BETULIN-RELATED ESTERS FROM BIRCH BARK TAR: IDENTIFICATION, ORIGIN AND ARCHAEOLOGICAL SIGNIFICANCE

J. Perthuison[1], P. Schaeffer[1*], P. Debels[2], P. Galant[3], P. Adam[1]

[1] University of Strasbourg, CNRS – UMR 7177, Strasbourg, France

[2] University Paul Valéry Montpellier 3 – UMR 5140 and UMR 8215, Montpellier and Paris, France; [3] Regional Service of Archaeology – Occitania, Montpellier, France.

[*] Corresponding Author: Tel: +33 (0)3 68 85 28 05

E-mail address: p.schaef@unistra.fr

Abstract

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

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

Declarations of interest: none

1. Introduction

Natural plant resins and resinous materials have played an important role 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

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

3. Experimental

3.1. Extraction

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

3.2. Fractionation of the solvent extract

3.2.1. Protocol A

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

3.2.2. Protocol B

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

3.3. GC-MS

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

Two oven temperature programs were used:

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

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

3.4. NMR

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

3.5. LiAlH4 hydrogenolysis of ester bonds

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

3.6. Synthesis of reference compounds

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

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

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

in THF (3-4 mL), triphenylphosphine (237 mg, 4 eq) and 3,3-dimethylglutarimide (129 mg, 4 eq) at 0 °C under Ar atmosphere. The mixture was left for 3 h at room temperature. After removal of the solvent under reduced pressure, the crude residue was fractionated by silica gel chromatography with a mixture of EtOAc/CH_2Cl_2 (5:95 v/v) to obtain 68 mg of pure Δ^2-betulin **T5** (respectively 46 mg of Δ^2-dihydrobetulin **T7**) with a yield of 71% (respectively 48%).

*Δ^2-betulin **T5*** (Fig. 3a)*:* GC-MS (acetate derivative of **T5**) (EI, 70 eV) *m/z* (rel. intensity) 466 ($M^{+\cdot}$, 10%), 406 (8), 393 (20), 229 (12), 216 (21), 203 (40), 189 (100), 187 (55), 173 (27), 159 (32), 147 (35), 133 (42), 119 (56), 107 (48), 93 (40). ^{1}H NMR (500 MHz; $CDCl_3$): 0.86 (3H, s), 0.87 (3H, s), 0.94 (3H, s), 0.99 (3H, 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 (1H, d, *J* = 10.5 Hz, H-28), 3.82 (1H, d, *J* = 10.5 Hz, H-28), 4.59 (1H, s, H-29), 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; 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.9, 15.8, 16.5, 19.2, 19.6, 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, 42.9, 47.9, 48.0, 48.9, 49.2, 52.2, 60.7, 109.8, 121.7, 138.1, 150.7.

*Δ^2-dihydrobetulin **T7*** (Fig. 3b)*:* GC-MS (acetate derivative of **T7**) (EI, 70 eV) *m/z* (rel. intensity) 468 ($M^{+\cdot}$, 9%), 408 (3), 395 (54), 229 (10), 217 (13), 204 (26), 191(41), 189 (100), 177 (36), 161 (22), 147 (27), 135 (47), 122 (62), 119 (47), 107 (43), 95 (35). ^{1}H NMR (500 MHz; $CDCl_3$): 0.77 (3H, d, *J* = 7.0 Hz, H-29 or 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 (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;

$CHCl_3$): 14.2, 14.9, 15.9, 16.5, 19.3, 19.6, 21.3, 22.8, 22.8, 25.3, 25.5, 27.2, 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, 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. *Compound **E2a*** (Fig. 3d)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 566 ($M^{+\cdot}$, 5%), 408 (8), 395 (47), 229 (16), 217 (14), 204 (39), 191 (43), 189 (100), 177 (37), 161 (23), 159 (24), 149 (28), 147 (30), 135 (50), 122 (51), 107 (42), 95 (42), 81 (30). ^{1}H NMR (500 MHz; $CDCl_3$): 0.77 (3H, d, *J* = 6.5 Hz, H-29 or H-30), 0.84 (3H, d, *J* = 7.0 Hz, H-29 or H-30), 0.87 (3H, s), 0.88 (3H, s), 1.07 (3H,s), 2.31 (2H, t, *J* = 8.0 Hz, H-2'), 3.83 (1H, d, *J* = 11.0 Hz, H-28), 4.26 (1H, d, *J* = 11.0 Hz, H-28), 5.36 (1H, dd, *J* = 2.0; 10.0 Hz, H-3), 5.41 (1H, ddd, *J*= 1.0; 6.0; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.2, 14.8, 15.1, 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, 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, 46.7, 48.3, 48.9, 52.2, 62.7, 121.7, 138.1, 174.5.

Synthesis of **E3a** and **E4a** (Fig. 2b)

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

removal of the solvent under reduced pressure was fractionated by silica gel chromatography with a mixture of EtOAc/CH_2Cl_2 (5:95 v/v) to obtain 8.9 mg (49 % yield) of pure **E3a** (respectively 10.1 mg of **E4a**, 55 % yield).

Compound ***E3a*** (Fig. 3e)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 594 ($M^{+\cdot}$, 2%), 406 (31), 391 (13), 363 (7), 229 (15), 215 (23), 203 (41), 202 (41), 189 (100), 187 (62), 173 (28), 159 (32), 147 (36), 133 (41), 119 (51), 107 (47), 95 (39), 81 (26). ^{1}H NMR (500 MHz; $CDCl_3$): 0.86 (3H, s), 0.87 (3H, s), 0.94 (3H, 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' 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, H-19), 3.66 (3H, s, OCH_3), 3.86 (1H, d, *J* = 11.5 Hz, H-28), 4.27 (1H, d, *J* = 11.0 Hz, H-28), 4.59 (1H, s, H-29), 4.69 (1H, s, H-29), 5.35 (1H, dd, *J* = 2.0; 10.0 Hz, H-3), 5.39 (1H, ddd, *J* = 1.0; 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.9, 15.9, 16.5, 19.3, 19.6, 21.3, 22.8, 24.9, 25.0, 25.5, 27.2, 28.9, 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, 46.6, 47.9, 48.9, 49.2, 51.6, 52.3, 62.7, 110.0, 121.7, 138.1, 150.4, 174.3, 174.3.

Compound ***E4a*** (Fig. 3f)*:* GC-MS (EI, 70 eV) *m/z* (rel. intensity) 596 ($M^{+\cdot}$, 2%), 408 (15), 395 (16), 365 (8), 326 (7), 229 (13), 217 (11), 204 (39), 191 (38), 189 (100), 177 (27), 159 (22), 149 (24), 147 (25), 135 (43), 119 (40), 107 (37), 95 (38), ^{1}H NMR (500 MHz; $CDCl_3$): 0.77 (3H, d, *J* = 7.0 Hz, H-29 or H-30), 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), 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* = 7.5 Hz, H-2' or H-7'), 3.66 (3H, s, OCH_3), 3.83 (1H, d, *J* = 11.0 Hz, H-28),

4.26 (1H, d, J = 11.0 Hz, H-28), 5.36 (1H, dd, J = 2.0; 10.0 Hz, H-3), 5.40 (1H, ddd, J = 1.0; 5.5; 10.0 Hz, H-2). ^{13}C NMR (500 MHz; $CHCl_3$): 14.8, 15.1, 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, 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, 48.3, 48.9, 51.6, 52.2, 62.8, 121.7, 138.1, 174.3, 174.3.

4. Results and discussion

4.1. Triterpenoids as markers of birch bark tar

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

biomarkers of birch bark tar (Bosquet et al., 2001; Modugno et al., 2006; Regert et al., 2006; Courel et al., 2018; Rageot et al., 2019).

Δ^2-triterpenoids are formed by elimination of the oxygenated function at C-3 of lupeol **T2** and betulin **T1**, this reaction being generally induced by a thermal treatment as is the case for the preparation of birch bark tar (Courel et al., 2018; Rageot et al., 2019). Similarly, it was shown that the same thermal treatment leads to the formation of allobetulane derivatives (**T10**-**T13)** which results from an acid-catalyzed intramolecular rearrangement of the ring *E* of betulin and by-products (Davy et al., 1951a, 1951b; Green et al., 2007; Salvador et al., 2009). In some cases, both reactions may co-occur, resulting in the formation of Δ^2-allobetulin derivatives such as **T10** and **T11**.

Similar observations regarding birch bark tar composition, in which alteration products dominate over genuine ones, are reported in the literature in the case of archaeological samples (e.g., Urem-Kotsou et al., 2002; Regert et al., 2003), and were interpreted as being the result of an intense heating of the material upon tar preparation. Such seems to be the case with our sample as well which was likely submitted to an abnormally high thermal stress, resulting in the almost complete transformation of the genuine triterpenoids (Fig. 4). In addition to these triterpenoids closely related to birch bark tar, four unusual late-eluted compound series were detected (named "series **E1**-**E4**"; Fig. 4), which present fragmentation 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

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

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

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

hydrogenolysis of the acyl chains (Fig. 8c) was compared to that of the free fatty acids from the same sample (Fig. 8b). It appeared from this experiment that the free fatty acids and the alcohols shared the same type of predominance, with $C_{16} > C_{18} > C_{22} > C_{20} = C_{21}$, confirming an origin from fatty acids from suberin for the esterifying moieties.

The esters **E3** and **E4** might correspond to the oxidation products of triterpenoids **T5** and **T7** originally esterified with mid-chain unsaturated fatty acid such as those occurring in birch bark suberin (Ekman, 1983; Ferreira et al., 2013) and which have been oxidized during ageing of the material or during tar preparation (Fig. 7). In this respect, it is worth noting that oleic and linoleic acids (both bearing a Δ^9 double bond) are predominant among the unsaturated acids present in suberin. Interestingly, it has been shown that oleic acid can form C_2-C_{12} dicarboxylic acids during photochemical degradation (Rontani, 1998; Tedetti et al., 2007), with azelaic acid (C_9) predominating (Passi et al., 1993; Tedetti et al., 2007), as is the case with the distribution of compounds from series **E3** and **E4** dominated by homologues esterified with a C_9 diacid moiety.

4.3. Mode of preparation of the archaeological birch bark tar

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

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

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

4.4. Triterpenoid esters as new biomarkers of birch bark tar

Dudd and Evershed (1999) originally reported on the presence of fatty acyl esters of betulin-related triterpenoids from archaeological samples. These samples, found in Roman archaeological sites in Great Britain, were interpreted as being the result of the condensation between birch bark tar triterpenoids and fatty acids from animal fat upon strong heating. Since such compounds were never previously reported in birch bark tar, they were considered by the authors as being molecular indicators of a composite material made of birch bark tar and fat. Based on the present work, it seems that esters of triterpenoids can (also) be formed during the preparation of birch bark tar by esterification reactions between birch triterpenoids and thermal degradation products of suberin (i.e., fatty acids, α,ω-dicarboxylic acids) during heating, without the presence of an additional ingredient (i.e., fat; Fig. 7). In this respect, it is interesting to note that recently, Urem-Kotsou et al. (2018) reported the occurrence of the same fatty acyl triterpenoids than those from the study of Dudd and Evershed (1999), which were interpreted as the result of a mixture between birch bark tar and fat. However, these authors also reported, in the same samples, on the presence of free fatty acids, as well as long and short chain dicarboxylic acids with distributions typical of thermal degradation products of suberin, which could indicate that triterpenoid esters occurring in these samples may also have been directly formed from birch bark during tar preparation. In this case, triterpenoid esters **E1**-**E4** should be considered as molecular indicators 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
479 **Acknowledgements**
480 Mrs E. Motsch (University of Strasbourg) is thanked for the GC-MS
481 analyses and Dr L. Allouche (University of Strasbourg) for the measurement
482 of the NMR spectra. J.P. has benefited from a doctoral fellowship from the
483 “French Ministère de l’Enseignement Supérieur et de la Recherche”.

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Figure Captions

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

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

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

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

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

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.
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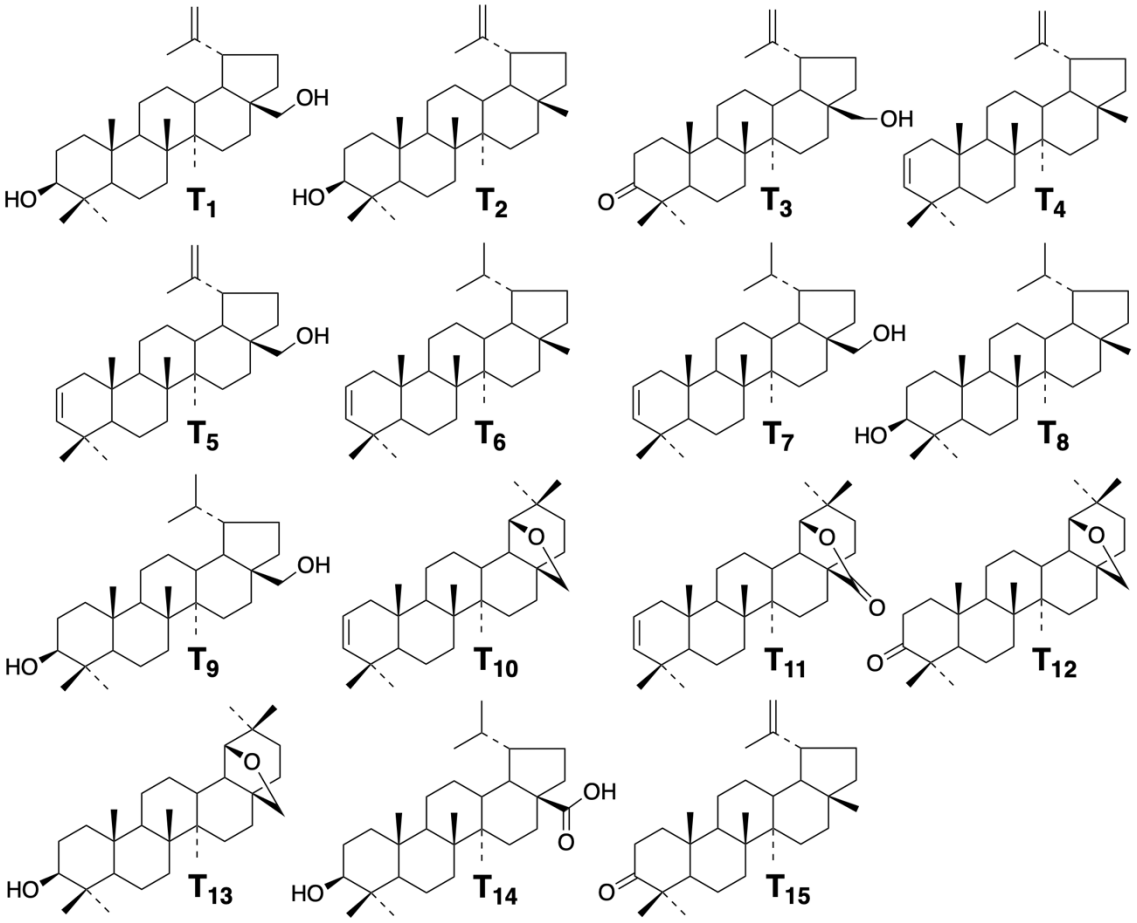
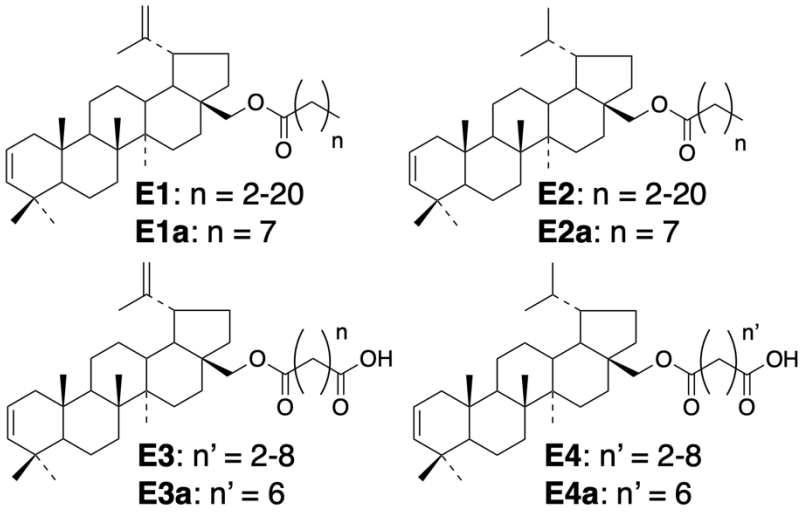
Appendix: structures cited in the text





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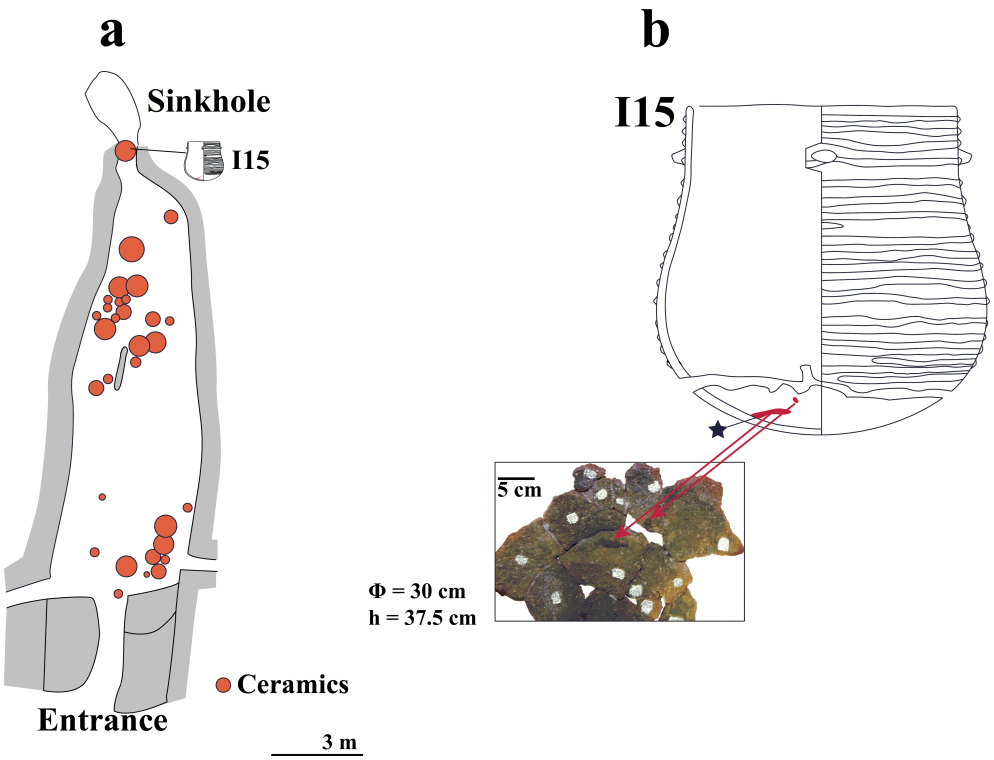

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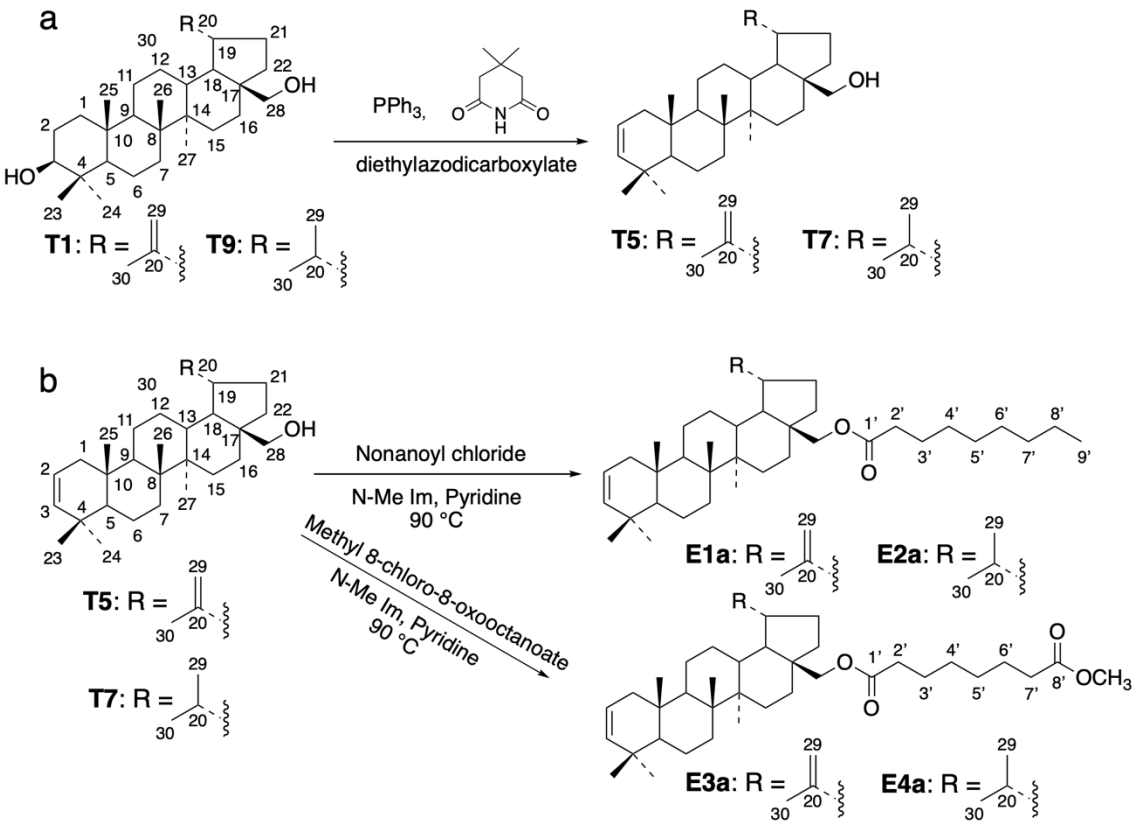

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711 Figure 2

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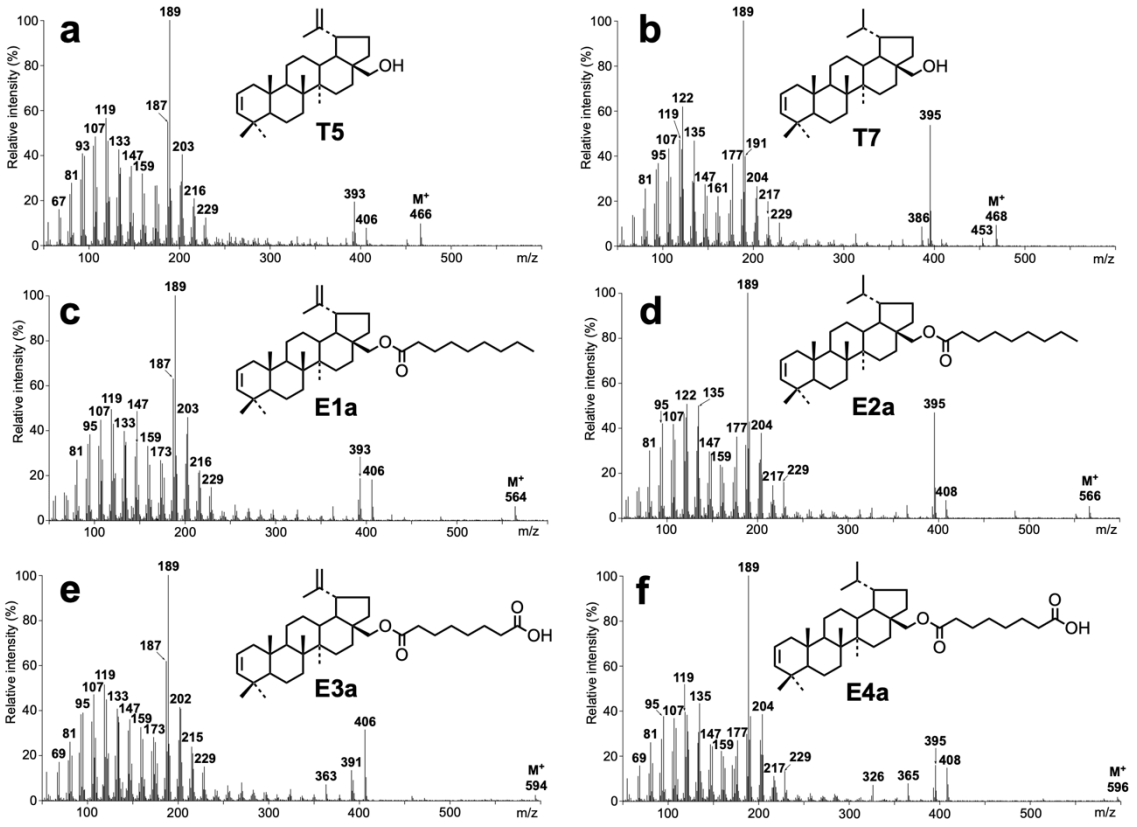

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717 Figure 3

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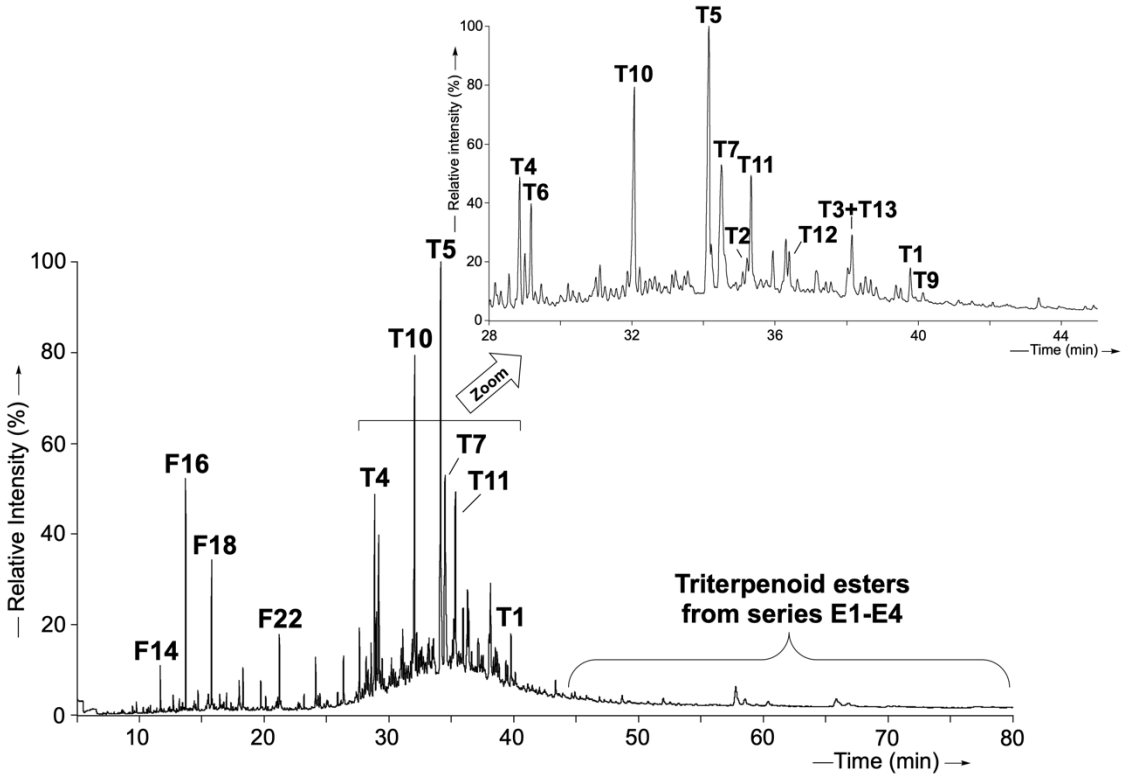

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723 Figure 4

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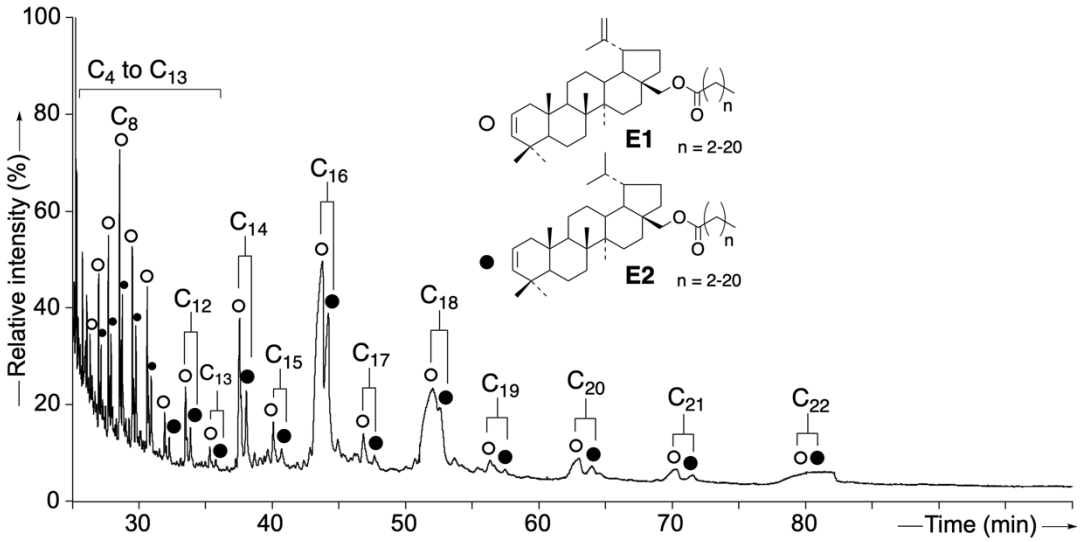

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728 Figure 5

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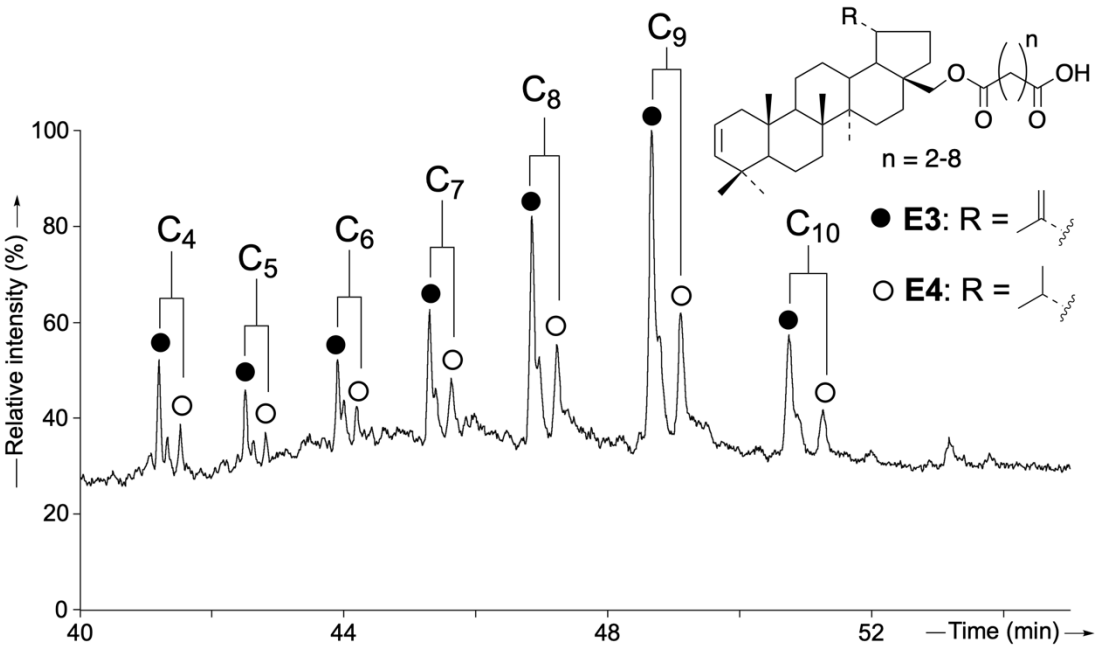

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733 Figure 6

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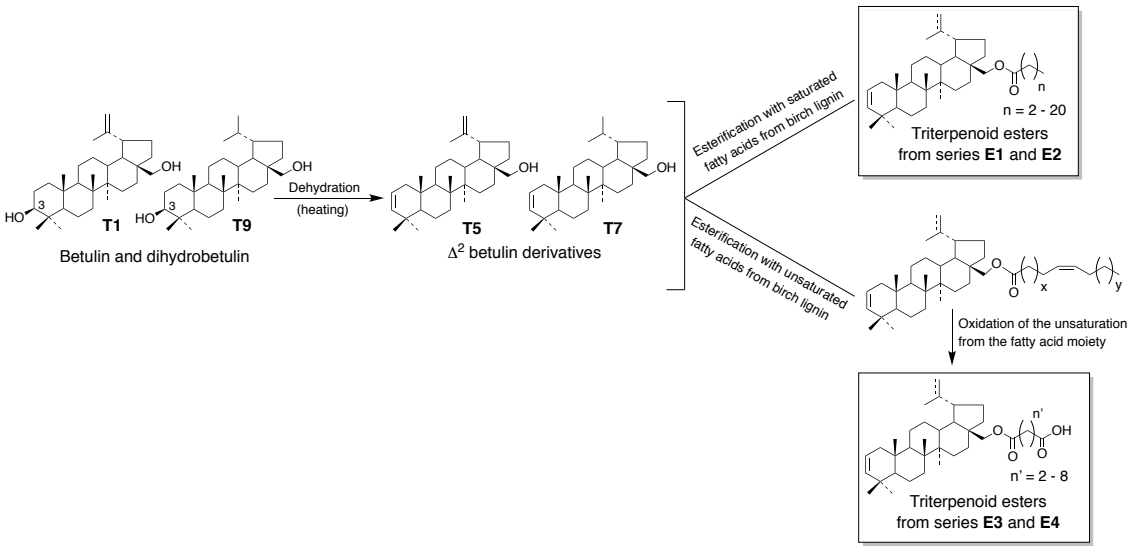

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739 Figure 7

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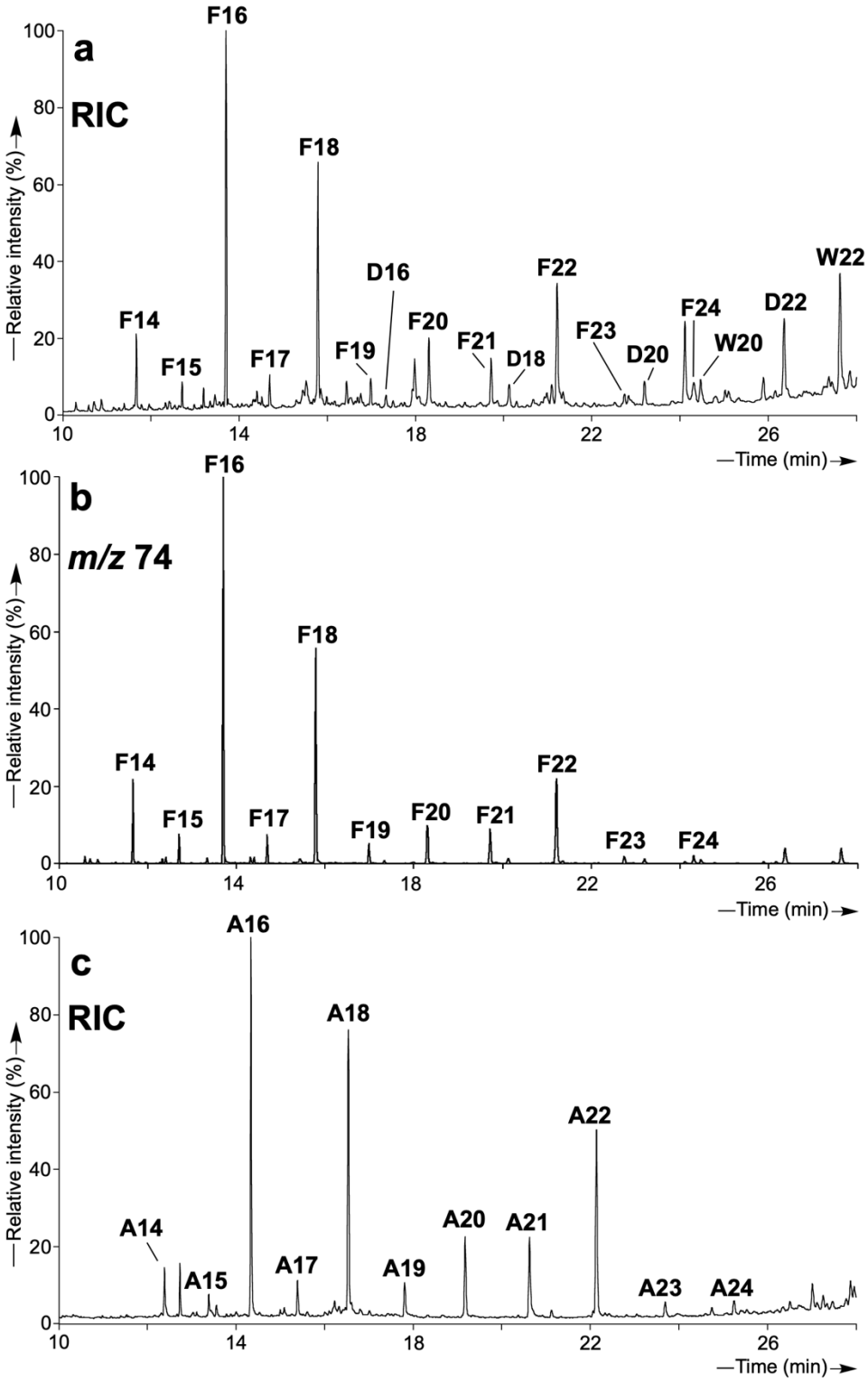

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744 Figure 8