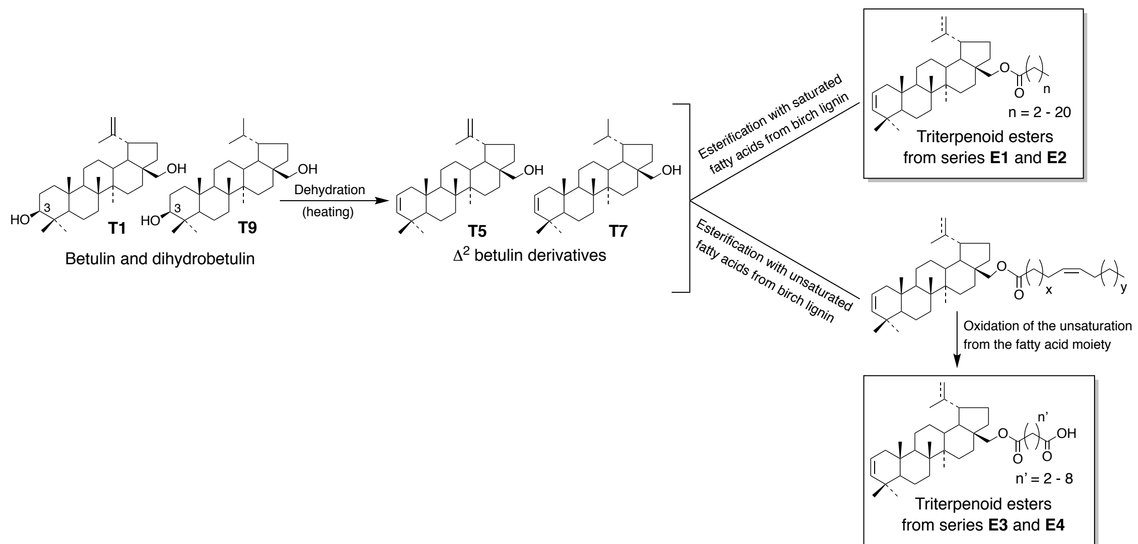

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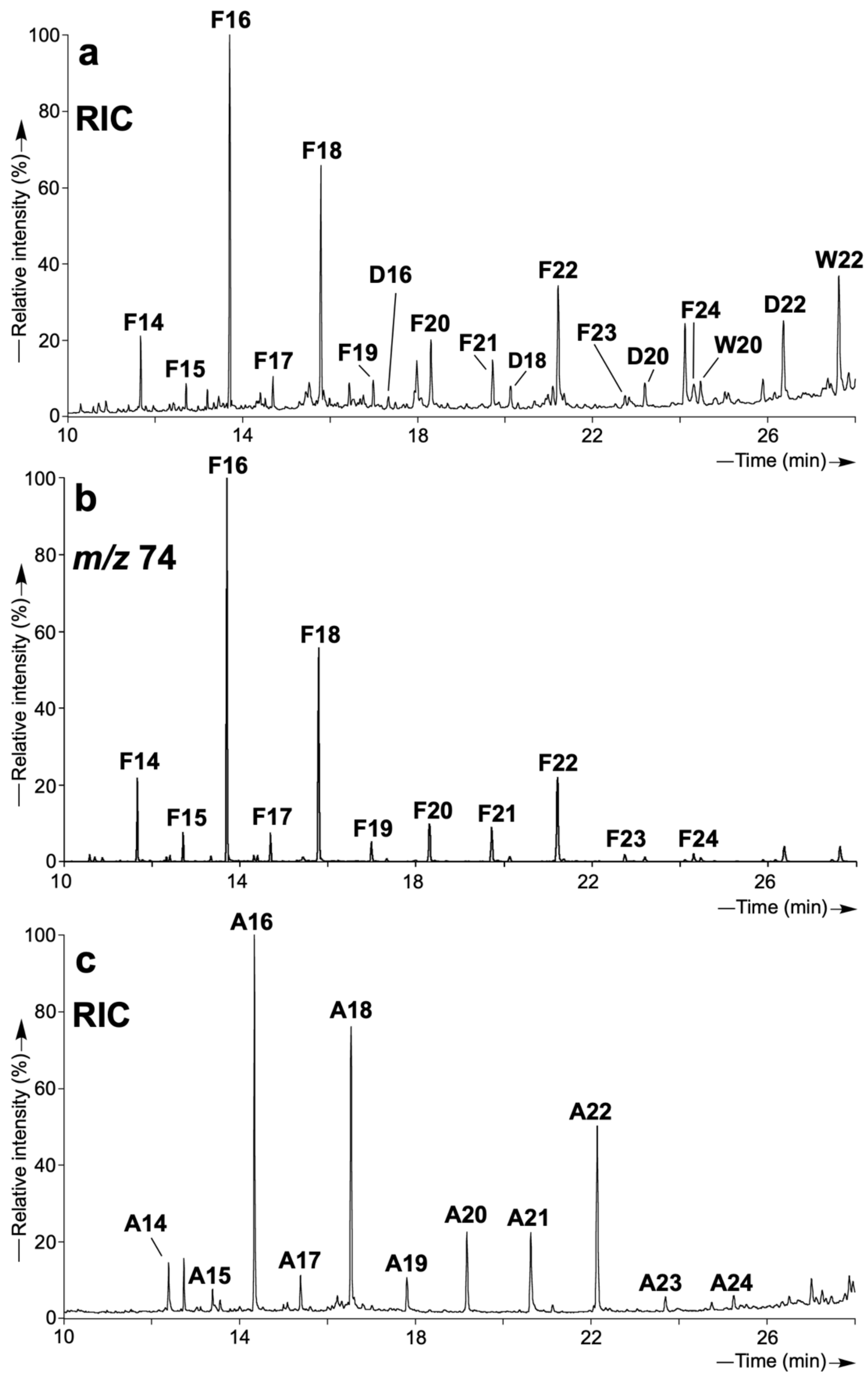

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744 Figure 8