Manuscript Number: OG-4284R1

Title: Betulin-related esters from birch bark tar: identification, origin and archaeological significance

Article Type: Research Paper

Keywords: Archaeology; Birch bark tar; Lupane-related triterpenoids; Triterpenoid esters; Neolithic

Corresponding Author: Dr. Philippe Schaeffer, PhD

Corresponding Author's Institution: Laboratoire de Biogéochimie Moléculaire, UMR 7177 du CNRS, Université de Strasbourg

First Author: Philippe Schaeffer, PhD

Order of Authors: Philippe Schaeffer, PhD; Julien Perthuisson, PhD student; Pauline Debels, PhD student; Philippe Galant; Pierre Adam, PhD

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.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:

Data can be available upon request upon agreement of all co-authors

Highlights

Betulin-related esters in archaeological samples identified via synthesis

Formed by reaction of suberin fatty acids and betulin-related triterpenoids

Potential new biomarkers of strong heating upon tar preparation

**Betulin-related esters from birch bark tar: identification, origin and
archaeological significance**

J. Perthuisson¹, P. Schaeffer^{1*}, P. Debels², P. Galant³, P. Adam¹

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

archaeological sites. Among them, birch bark tar is a vegetal substance produced by dry distillation of birch bark (Aveling and Heron, 1998; Urem-Kotsou et al., 2002; Rageot et al., 2016; Courel et al., 2018). This sticky and hydrophobic material has been used since the Paleolithic (Koller et al., 2001; Grünberg, 2002) for various purposes, like the hafting of lithic tools or jewellery (Sauter et al., 2000; Bosquet et al., 2001; Koller et al., 2001; Courel et al., 2018), the repairing or caulking of ceramics (Charters et al., 1993; Connan et al., 2000; Bosquet et al., 2001; Rageot et al., 2016; Reunanen et al., 1993; Urem-Kotsou et al., 2002), the decorating of ceramic vessels (Vogt, 1949) and has even been used as chewing-gum (Aveling and Heron, 1999; Karg et al., 2014). Birch bark tar has a typical molecular composition, making it easy to identify by detection of predominant lupane-related triterpenoids using gas chromatography coupled to mass spectrometry - GC-MS - (Hayek et al., 1989, 1990; Reunanen et al., 1996; Aveling and Heron, 1998; Schnell et al., 2014).

As part of a study dedicated to the use of pottery during the Neolithic, we have investigated an organic black substance having most likely served to repair different ceramics (Fig. 1) found at the site of La Rouvière (Rogues, Gard, Occitanie, Southern France) and dated to the late Neolithic (ca. 3000 BCE). GC-MS analysis of this substance led to its identification as birch bark tar. Along with typical triterpenoid markers from the lupane series, four series of uncommon compounds eluted late on the gas chromatogram 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 CH₂Cl₂/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 (Fig. 2)

An aliquot (ca. 4.2 mg) of the extract was acetylated (Ac₂O, 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 CH₂Cl₂/EtOAc (8:2, v/v; 3 dead volumes - D_{vol} -; 3.6 mg) and a more polar fraction (F_{A.2}) not further investigated eluted with CH₂Cl₂/MeOH (1:1, v/v; 2 D_{vol}; ca. 1.4 mg). F_{A.1} was analyzed by GC-MS. Note that fraction FA.2 being eluted using MeOH, it may contain some dissolved silica gel.

3.2.2. Protocol B (Fig. 2)

An aliquot of the extract (ca. 20.8 mg) was fractionated by liquid chromatography (LC) on silica gel without derivatization. A first fraction ($F_{B.1}$; 2.3 mg) containing the fatty acyl esters from series **E1** and **E2** was recovered by elution with CH_2Cl_2 (1.4 D_{vol}). An aliquot of the second fraction ($F_{B.2}$; 18.9 mg) 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 $F_{B.2}$ (9.8 mg) was re-fractionated into three fractions. The first fraction ($F_{B.2.1}$ eluted with CH_2Cl_2 ; 1.25 D_{vol} ; 0.6 mg) was shown to contain mainly methylated fatty acids, and the second fraction ($F_{B.2.2}$; 1.2 mg), also eluted with CH_2Cl_2 (2 D_{vol}), comprised dicarboxylic esters from series **E3** and **E4**. The last eluted one ($F_{B.2.3}$; 8.0 mg), 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 ; 0.1 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 (CDCl₃: δ¹H 7.26 ppm ; δ¹³C 77.16 ppm).

3.5. LiAlH₄ hydrogenolysis of ester bonds (Fig. 2)

Fraction F_{B,1} (cf. § 3.2.2.) was submitted to hydrogenolysis using LiAlH₄. A small amount of LiAlH₄ (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 (Ac₂O/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. 3a)

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/CH₂Cl₂ (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**: GC-MS data (acetate derivative of **T5**; EI, 70 eV): see Fig. 4a. ¹H NMR (500 MHz; CDCl₃): 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). ¹³C NMR (500 MHz; CHCl₃): 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**: GC-MS data (acetate derivative of **T7**; EI, 70 eV): see Fig. 4b. ¹H NMR (500 MHz; CDCl₃): 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, $J = 11.0$ Hz, H-28), 5.36 (1H, dd, $J = 2.5; 10.0$ Hz, H-3), 5.40 (1H, ddd, $J = 1.5; 5.5; 10.0$ Hz, H-2). ^{13}C NMR (500 MHz; CHCl_3): 14.8, 15.1, 15.8, 16.5, 19.6, 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, 41.2, 41.4, 43.0, 44.7, 48.1, 48.2, 48.9, 52.2, 60.8, 121.7, 138.1.

3.6.2. Esterification of betulin derivatives

Synthesis of **E1a** and **E2a** (Fig. 3b)

A solution containing Δ^2 -betulin **T5** (14.7 mg) (respectively Δ^2 -dihydrobetulin **T7**, 17.9 mg), nonanoyl chloride (50 μL , ca. 7 eq), N-methylimidazole (N-Me Im, one drop) and pyridine (0.6 mL) was placed in a vial at 90 °C for 4 h. After transfer of the mixture into a separatory funnel and addition of an aqueous solution of CuSO_4 (3 mL, 9/1 wt:wt), the organic layer was recovered using CH_2Cl_2 . The organic phase was washed with distilled water, and the solvent removed under reduced pressure. The crude residue was fractionated by silica gel chromatography with toluene to obtain 4.3 mg of pure **E1a** with a yield of 22% (resp. 4.4 mg of **E2a**; 19%, yield).

Compound E1a: GC-MS data (EI, 70 eV): see Fig. 4c. ^1H NMR (500 MHz; CDCl_3): 0.94 (3H, s), 0.99 (3H, s), 1.06 (3H, s), 1.69 (3H, s, H-30), 2.32 (2H, t, $J = 7.5$ Hz, H-2'), 2.46 (1H, td, $J = 6.0; 11.5$ Hz, H-19), 3.86 (1H, d, $J = 11.0$ Hz, H-28), 4.26 (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), 5.40 (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,

202 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,
203 121.7, 138.1, 150.4, 174.5.
204 *Compound E2a*: GC-MS data (EI, 70 eV): see Fig. 4d. ¹H NMR (500 MHz;
205 CDCl₃): 0.77 (3H, d, *J* = 6.5 Hz, H-29 or H-30), 0.84 (3H, d, *J* = 7.0 Hz, H-29
206 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'),
207 3.83 (1H, d, *J* = 11.0 Hz, H-28), 4.26 (1H, d, *J* = 11.0 Hz, H-28), 5.36 (1H, dd,
208 *J* = 2.0; 10.0 Hz, H-3), 5.41 (1H, ddd, *J* = 1.0; 6.0; 10.0 Hz, H-2). ¹³C NMR
209 (500 MHz; CHCl₃): 14.2, 14.8, 15.1, 15.9, 16.5, 19.6, 21.3, 21.8, 22.8, 22.8,
210 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,
211 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,
212 138.1, 174.5.

213 Synthesis of **E3a** and **E4a** (Fig. 3b)

214 A solution containing 12.9 mg of Δ²-betulin **T5** (resp. 13 mg of Δ²-
215 dihydrobetulin **T7**), methyl 8-chloro-8-oxooctanoate (50 μL, 11 eq), N-methyl
216 imidazole (N-Me Im) (one drop) and pyridine (0.6 mL, was placed in a vial at
217 90 °C for 4 h. To remove pyridine and N-Me Im, the mixture was transferred
218 into a separatory funnel, shaken with 3 mL of an aqueous solution of CuSO₄
219 (9/1 wt:wt) and extracted 2x with CH₂Cl₂ and 2x with EtOAc. The combined
220 organic extracts were evaporated under reduced pressure, dissolved in
221 EtOAc and washed with distilled water. The crude residue obtained after
222 removal of the solvent under reduced pressure was fractionated by silica gel
223 chromatography with a mixture of EtOAc/CH₂Cl₂ (5:95 v/v) to obtain 8.9 mg
224 (49 % yield) of pure **E3a** (respectively 10.1 mg of **E4a**, 55 % yield).

225 *Compound E3a*: GC-MS (EI, 70 eV): see Fig. 4e. ¹H NMR (500 MHz; CDCl₃):
226 0.86 (3H, s), 0.87 (3H, s), 0.94 (3H, s), 0.98 (3H, s), 1.06 (3H, s), 1.68 (3H, s,
227 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
228 H-7'), 2.45 (1H, td, $J = 5.5; 11.5$ Hz, H-19), 3.66 (3H, s, OCH₃), 3.86 (1H, d, J
229 = 11.5 Hz, H-28), 4.27 (1H, d, $J = 11.0$ Hz, H-28), 4.59 (1H, s, H-29), 4.69
230 (1H, s, H-29), 5.35 (1H, dd, $J = 2.0; 10.0$ Hz, H-3), 5.39 (1H, ddd, $J = 1.0; 5.5;$
231 10.0 Hz, H-2). ¹³C NMR (500 MHz; CHCl₃): 14.9, 15.9, 16.5, 19.3, 19.6, 21.3,
232 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,
233 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,
234 121.7, 138.1, 150.4, 174.3, 174.3.

235 *Compound E4a*: GC-MS data (EI, 70 eV): see Fig. 4f. ¹H NMR (500 MHz;
236 CDCl₃): 0.77 (3H, d, $J = 7.0$ Hz, H-29 or H-30), 0.84 (3H, d, $J = 7.0$ Hz, H-29
237 or H-30), 0.87 (3H, s), 0.88 (3H, s), 0.94 (3H, s), 0.96 (3H, s), 1.07 (3H, s),
238 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'),
239 3.66 (3H, s, OCH₃), 3.83 (1H, d, $J = 11.0$ Hz, H-28), 4.26 (1H, d, $J = 11.0$ Hz,
240 H-28), 5.36 (1H, dd, $J = 2.0; 10.0$ Hz, H-3), 5.40 (1H, ddd, $J = 1.0; 5.5; 10.0$
241 Hz, H-2). ¹³C NMR (500 MHz; CHCl₃): 14.8, 15.1, 15.9, 16.5, 19.6, 21.3, 21.8,
242 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,
243 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,
244 121.7, 138.1, 174.3, 174.3.

245 4. Results and discussion

246 4.1. Triterpenoids as markers of birch bark tar

247 The gas chromatogram of fraction F_{A.1} (cf. § 3.2.1.) isolated from the
248 archaeological sample is shown in Fig. 5. Its lipid distribution was
249 dominated by triterpenoids from the lupane series, indicating a major
250 contribution from a vegetal source derived from angiosperms (Fig. 5). The
251 triterpenoids notably comprised betulin **T1** (see the appendix for the
252 structures of the compounds cited in the text), lupeol **T2** and betulone **T3**
253 which are native triterpenoids occurring in birch bark (Hayek et al., 1989,
254 1990; Schnell et al., 2014). However, these compounds represent generally
255 by far the predominant triterpenoids in the case of fresh (or even weathered)
256 birch bark (Hayek et al., 1990; Aveling and Heron, 1998; Schnell et al.,
257 2014; Courel et al., 2018), whereas they appear only as minor constituents
258 in the archaeological sample. In the latter case, the distribution is
259 dominated by compounds **T4**, **T5**, **T10**, **T7** and **T11**, which all belong to the
260 series of Δ^2 -betulin-related triterpenoids. These compounds, together with
261 the allobetulane derivatives **T10-T13**, are known from the literature to be
262 biomarkers of birch bark tar (Bosquet et al., 2001; Modugno et al., 2006;
263 Regert et al., 2006; Courel et al., 2018; Rageot et al., 2019).
264 Δ^2 -triterpenoids are formed by elimination of the oxygenated function at C-3
265 of lupeol **T2** and betulin **T1**, this reaction being generally induced by a
266 thermal treatment as is the case for the preparation of birch bark tar
267 (Courel et al., 2018; Rageot et al., 2019). Similarly, it was shown that the
268 same thermal treatment leads to the formation of allobetulane derivatives
269 (**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 original ones, are reported in the literature in the case of archaeological samples (e.g., Urem-Kotsou et al., 2002; Regert et al., 2003b), 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 huge predominance of triterpenoids formed by thermal processes and the almost absence of the triterpenoids originally occurring in birch bark like betulin **T1**, the by far predominant triterpenoid in fresh material (Fig. 5). In addition to these triterpenoids closely related to birch bark tar, four unusual late-eluted compound series were detected (named “series **E1-E4**”; Fig. 5), which present fragmentation patterns in MS (Fig. 4c-4f) very similar to those of Δ^2 -betulin **T5** (Fig. 4a) and Δ^2 -dihydrobetulin **T7** (Fig. 4b) but with quite higher molecular masses in the range 494-748 Da. In order to have a more detailed look to these different compounds, a fractionation procedure of the lipid extract was developed and led to obtaining chromatographic fractions considerably enriched in triterpenoids from series **E1-E4** (cf. § 3.2.2).

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

4.2.1. Identification of triterpenoid esters from series E1-E2

Detailed investigation of fraction F_{B.1} (cf. § 3.2.2.) revealed the occurrence of compounds belonging to the series **E1** and **E2** (Fig. 6). Homologues from series **E1** have a mass fragmentation pattern very similar to that of Δ^2 betulin **T5** (Fig. 4a and 4c) and a molecular weight of $494 + n \times 14$ ($n = 0-18$), while those from series **E2** have mass spectra close to that of Δ^2 dihydrobetulin **T7** (Fig. 4b and 4d) and with a molecular weight shifted upwards by 2 mass units compared to series **E1** (i.e., M^+ of $496 + n \times 14$, $n = 0-18$). According to the literature, the generic structure of compounds from series **E1** has been previously proposed to correspond to Δ^2 betulin **T5** esterified at C-28 with saturated monocarboxylic acids of different chain lengths (Dudd and Evershed, 1999). By analogy, it can be proposed that compounds from the series **E2** have the same generic structure, but without the $\Delta^{20(29)}$ unsaturation. For both series, the fatty acyl moiety is ranging from C₄ to at least C₂₂ (Fig. 6). However, since the original structural identification of compounds from series **E1** was based on MS interpretations and hydrolysis experiments (Dudd and Evershed, 1999), thus remaining tentative, we have carried out the synthesis of one reference compound from each series **E1** and **E2** (Fig. 3 and § 3.6.) for firm identification. Briefly, the hydroxy group at C-3 from betulin **T1** (resp. dihydrobetulin **T9**) was selectively dehydrated following the method reported by Sun et al. (1998b), leading to the formation of Δ^2 -betulin **T5** (resp. Δ^2 -dihydrobetulin **T7**). The remaining hydroxy group at C-28 from **T5** (resp. **T7**) was then esterified

with nonanoyl chloride to yield the C₉ ester of Δ²-betulin **E1a** (resp. **E2a**). Since the mass spectra and retention times in GC of the synthetic and naturally-occurring compound **E1a** (resp. **E2a**) were identical as shown by co-injection experiments, it can be considered that both compound series have been successfully identified.

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

The fraction F_{B,2,2} recovered after purification of the solvent extract (cf. § 3.2.2.) revealed the occurrence of late eluting compounds from series **E3** and **E4** (Fig. 7). Like compounds from series **E1**, the mass spectra of the homologues from series **E3** (Fig. 4e) showed close similarities with those of Δ²-betulin **T5** (Fig. 4a) but with molecular ions at $538 + n \times 14$ ($n = 0-6$). Similarly, the mass spectra of compounds from series **E4** (Fig. 4f) closely resemble that of Δ²-dihydrobetulin **T7** (Fig. 4b), with a molecular weight of $540 + n \times 14$ ($n = 0-6$). Based on these data, it was proposed that compounds from series **E3** (resp. **E4**) could correspond to dicarboxylic acids esterified at the C-28 hydroxy group of Δ²-betulin **T5** (resp. Δ²-dihydrobetulin **T7**). For both series, the dicarboxylic acid moieties comprised C₄-C₁₀ homologues with a predominance of the C₈ and C₉ 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. 3b). Mass spectra of the synthetic references and of the archaeological sample

were in good agreement, as were retention times (co-injection experiments), 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. 8), 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 C₁₆, C₁₈ and C₂₂ homologues, together with that of C₂₀-C₂₂ α,ω -hydroxyacids and C₁₆-C₂₂ α,ω -diacids (Fig. 9a). 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 LiAlH₄, and the distribution of the resulting alcohols released by hydrogenolysis of the acyl chains (Fig. 9c) was compared to that of the free fatty acids from the same sample (Fig. 9b). 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. 8). 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.

It should be noted that Dudd and Evershed (1999) report on the occurrence of an additional series of triterpenoid esters which could correspond to lupeol derivatives esterified with fatty acids at the C-3 position - and not at C-28 as are the triterpenoids from series **E1**-**E4** -, this identification relying on mass spectrometry data and on the presence of lupeol **T2** among the triterpenoids released after KOH/MeOH methanolysis. In our sample, this C-3-esterified series could not be detected, and this is in line with the absence of C-3 oxygenated lupane triterpenoids among the hydrogenolysis products recovered after $LiAlH_4$ treatment (Δ^2 -triterpenoids were almost

exclusively obtained after hydrogenolysis). Furthermore, given the presence of betulin **T1** within the saponification products, Dudd and Evershed (1999) proposed that triterpenoids “bis-esters” could also be formed by esterification of betulin **T2** with fatty acids both at the C-3 and C-28 positions. In our sample, the presence of such “bis-esters” is unlikely given the absence of betulin among the free-occurring triterpenoids as well as among the triterpenoids obtained after LiAlH₄ treatment.

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 original 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., 2003b), 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., 2003b; Trintignac, 2003; Burri, 2009, 2010; Burri et al., 2018). According to the distribution of the triterpenoids, and particularly given the

almost absence of original 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 would be 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. 8). 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 expected to occur in the “single-pot” procedure. The condensation/esterification reactions between the triterpenoid alcohols and the compounds bearing a carboxylic acid functionality may have been favored by an acid catalysis, possibly provided by phenols formed during the pyrolytic degradation of lignin (Faix et al., 1990; Reunanen et al., 1996; Dudd and Evershed, 1999; Regert et al., 2006; Colombini et al., 2009; Orsini et al., 2015).

It can be noted that in several cases reported in the literature (e.g., Charters et al., 1993; Regert et al., 2003a; Lucquin et al., 2007; Mitkidou et al., 2008; Mirabaud et al., 2015), the presence of a hump and/or of compounds eluted

after betulin **T1** can be observed on the gas chromatograms of the lipid fraction from birch bark tar, and it is possible that triterpenoid esters could be part of these late-eluted compounds. Further chemical investigations of archaeological samples and birch bark tar production experiments should be undertaken to identify these novel compound series in a wider sample set and to further clarify under which conditions esters of triterpenoids may be formed, the latter representing potentially new molecular tools allowing different modes of preparation of birch bark tar to be distinguished.

5. Conclusion

The set of lipids identified in an organic material found on an ancient crack on a jar from the late Neolithic led to its identification as birch bark tar that has been used to repair the ceramic. Further chemical investigation of the sample allowed identification of four unusual series of triterpenoid esters by synthesis of reference compounds, three of them being reported here for the first time. The investigation of these compounds allowed a new interpretation of their mode of formation to be proposed. We suggest that these markers represent the esterification products between triterpenoid alcohols related to betulin and carboxylic acids from suberin formed upon heating of birch bark tar. They could be indicators of a high level of heating during birch bark tar preparation using the “single pot” procedure rather than biomarkers resulting from the mixture between birch bark tar and

animal fat, as envisaged previously (cf. Dudd and Evershed, 1999; Urem-Kotsou et al., 2018).

Acknowledgements

Mrs E. Motsch (University of Strasbourg) is thanked for the GC-MS analyses and Dr L. Allouche (University of Strasbourg) for the measurement of the NMR spectra. J.P. has benefited from a doctoral fellowship from the “French Ministère de l’Enseignement Supérieur et de la Recherche”. We thank the two anonymous reviewers for their comments and suggestions.

References

- Aveling, E.M., Heron, C., 1998. Identification of birch bark tar at the Mesolithic site of Star Carr. *Ancient Biomolecules* 2, 69–80.
- Aveling, E.M., Heron, C., 1999. Chewing tar in the early Holocene: an archaeological and ethnographic evaluation. *Antiquity* 73, 579–584.
- Balsan, L., 1951. L’industrie de la résine dans les Causses et son extension dans l’Empire romain. *Gallia* 9, 53–55.
- Bosquet, D., Regert, M., Dubois, N., Jadin, I., 2001. Identification de brai de bouleau sur quatre vases du site rubané de Fexhe-le-Haut-Clocher “Potri l’Cortri”. *Premiers résultats. Notae Praehistoricae* 21, 119–127.
- Burri, S., 2009. La fouille programmée d’un site de distillation du bois à la Roche Redonne (Le Castellet, Var). *Revue du Centre Archéologique du Var*, 31–34.

518 Burri, S., 2010. Production et commerce de la poix et de l'huile de cade en
 519 basse Provence au Moyen Âge. *Anthropobotanica* 1, 3–17.

520 Burri, S., Alifriqui, M., Bun, S., Cençon-Salvayre, C., Ciğerci, İ., Cloarec-
 521 Quillon, A., Corbineau, R., Delgado Robles, A.A., Durand, A., El Jemli, M.,
 522 Fernandez, X., Genin, D., Ghanmi, M., Konuk, M., Le Maguer-Gillon, S.,
 523 Liman, R., Marmouzi, I., Mazuy, A., Ollivier, D., Yılancıoğlu, K., 2018. Des
 524 ressources naturelles à la santé: Approche interdisciplinaire de la
 525 production des goudrons de conifères et de leur usage médicinal en
 526 Méditerranée sur la longue durée. *Les Nouvelles de l'Archéologie* 152, 62–69.

527 Charters, S., Evershed, R.P., Goad, L.J., Heron, C., Blinkhorn, P., 1993.
 528 Identification of an adhesive used to repair a Roman jar. *Archaeometry* 35,
 529 91–101.

530 Colombini, M.P., Lucejko, J.J., Modugno, F., Orlandi, M., Tolppa, E.-L., Zoia,
 531 L., 2009. A multi-analytical study of degradation of lignin in archaeological
 532 waterlogged wood. *Talanta* 80, 61–70.

533 Connan, J., Adam, P., Dessort, D., Albrecht, P., 2000. Apport de la chimie
 534 moléculaire à la connaissance des enduits utilisés pour le traitement des
 535 bois et le calfatage des bateaux romains de la Saône. In: *Archéologie des*
 536 *fleuves et des rivières*. Bonnamour, L. (Ed.), Errance, Paris, pp. 40–47.

537 Connan, J., Maurin, B., Long, L., Sebire, H., 2002. Identification de poix et
 538 de résine de conifère dans des échantillons archéologiques du lac de
 539 Sanguinet : exportation de poix en Atlantique à l'époque gallo-romaine.
 540 *Revue d'Archéométrie* 26, 177–196.

541 Courel, B., 2016. Caractérisation de substances naturelles en contexte
 542 archéologique: apport des études moléculaires, isotopiques et de la datation
 543 au ^{14}C . PhD thesis, Université de Strasbourg, France.

544 Courel, B., Schaeffer, P., Féliu, C., Thomas, Y., Adam, P., 2018. Birch bark
 545 tar and jewellery: The case study of a necklace from the Iron Age
 546 (Eckwersheim, NE France). *Journal of Archaeological Science: Reports* 20,
 547 72–79.

548 Dal Ri, L., Tecchiati, U., 2003. Una distilleria per la pece della fine dell'età
 549 del Bronzo dal lago di Ledro nel Trentino (loc. Longhini-Assat), in:
 550 Attraverso Le Alpi. Stuttgart: Uomini, Vie, Beni, Konrad Theiss Verlag, pp.
 551 175–181.

552 Davy, G.S., Halsall, T.G., Jones, E.R.H., Meakins, G.D., 1951a. The
 553 chemistry of the triterpenes. Part IX. Elucidation of the betulin-oleanolic
 554 acid relationship. *Journal of the Chemical Society*, 2696-2702.

555 Davy, G.S., Halsall, T.G., Jones, E.R.H., Meakins, G.D., 1951b. The
 556 chemistry of the triterpenes. Part X. The structures of some isomerization
 557 products from betulin and betulinic acid. *Journal of the Chemical Society*,
 558 2702-2705.

559 Dudd, S.N., Evershed, R.P., 1999. Unusual triterpenoid fatty acyl ester
 560 components of archaeological birch bark tars. *Tetrahedron Letters* 40, 359–
 561 362.

562 Ekman, R., 1983. The suberin monomers and triterpenoids from the outer
 563 bark of *Betula verrucosa* Ehrh. *Holzforschung* 37, 205–211.

564 Faix, O., Meier, D., Fortmann, I., 1990. Thermal degradation products of
 565 wood. *Holz als Roh- und Werkstoff* 48, 281–285.

566 Ferreira, R., Garcia, H., Sousa, A.F., Freire, C.S.R., Silvestre, A.J.D., Rebelo,
 567 L.P.N., Silva Pereira, C., 2013. Isolation of suberin from birch outer bark
 568 and cork using ionic liquids: A new source of macromonomers. *Industrial*
 569 *Crops and Products* 44, 520–527.

570 Galant, P., Halgand, J., Camus, H., Delaporte, S., 2000. L’aven de la
 571 Rouvière: de la découverte spéléologique à l’étude archéologique. *Grands*
 572 *Causses – Préhistoire et Archéologie* 1, 32-63.

573 Galant, P., Halgand, J., 2004. Rogues - Aven de la Rouvière-. ADLFI.
 574 *Archéologie de la France - Informations, Languedoc-Roussillon*, 2-11.
 575 <http://journals.openedition.org/adlfi/11582>

576 Green, B., Bentley, M.D., Chung, B.Y., Lynch, N.G., Jensen, B.L., 2007.
 577 Isolation of betulin and rearrangement to allobetulin. A biomimetic natural
 578 product synthesis. *Journal of Chemical Education* 84, 1985–1987.

579 Grünberg, J.M., 2002. Middle Palaeolithic birch-bark pitch. *Antiquity* 76,
 580 15–16.

581 Hayek, E.W.H., Jordis, U., Moche, W., Sauter, F., 1989. A bicentennial of
 582 betulin. *Phytochemistry* 28, 2229–2242.

583 Hayek, E.W.H., Krenmayr, P., Lohninger, H., Jordis, U., Moche, W., Sauter,
 584 F., 1990. Identification of archaeological and recent wood tar pitches using
 585 gas chromatography/mass spectrometry and pattern recognition. *Analytical*
 586 *Chemistry* 62, 2038–2043.

587 Holloway, P.J., 1972. The composition of suberin from the corks of *Quercus*
 588 *suber* L. and *Betula pendula* roth. *Chemistry and Physics of Lipids* 9, 158–
 589 170.

590 Karg, S., Hansen, U.L., Walldén, A.M., Glastrup, J., Ærenlund Pedersen, H.,
 591 Sonne Nielsen, F.O., 2014. Vegetal grave goods in a female burial on
 592 Bornholm (Denmark) from the Late Roman Iron Age period interpreted in a
 593 comparative European perspective. *Danish Journal of Archaeology* 3, 52–60.

594 Koller, J., Baumer, U., Mania, D., 2001. High-tech in the Middle
 595 Palaeolithic: Neandertal-manufactured pitch identified. *European Journal*
 596 *of Archaeology* 4, 385–397.

597 Kozowyk, P.R.B., Soressi, M., Pomstra, D., Langejans, G.H.J., 2017.
 598 Experimental methods for the Palaeolithic dry distillation of birch bark:
 599 implications for the origin and development of Neandertal adhesive
 600 technology. *Scientific Reports* 7. doi:10.1038/s41598-017-08106-7

601 Kurt, Y., Suleyman Kaçar, M., Isik, K., 2008. Traditional tar production
 602 from *Cedrus libani* A. Rich on the Taurus mountains in Southern Turkey.
 603 *Economic Botany* 62, 615–620.

604 Lucquin, A., March, R.J., Cassen, S., 2007. Analysis of adhering organic
 605 residues of two “coupes-à-socles” from the Neolithic funerary site “La
 606 Hougue Bie” in Jersey: evidences of birch bark tar utilisation. *Journal of*
 607 *Archaeological Science* 34, 704-710.

608 Mirabaud, S., Petrequin, A.M., Petrequin, P., Regert M., 2015. Système de
 609 production des adhésifs exploités à Clairvaux VII et à Clairvaux XIV, in :
 610 Clairvaux et le « NMB ». Besançon, PUF et CRAVA, tome 2, 1001-1021.

611 Mitkidou, S., Dimitrakoudi, E., Urem-Kotsou, D., Papadopoulou, D.,
 612 Kotsakis, K., Stratis, J.A., Stephanidou-Stephanatou, I., 2008. Organic
 613 residue analysis of Neolithic pottery from North Greece. *Microchimica Acta*
 614 160, 493-498.

615 Modugno, F., Ribechini, E., Maria Perla Colombini, 2006. Chemical study of
 616 triterpenoid resinous materials in archaeological findings by means of direct
 617 exposure electron ionisation mass spectrometry and gas
 618 chromatography/mass spectrometry. *Rapid Communications in Mass*
 619 *Spectrometry* 20, 1787–1800.

620 Orsini, S., Ribechini, E., Modugno, F., Klügl, J., Di Pietro, G., Colombini, M.,
 621 2015. Micromorphological and chemical elucidation of the degradation
 622 mechanisms of birch bark archaeological artefacts. *Heritage Science* 3, 2. doi
 623 10.1186/s40494-015-0032-7

624 Passi, S., Picardo, M., De Luca, C., Nazzaro-Porro, M., Rossi, L., Rotilio, G.,
 625 1993. Saturated dicarboxylic acids as products of unsaturated fatty acid

626 oxidation. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid*
 627 *Metabolism* 1168, 190–198.

628 Pietrzak, S., 2012. Wood tar in the Dnieper and Elbe communities: VI - II
 629 millenium BC. In: *Baltic-Pontic studies* 17, Kosko, A. (Ed), p. 44.

630 Rageot, M., Pêche-Quilichini, K., Py, V., Filippi, J.-J., Fernandez, X., Regert,
 631 M., 2016. Exploitation of beehive products, plant exudates and tars in
 632 Corsica during the early Iron Age. *Archaeometry* 58, 315–332.

633 Rageot, M., Théry-Parisot, I., Beyries, S., Lepère, C., Carré, A., Mazuy, A.,
 634 Filippi, J.-J., Fernandez, X., Binder, D., Regert, M., 2019. Birch bark tar
 635 production: Experimental and biomolecular approaches to the study of a
 636 common and widely used prehistoric adhesive. *Journal of Archaeological*
 637 *Method and Theory* 26, 276-312.

638 Regert, M., Alexandre, V., Thomas, N., Lattuati-Derieux, A., 2006.
 639 Molecular characterisation of birch bark tar by headspace solid-phase
 640 microextraction gas chromatography–mass spectrometry: A new way for
 641 identifying archaeological glues. *Journal of Chromatography A* 1101, 245–
 642 253.

643 Regert, M., Garnier, N., Decavallas, O., Cren-Olivé, C., Rolando, C., 2003a.
 644 Structural characterization of lipid constituents from natural substances
 645 preserved in archaeological environments. *Measurement Science and*
 646 *Technology* 14, 1620-1630.

647 Regert, M., Vacher, S., Moulherat, C., Decavallas, O., 2003b. Adhesive
648 production and pottery function during the Iron Age at the site of Grand
649 Aunay (Sarthe, France). *Archaeometry* 45, 101–120.

650 Reunanen, M., Ekman, R., Hafizoğlu, H., 1996. Composition of tars from
651 softwoods and birch. *Holzforschung* 50, 118–120.

652 Reunanen, M., Holmbom, B., Edgren, T., 1993. Analysis of archaeological
653 birch bark pitches. *Holzforschung* 47, 175–177.

654 Rontani, J.-F., 1998. Photodegradation of unsaturated fatty acids in
655 senescent cells of phytoplankton: photoproduct structural identification and
656 mechanistic aspects. *Journal of Photochemistry and Photobiology A:*
657 *Chemistry* 114, 37–44.

658 Salvador, J.A.R., Pinto, R.M.A., Santos, R.C., Le Roux, C., Beja, A.M.,
659 Paixão, J.A., 2009. Bismuth triflate-catalyzed Wagner-Meerwein
660 rearrangement in terpenes. Application to the synthesis of the 18 α -oleanane
661 core and A-*neo*-18 α -oleanene compounds from lupanes. *Organic and*
662 *Biomolecular Chemistry* 7, 508–517.

663 Sauter, F., Jordis, U., Graf, A., Werther, W., Varmuza, K., 2000. Studies in
664 organic archaeometry I: Identification of the prehistoric adhesive used by
665 the “Tyrolean Iceman” to fix his weapons. *Arkivoc* 2000, 735–747.

666 Schenck, T., Groom, P., 2018. The aceramic production of *Betula pubescens*
667 (downy birch) bark tar using simple raised structures. A viable Neanderthal
668 technique? *Archaeological and Anthropological Sciences* 10, 19–29.

669 Schnell, G., Schaeffer, P., Tardivon, H., Motsch, E., Connan, J., Ertlen, D.,
 670 Schwartz, D., Schneider, N., Adam, P., 2014. Contrasting diagenetic
 671 pathways of higher plant triterpenoids in buried wood as a function of tree
 672 species. *Organic Geochemistry* 66, 107–124.

673 Sun, I.-C., Shen, J.-K., Wang, H.-K., Cosentino, L.M., Lee, K.-H., 1998a.
 674 Anti-AIDS agents. 32.¹ synthesis and anti-HIV activity of betulin
 675 derivatives. *Bioorganic & Medicinal Chemistry Letters* 8, 1267–1272.

676 Sun, I.-C., Wang, H.-K., Kashiwada, Y., Shen, J.-K., Cosentino, L.M., Chen,
 677 C.-H., Yang, L.-M., Lee, K.-H., 1998b. Anti-AIDS agents. 34.[†] Synthesis and
 678 structure–activity relationships of betulin derivatives as anti-HIV agents.
 679 *Journal of Medicinal Chemistry* 41, 4648–4657.

680 Tedetti, M., Kawamura, K., Narukawa, M., Joux, F., Charrière, B., Sempéré,
 681 R., 2007. Hydroxyl radical-induced photochemical formation of dicarboxylic
 682 acids from unsaturated fatty acid (oleic acid) in aqueous solution. *Journal of*
 683 *Photochemistry and Photobiology A: Chemistry* 188, 135–139.

684 Trintignac, A., 2003. La production de poix dans la cité des Gabales (Lozère)
 685 à l'époque gallo-romaine. *Revue Archéologique de Picardie* 1, 239–248.

686 Urem-Kotsou, D., Mitkidou, S., Dimitrakoudi, E., Kokkinos, N., Ntinou, M.,
 687 2018. Following their tears: Production and use of plant exudates in the
 688 Neolithic of North Aegean and the Balkans. *Quaternary International* 496,
 689 68–79.

690 Urem-Kotsou, D., Stern, B., Heron, C., Kotsakis, K., 2002. Birch-bark tar at
691 Neolithic Makriyalos, Greece. *Antiquity* 76, 962–967.

692 Vogt, E., 1949. The Birch as a source of raw material during the Stone Age.
693 *Proceedings of the Prehistoric Society* 15, 50–51.

694

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. Flow chart summarizing the separation protocols used for the analysis of the birch bark tar sample (protocol A) and for the characterization of the ester triterpenoids from series **E1-E4** (protocol B).

Fig. 3. Synthesis of **(a)** Δ^2 -betulins **T5** and Δ^2 -dihydrobetulin **T7**; **(b)** triterpenoid esters **E1a-E4a**.

Fig. 4. Mass spectra (EI, 70 eV) of Δ^2 -betulins **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. 5. 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. F_x : fatty acid, x: number of carbon atoms of the hydrocarbon skeleton.

Fig. 6. Partial gas chromatogram (RIC) showing the distribution of the triterpenoid fatty acyl esters from series **E1** and **E2** present in fraction $F_{B.1}$.

Fig. 7. Partial gas chromatogram (RIC) showing the distribution of the triterpenoid dicarboxylic esters from series **E3** and **E4** present in fraction $F_{B.2.2}$. Carboxylic acids are analyzed as methyl esters.

Fig. 8. Hypothetical pathway leading to the formation of the triterpenoid esters from series **E1-E4**.

Fig. 9. (a) Partial gas chromatogram (RIC) showing the aliphatic compounds from fraction $F_{A.1}$. F_x : Monocarboxylic fatty acid, D_x : dicarboxylic acid, W_x : ω -hydroxyacid. x : number of carbon atoms. **(b)** Partial mass chromatogram m/z 74 showing the distribution of the fatty acids from fraction $F_{A.1}$. **(c)** Partial gas chromatogram (RIC) showing the distribution of the fatty alcohols released by hydrogenolysis using $LiAlH_4$ of the triterpenoid esters **E1-E2** present in fraction $F_{B.1}$. A_x : Alcohol. x : number of carbon atoms. Alcohols are analyzed as acetates.

Figure

[Click here to download high resolution image](#)

Appendix: structures cited in the text

Figure
[Click here to download high resolution image](#)

Figure

[Click here to download high resolution image](#)

Figure 2

Figure
[Click here to download high resolution image](#)

Figure

[Click here to download high resolution image](#)

Figure 4

Figure

[Click here to download high resolution image](#)

Figure 5

[Click here to download high resolution image](#)

Figure 6

Figure

[Click here to download high resolution image](#)

Figure 7

Figure

[Click here to download high resolution image](#)

Figure 8

Figure 9