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Organic substances from Egyptian jars of the Early Dynastic period (3100–2700 BCE): Mode of preparation, alteration processes and botanical (re)assessment of “cedrium”

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► To cite this version:

Mathilde Sarret, Pierre Adam, Philippe Schaeffer, Quentin Ebert, Julien Perthuison, et al.. Organic substances from Egyptian jars of the Early Dynastic period (3100–2700 BCE): Mode of preparation, alteration processes and botanical (re)assessment of “cedrium”. *Journal of Archaeological Science: Reports*, 2017, 14, pp.420-431. 10.1016/j.jasrep.2017.06.021 . hal-03070756

HAL Id: hal-03070756

<https://hal.science/hal-03070756v1>

Submitted on 15 Dec 2020

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1 **Organic substances from Egyptian jars of the Early Dynastic**
2 **period (3100-2700 B.C.): mode of preparation, alteration**
3 **processes and botanical (re)assessment of “cedrium”**

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25 **ABSTRACT**

26 Organic substances from three jars found at Abydos in the tombs of Kings from the two first
27 Egyptian dynasties (3100-2700 B.C.) were investigated using gas chromatography-mass
28 spectrometry in order to assess their biological origin, use and mode of preparation. The organic
29 material was found to correspond to essential oils from conifers severely altered/oxidized. A
30 predominant contribution from *Cedrus libani* was determined from the identification of
31 sesquiterpenoid derivatives of the himachalene series which constitute new molecular tools for
32 the identification of ancient oils made from real cedars. The presence of cuparene-related
33 terpenoids suggested an additional contribution of members from the Cupressaceae family
34 (junipers, cypresses). The genuine substances were most likely prepared in the Levant since
35 neither *C. libani* nor junipers or cypresses are native from Egypt. Despite obvious analogies
36 with "cedrium" used for embalming during later periods, their use as ingredients for embalming
37 remains uncertain because true embalming only begun in Egypt after 2500 BC. Instead, it is
38 proposed that these substances have been used as cosmetics, medicinal substances or for cultural
39 purposes. The predominance of low molecular weight sesquiterpenoids and the absence of
40 typical markers for wood tars indicate that the substances have possibly been prepared by
41 steam-distillation of odoriferous plant material rather than by pyrolysis. Our study gives clues
42 to the controversy concerning the composition of "cedrium", indicating that it most likely
43 originates from mixed true cedar and junipers or cypresses, and not exclusively from real cedar
44 vs. juniper oil.

45 Key words : Ancient Egypt, Sesquiterpenoids, Cedar, Juniper, Oxidation, Archaeometry,
46 Cedrium.

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50 **1. Introduction**

51 Archaeological excavations of the tombs of Kings from the two first Egyptian dynasties
52 (3100-2700 B.C.) in Abydos (Egypt) in the late 19th century directed by Emile Amélineau
53 (Amélineau, 1901, 1902, 1904) led to the discovery of a series of ceramics. Several of the jars
54 discovered are presently located in the Louvre Museum in Paris. They were postulated having
55 been imported from the Levant based on the typology relating them to the so-called “Abydos
56 ware” (Hennessy and Millett, 1963; Serpico and White, 1996; Braun, 2009). Three of them, of
57 similar typology, contain a material made of sand particles and black amorphous, resin-like,
58 grains, likely of organic nature, of unknown origin and function. Although several hypotheses
59 have been proposed concerning the content of this type of jars, postulated to correspond to oil,
60 perfume or coniferous resin (Serpico and White, 1996), there is, to our knowledge, only one
61 molecular study dedicated to the investigation of the organic content of potteries from the same
62 period (Serpico and White, 1996). The latter, discovered by Petrie (1901) in the tomb of Djer
63 at Abydos, correspond to ceramics now being part of the collections of the British Museum, of
64 the Petrie Museum of London, and of the Ashmolean Museum. The present molecular study
65 aims at identifying the molecular composition of the resin-like material from the jars from
66 Abydos using gas-chromatography-mass spectrometry (GC-MS), in order to assess its
67 biological and geographical origin, as well as its use and mode of preparation. In addition,
68 laboratory experiments involving essential oils from conifers were conducted in order to
69 reproduce the oxidative alteration processes affecting sesquiterpenoids when exposed to air
70 atmosphere upon ageing.

71

72 **2. Experimental**

73 *2.1. Samples*

74 The samples were collected in three jars found in the tombs of Kings from the two first
75 Egyptian dynasties (3100-2700 B.C.) at Abydos (Egypt) by Emile Amélineau in 1895-1896.
76 The typology of the jars (Fig. 1) relates them to the so-called “Abydos ware” which comprises
77 pottery vessels, jugs, and jars initially found in Abydos but later also in other places in Egypt
78 (Hennessy and Millett, 1963). The jars are presently located in the Musée du Louvre in Paris
79 with the reference numbers E21727, E21809 and E28010.

80 In the case of jar E21727, sampling has been performed on the mixture of sand and
81 organic material sticking on the neck of the jar. For the other two jars (E21809, E28010), the
82 samples were collected by pouring the jars content on an aluminum foil. The samples obtained
83 had a similar aspect and consisted of sand particles mixed with black amorphous grains, the
84 latter being almost completely soluble in organic solvents.

85 *2.2. Extraction and fractionation*

86 Two distinct extraction protocols have been developed for the analysis of the resin-like material,
87 the first one (protocol A, see below) involving derivatization which allows investigation of
88 polar lipids like alcohols and carboxylic acids, but results in the loss of the more volatile
89 compounds from the extract, and the second one (protocol B), devoid of derivatization steps to
90 avoid evaporation steps, giving access to volatile sesquiterpenoids, but not to the more polar
91 compounds.

92 *2.2.1. Extraction using protocol A*

93 The samples (70 mg, 80 mg, 606 mg, for E21727, E21809 and E28010 respectively) were
94 extracted by sonication with a mixture of CH₂Cl₂/CH₃OH (1:1, v/v). An aliquot of each crude

95 extract obtained by filtration on celite was successively acetylated (Ac₂O/Pyridine 1:1 v/v,
96 60°C, 2 h) and, after removal of the solvent and excess reagent under reduced pressure,
97 esterified using a solution of diazomethane in Et₂O. The derivatized extracts were fractionated
98 by liquid chromatography (LC) on silica gel eluting successively with a mixture of
99 CH₂Cl₂/EtOAc (8:2 v/v) and CH₂Cl₂/CH₃OH (6:4 v/v), leading, respectively, to a moderately
100 polar fraction **F1** analysed using GC-flame ionization detection (GC-FID) and GC-MS, and a
101 polar fraction **F2** not being investigated further.

102 *2.2.2. Extraction using protocol B*

103 The samples (573 mg, 587 mg, 426 mg, for E21727, E21809 and E28010 respectively) were
104 extracted by sonication with CH₂Cl₂. The crude extracts obtained by filtration on celite were
105 concentrated under reduced pressure, avoiding complete solvent removal to preserve the
106 volatile compounds. The extract was chromatographed over silica gel using CH₂Cl₂ as eluent
107 in order to recover an apolar fraction (**F1_{volatile}**) and remove the polar constituents of the extract,
108 and **F1_{volatile}** was concentrated under reduced pressure and analysed by GC-FID and GC-MS.

109 *2.3. Identification of key components*

110 Several aromatic (poly)carboxylic acids and sesquiterpenic structures have been identified
111 using reference compounds. Details on the preparation and characterisation of these standards
112 are described in detail in the Supplementary data.

113 *2.4. Oxidation of essential oil of Cedrus atlantica*

114 Essential oil of *Cedrus atlantica* (Laboratoire Sirius; 15 mg), *N*-hydroxyphthalimide
115 (NHPI, 15 mg), cobalt (II) acetylacetonate (Co(acac)₂, 2 mg) and H₂O (20 µl) in AcCN (3 ml)
116 were heated at 70 °C under an atmosphere of O₂ in a glass tube (10 ml) closed with a screwcap.
117 After 20 h, H₂O (400 µl) was added to an aliquot (500 µl) of the reaction mixture. The organic

118 compounds were extracted with pentane (400 μ l) and analyzed by GC-MS without further
119 purification.

120 *2.5. Analytical methods*

121 *2.5.1. GC-FID*

122 GC-FID was carried out with a Hewlett Packard 6890 gas chromatograph equipped with an on-
123 column injector, FID and a HP-5 fused silica capillary column (30 m x 0.32 mm; 0.25 μ m film
124 thickness). H₂ was used as carrier gas (constant flow mode, 2.5 ml/min), and the oven was
125 programmed as follows: Temperature program: 40 °C (10 min⁻¹), 40 °C-300 °C (5 °C min⁻¹),
126 isothermal at 300 °C for 30 min.

127 *2.5.2. GC-MS*

128 GC-MS was carried out with a Thermo Scientific Trace Ultra gas chromatograph coupled to a
129 Thermo Scientific TSQ Quantum mass spectrometer equipped with an autosampler Tri Plus
130 and a programmed temperature vaporizing (PTV) injector. The temperature of the source was
131 set at 220 °C. The mass spectrometer was operating in the electron impact (EI) mode at 70 eV
132 and scanning m/z 50 to 700. Gas chromatographic separations were performed on a HP5-MS
133 column (30 m x 0.25 mm; 0.1 μ m film thickness) using He as carrier gas. Temperature program:
134 40 °C (10 min⁻¹), 40 °C-300 °C (5 °C min⁻¹), isothermal at 300 °C for 30 min. The data were
135 investigated using the Xcalibur Software.

136 *2.5.3. Nuclear Magnetic Resonance (NMR)*

137 The NMR spectra were recorded on a Bruker Avance I 500 MHz spectrometer (500 MHz for
138 ¹H; 125 MHz for ¹³C). The chemical shifts are reported in ppm relative to tetramethylsilane
139 with the solvent used as internal standard (CHDCl₂: δ^1 H 5.32 ppm; CD₂Cl₂: δ^{13} C 53.8 ppm)
140 and the coupling constants are expressed in Hz.

141 **3. Results and discussion**

142 *3.1. GC-MS investigation of the lipid extracts*

143 The distributions of the apolar fraction (**F1**) from the three samples obtained using protocol
144 A (cf. § 2.3) revealed the presence of a highly complex assemblage of compounds (Fig. 2a for
145 sample E21727, and Fig. S1 in Supplementary data for samples E21809 and 28010). Four main
146 series of compounds could be detected comprising terpenic structures, aromatic carboxylic
147 acids, fatty acids and phenolic compounds. Since most of these compounds had carbon skeleton
148 ≤ 15 C atoms, a second protocol (protocol B) optimized to avoid the loss of the more volatile
149 constituents has been used and the recovered apolar fractions (**F1_{volatile}**) were analyzed by GC-
150 MS. Molecular distributions of the **F1_{volatile}** fractions were again very similar to each other with
151 terpenic structures predominating (Fig. 2b and Fig. S2 in Supplementary data). Several
152 compounds from fractions **F1** and **F1_{volatile}** could be firmly identified by comparison of mass
153 spectra and GC behavior with those of commercial standards or compounds obtained by
154 preparation of references (cf. Supplementary data).

155 *3.1.1. Sesquiterpenic structures*

156 The distributions obtained (Fig. 2a,b) are dominated by four main families of
157 sesquiterpenoid derivatives:

158 (a) tricyclic sesquiterpenoids comprising, notably, longiborneol **1** (numbers refer to
159 structures in the Appendix), its related ketone (longicamphor) **2**, and longicamphenilone **3**
160 related to longifolene **4** (cf. Fig S.3 and Section 2.1 from Supplementary data).

161 (b) sesquiterpenoids from the cuparene series (cf. Fig. S4d and Section 2.2. from
162 Supplementary data) which include cuparene **5**, cuparenic acid **6** (Enzell and Erdtman, 1958)
163 identified by comparison with its mass spectrum published by Grantham and Douglas (1980)

164 and a ketone **7** (Fig. S4c in Supplementary data), the mass spectrum of which being identical
165 to that of α -cuparenone **7a** published in the NIST library. Since it is not possible to infer the
166 exact position of the carbonyl group on the cyclopentane ring using MS, this compound might
167 also correspond to β -cuparenone **7b**. A further compound having a mass spectrum very similar
168 to that of cuparenic acid **6** (presence of an even fragment at m/z 176 for the related methyl ester)
169 but with a molecular ion shifted upwards by 14 Da (Fig. S4d in Supplementary data) could
170 correspond to the analogue **8** of cuparenic acid **6** bearing a carbonyl group on the cyclopentane
171 ring at a position which could not be determined but possibly similar to that of compound **7a**
172 or **7b**.

173 (c) cadalene **9** which might be formed from the various sesquiterpenoids of the cadinene
174 series (e.g. **10**, **11**) by an aromatization process.

175 (d) a series of aromatic and oxygenated sesquiterpenoids (**12-15**) related to himachalenes
176 (**16-18**). Sesquiterpenoids **12-15** were unambiguously identified by comparison of mass spectra
177 (Fig. 3) and GC behavior with those of reference compounds prepared by oxidation of
178 himachalene-related sesquiterpenoids from the essential oil of *Cedrus atlantica* (Fig S.5 and
179 Section 2.3 from Supplementary data). Firm determination of the structure of these compounds
180 using NMR spectroscopy of isolated compounds constitutes the first unambiguous
181 identification of himachalene-related biomarkers in archaeological samples.

182 3.1.2. Diterpenoids

183 Diterpenoids were detected in low relative amounts (Fig. 2a,b), and comprised mainly 7 oxo-
184 dehydroabietic acid **19** and 7-oxo-dehydroabietane **20** identified on the basis of their mass
185 spectra (Van den Berg, 2003; Bhar and Ramana, 2004). A phenolic diterpenoid, totarol **21**,
186 identified using an authentic standard, was also present in one sample (E21809; Fig S2 in
187 Supplementary data).

188 3.1.3. Benzene polycarboxylic acids and acetophenone derivatives

189 Benzene(poly)carboxylic acids were predominant in the organic extracts of samples E21727
190 and E21809, but were relatively less abundant in sample E28010 (Fig. 2a; Fig. S1 in
191 Supplementary data). Compounds **22-26** were unambiguously identified using prepared
192 standards (cf. Section 2.4 from Supplementary data), allowing the precise position of the
193 substituents on the aromatic ring to be determined. Their formation can be explained by
194 autoxidation at the oxygen-sensitive benzylic positions (e.g. Mayo, 1968; Charrié-Duhaut et
195 al., 2000) from aromatic terpenic structures deriving from biological unsaturated sesquiterpenes
196 and/or monoterpenoids as illustrated in Fig. 4 (cf. Section 3.2 and Fig. 5 and 6 below dedicated
197 to oxidation processes of terpenoids). The occurrence of compounds **25-26** has been reported
198 previously from the organic residue of a broken jar found in a tomb of the XXVIth Dynasty by
199 Seher et al. (1980), these compounds being interpreted as oxidation products of terpenoids. *p*-
200 Methylacetophenone **27** detected in the volatile fraction (Fig. 2b) can similarly be the result of
201 the oxidation of monocyclic mono- and sesquiterpenoids. Vanillic acid **28** has likely another
202 origin which will be discussed in Section 3.4.

203 3.1.4. Fatty acids

204 Fatty acids (FA) were minor constituents of the samples (cf Fig. 2a; Fig. S1). Their
205 distribution was dominated by palmitic (C₁₆) and stearic (C₁₈) acid. Longer chain FA (C₂₂-C₂₈)
206 indicating a contribution from plants appear in trace amounts. No typical oxidation products of
207 unsaturated FA such as carboxylic acid diols or diacids (Colombini et al., 2005; Hansel and
208 Evershed, 2009) were detected. The relatively low proportion of these compounds as compared
209 to the sesquiterpenic structures and to the aromatic (poly)carboxylic acids suggests that oil or
210 fat are not part of the ingredients used to prepare the substances from the jars.

211 3.2 Oxidation processes of terpenoids

212 The predominance of oxidized analogues of sesquiterpenoids from the himachalene and
213 cuparene series, together with the occurrence of longicamphenylone **3** and longicamphor **2** -
214 possible oxidation products of longifolene **4** and longiborneol **1**, respectively - (Fig. 5a), as well
215 as the predominance of benzene polycarboxylic acids and acetophenone derivatives (§ 3.1),
216 attest of the intense oxidative alteration processes to which the organic substances in the jars
217 were submitted to since their preparation. The intensity of such an alteration is further
218 underlined by the absence of sesquiterpenoids with non-conjugated double bonds, which
219 generally predominate among genuine plant terpenoids, but are particularly sensitive to
220 oxidative alteration (e.g. Ngo et al., 2000; Huang et al., 2015). This is the case of
221 sesquiterpenoids of the himachalene series, naturally-occurring as himachalene dienes (**16-18**)
222 which could not be detected. All the himachalene derivatives found in the extracts (**12-15**) most
223 likely corresponded to products formed by alteration processes (Fig. 5b), although *ar*-
224 himachalene **12** sometimes occurs in small amounts in plant extracts (e.g. Bredenberg and
225 Erdtman, 1961; Panday and Dev, 1968; Baser and Demirçakmak, 1995; Loizzo et al., 2007). It
226 thus appears that the original sesquiterpenic distributions of the investigated organic substances
227 have been severely altered, most of the sesquiterpenoids initially present have completely
228 disappeared or have been converted into oxidized analogues.

229 The origin of the oxidized cuparenes **6-8** can only partly be ascribed to autoxidation
230 processes (Fig. 5c). If cuparenic acid **6** likely results from the autoxidation at the benzylic
231 methyl group of cuparene **5**, abiotic oxidative processes are, however, not expected to be
232 involved in the formation of cuparenone **7** since such processes would involve autoxidation at
233 a non-activated methylenic position. Therefore, it has to be envisaged that cuparenone **7** is a
234 naturally-occurring compound directly originating from a vegetal source used to prepare the
235 organic substances from the jars. α - and β -cuparenones **7a** and **7b** have been reported only from
236 tree species not native from the Eastern Mediterranean region (e.g. *Thuja orientalis*; Dev and

237 Chetty, 1964) and which were probably not available in ancient Egypt and in the Levant.
238 Alternatively, it can be proposed that incorporation of the keto group on the cyclopentane ring
239 of cuparene **5** was induced by biotic oxidation. In this respect, the biotransformation of cuparene
240 by *Aspergillus niger* has been shown to result in the incorporation of oxygenated functions
241 (alcohols, carbonyls) on the cyclopentane ring of cuparene (Asakawa and Noma, 2010) and in
242 the oxidation of the aromatic methyl group into a carboxylic acid, thus providing an alternative
243 biotic (as opposed to abiotic) formation pathway for both cuparenic acid **6**, compound **8** and
244 possibly compound **7**.

245 In order to evaluate in greater detail the impact of abiotic oxidation processes on the
246 distribution of sesquiterpenoids from the himachalene series, a laboratory experiment involving
247 the essential oil of *C. atlantica*, a radical initiator (NHPI and Co(acac)₂) under an oxygen
248 atmosphere (Ishii et al., 1996; Matsushita et al., 2010) has been performed to simulate abiotic
249 autoxidation processes. Under these conditions, himachalenes **16-18**, the predominant
250 sesquiterpenoids from the original essential oil (Fig. 6a), have completely disappeared after 20
251 h of reaction at 70 °C (Fig. 6b). Concomitantly, the relative abundance of several compounds,
252 initially present in trace amounts, comprising *ar*-himachalene **12** and its unsaturated analogues
253 (**29, 30**), increased significantly (Fig. 6b). Given their low initial concentration, they most likely
254 formed by dehydrogenation processes, involving, for instance, the successive incorporation of
255 oxygen followed by elimination of water or hydrogen peroxide. In addition, it was also possible
256 to observe the formation of oxygenated derivatives of *ar*-himachalene, **13-15** (Fig. 5b), which
257 were also detected among the terpenoid derivatives from the three jars (Fig. 2b). These
258 compounds can thus be interpreted as oxidation products of the unsaturated analogues of *ar*-
259 himachalene **29** and **30** (Fig. 5b).

260

261 *3.3 Botanical and geographical origin of the substances from the jars*

262 Several lipid constituents from the organic extracts of the jars could be unambiguously
263 related to terpenoids from conifers. Such is the case of longiborneol **1**, longicamphor **2**,
264 longicamphenylone **3** (Fig. 2b) most likely formed by oxidation of longifolene **4** - a non-specific
265 sesquiterpene from conifers -, cadalene **9** - an aromatized analogue of cadinene-type terpenoids
266 widely distributed in conifers (Otto and Wilde, 2001) -, as well as 7-oxo-dehydroabietic acid
267 **19** and of 7-oxo-dehydroabietatriene **20** - oxidized analogues of abietic acid **31** and of
268 abietadiene **32** or abietatriene **33** abundant in conifers (e.g.. Otto et al., 2007; Otto and Wilde,
269 2001). However, these compounds are not specific and cannot be related to any precise conifer
270 species or genus. The very low amounts of 7-oxo-dehydroabietic acid **19** detected in the
271 samples clearly indicate that conifer resins, generally dominated by diterpenic acids from the
272 abietane (**34**) and pimarane (**35**) series (e.g., Keeling and Bohlmann, 2006), can be ruled out as
273 ingredients. Himachalene (**12-15**) and cuparene (**5-8**) derivatives, as well as totarol **21**, are more
274 specific and allow some conclusions to be drawn regarding the nature of the conifers used to
275 prepare the substances from the jars. Thus, if we consider potential conifer sources from the
276 Eastern Mediterranean region (Berjaoui, 1952; Germer, 1985; Quézel, 1998; Serpico and
277 White, 2000; Hajar et al., 2010; Caudullo and de Rigo, 2016a,b,c,d), himachalenes are
278 biosynthesized in particularly high amounts by *Cedrus libani* (e.g. Agrawal and Rastogi, 1984;
279 Fleisher and Fleisher, 2000; Saab et al., 2005) and, to a more limited extent, by *Abies cilicica*
280 (Bagci et al., 1999). Additionally, members of the Cupressaceae family also produce
281 himachalenes, notably several juniper species (e.g., *Juniperus communis*: Gonny et al., 2006;
282 *J. phoenicea*: Chalchat et al., 1988; *J. oxycedrus*: Saab et al., 2012) but not cypresses such as
283 the Mediterranean cypress (*Cupressus sempervirens*). Cuparene derivatives are typical
284 sesquiterpenes from Cupressaceae such as cypresses (Piovetti et al., 1981; Otto and Wilde,
285 2001) and junipers (San Feliciano et al., 1995; Otto and Wilde, 2001; Seca and Silva, 2005;
286 Gonny et al., 2006; Robbat et al., 2011; Saab et al., 2012), and cuparene **5** is biosynthesized in

287 significant proportions relative to other sesquiterpenoids by both *Juniperus* sp. and *Cupressus*
288 sp. from the Eastern Mediterranean region comprising *J. oxycedrus* (Chalchat et al., 1988, Uçar
289 and Balaban, 2002), *J. phoenicea* (Chalchat et al., 1988), *J. drupacea* (Adams, 1997), *J. excelsa*
290 (Uçar and Balaban, 2002; Saab et al., 2012), *J. foetidissima* (Uçar and Balaban, 2002), *J.*
291 *communis* (Gonny et al., 2006), and *C. sempervirens* (Piovetti et al., 1981). To our knowledge,
292 except in two studies reporting the presence of cuparene **5** in trace amounts in *C. libani* (Baser
293 and Demirçakmak, 1995) and *C. atlantica* (Aberchane et al., 2004), cuparene derivatives are
294 not reported from real cedars (e.g., Agrawal and Rastogi, 1984; Fleisher and Fleisher, 2000;
295 Otto and Wilde, 2001; Loizzo et al., 2007; Paoli et al., 2011; Saab et al., 2012).

296 The predominance of aromatized and oxidized compounds such as **12-15** deriving from
297 himachalenes **16-18**, which are major constituents of essential oils from real cedars, led us to
298 propose that the organic material originated predominantly from *C. libani*. The occasional
299 occurrence of cuparene **5** in *C. libani* and *C. atlantica* (Baser and Demirçakmak, 1995;
300 Aberchane et al., 2004) in low amounts cannot account for the important proportions of
301 cuparene derivatives **5-8** relative to other sesquiterpenoids in the extracts from the jars (cf. Fig.
302 2a,b and Fig S1, S2 in Supplementary data), unless considering a marked concentration effect.
303 In the latter case, one may envisage that the structure of cuparene, which does not contain non-
304 conjugated double bonds and has a limited number of oxygen-sensitive non-quaternary benzylic
305 positions in contrast to himachalene derivatives, would confer to this compound an enhanced
306 relative resistance upon oxidative ageing. However, this enhanced resistance is unlikely to be
307 sufficient for resulting in such a clear predominance of cuparene-related compounds in the
308 altered material which only contained trace amounts (if any) of cuparene within the genuine
309 products. Therefore, we propose that cuparene-related compounds originate from vegetal
310 sources other than real cedars, such as junipers or cypresses (Cupressaceae) which
311 biosynthesize cuparene **5** in significant high relative amounts. This possibility is further

312 supported by the detection of totarol **21** in one sample (E21809, Fig. S1 in Supplementary data),
313 this phenolic compound being present in *Juniperus* sp. and *Cupressus* sp. (e.g. San Feliciano,
314 1992; Gonny et al., 2006; Dob et al., 2008; Herzi et al., 2013). An origin of totarol **21** from real
315 cedars might however be considered given the report of this compound in the leaves of *C.*
316 *atlantica* (Lercker et al., 1977, Agrawal and Rastogi, 1984), although our own investigations
317 could not confirm this finding, neither could we detect totarol in the leaves and twigs of *C.*
318 *libani*. In addition, this compound has never been reported from *C. libani* wood.

319 To conclude, we propose that the organic substances from the jars from the Early Dynastic
320 period predominantly originate from *C. libani* with a contribution from a member of the
321 Cupressaceae family (*Cupressus* sp or *Juniperus* sp.) to account for the presence of the various
322 cuparene derivatives and of totarol. Except *J. phoenicea* which grows in the Sinai (Germer,
323 1985; Serpico and White, 2000, Caudullo, G. and de Rigo, 2016a.), the various *Cedrus*, *Juniper*
324 and *Abies* species mentioned above do not grow in Egypt (Berjaoui, 1952; Germer, 1985;
325 Quézel, 1998; Serpico and White, 2000; Hajar et al., 2010; Caudullo and de Rigo, 2016a,b,c,d),
326 but are all native from the Levant. Therefore, the organic material from the jars has likely been
327 prepared in southern Levant, where the jars are postulated to originate from (Hennessy and
328 Millet, 1963; Serpico and White, 1996; Braun, 2009).

329

330 *3.4. Implications on the constitution of “cedrium”*

331 The organic substances from the jars present many molecular similarities with the material
332 referred to as “oil of cedar” or “cedrium” in the literature and used as an ingredient for
333 embalming (Buckley et al., 2004; Koller et al., 2003, 2005, Łucejko et al., 2012; Scholz-
334 Böttcher et al., 2013; Brettel et al., 2016). In particular, the present organic material and
335 “cedrium” are both characterized by the significant contribution of cuparene derivatives as

336 reported in the latter case by several authors (cf. Koller et al.; 2003, 2005; Łucejko et al., 2012;
337 Scholz-Böttcher et al., 2013; Brettel et al., 2016). Additionally, the “cedrium” described by
338 Koller et al. (2005) and Scholz-Böttcher et al. (2013) comprised several compounds having the
339 same fragmentation and molecular ions in MS as the compounds unambiguously identified for
340 the first time in the present study as himachalene derivatives (compounds **12**, **13**, **15**, **29**, **30**) by
341 comparison with prepared standards (Section 2.3 from Supplementary data). Thus, we propose
342 to reassess and/or refine the structures of some compounds reported by Koller et al. (2005) and
343 Scholz-Böttcher et al. (2013). Thus, we propose, based on the comparison of the mass spectra
344 of the himachalene derivatives identified in our samples (cf Fig. 3) with mass fragmentation
345 patterns reported in Figure 2 and 4 in Koller et al. (2005) and Table 4 in Scholz-Böttcher et al.
346 (2013), that “cadinatriene” most likely corresponds to *ar*-himachalene **12**, “anhydro-*a*-copaene”
347 to ketone **13**, “tetramethoxyphenol” to *ar*-himachalene-related unsaturated ketone **15**, and “2-
348 acetyl-6-methoxynaphtalene” to dehydro-*ar*-himachalene **29** or **30**. Identical mass spectra were
349 also reported by Hamm et al. (2004) to occur for lipids recovered using headspace solid phase
350 extraction of organic material from the embalming of a heart found in a blue vase from the
351 Louvre Museum (reference numbers E 11094, cf. Charrié et al., 2007). “Cedrium” is thus, from
352 a molecular point of view, very similar to the samples from the jars investigated, both being
353 characterized by predominant sesquiterpenoids from the cuparene and himachalene series
354 originating from a plant mixture comprising *Cedrus* sp. and a member of the Cupressaceae
355 family.

356 Our results and comparison with those obtained from previous molecular studies on
357 embalming material from more recent periods give clues to a controversy discussed by Buckley
358 et al. (2004) and Koller et al. (2003, 2005) concerning the use of cedar oil for embalming
359 mummies. This controversy begun when Lucas (1931) pointed out that the so called “cedar oil”
360 corresponds to a material extracted from juniper. Wisseman (2001) also mentioned that juniper

361 oil was probably used as an ingredient for mummification rather than cedar oil. This hypothesis,
362 which also appeared in more recent articles (e.g. Abdel-Maksoud and El-Amin; 2011) does,
363 however, not rely on detailed molecular investigation or on the detection of juniper-specific
364 molecular markers. Wisseman (2001) did not exclude that further investigations of embalming
365 material might show that the use of cedar vs. juniper oil may have changed with time, depending
366 on criteria such as fashion or cost. Other authors, on the other hand, favored the hypothesis of
367 the use of cedar oil originating from *Cedrus sp* based on molecular criteria, although the latter
368 have proven to be ambiguous and tenuous. Thus, based on MS interpretations, Buckley et al
369 (2004) have identified two oxidized derivatives of himachalenes, the predominant
370 sesquiterpenes of oils from *Cedrus sp*. (e.g. Agrawal and Rastogi, 1984; Fleisher and Fleisher,
371 2000; Otto and Wilde, 2001; Loizzo et al., 2007; Paoli et al., 2011; Saab et al., 2012) in the
372 material used to embalm a cat mummy, but the authors recognized that the identification was
373 only tentative and that they could unfortunately not firmly support the hypothesis that the
374 embalming material indeed contained true cedar oil. Koller et al. (2003, 2005) also discussed
375 the use of cedar vs. juniper oil for mummification. These authors proposed a contribution from
376 cedar and excluded the use of juniper given the absence of cedrol **36** among the
377 sesquiterpenoids from an unused embalming material unearthed at Deir el-Bahari (ca. 1500
378 BC), whereas this compound is reported to occur in fresh juniper extracts (e.g. Piovetti et al.,
379 1981; Uçar and Balaban, 2002). However, cedrol is not necessarily abundant and can even be
380 absent from junipers (e.g. Uçar and Balaban, 2002; Gonny et al., 2006). The contribution of
381 cedar was established from the identification by MS of cuparene **5**, cadalene **9**, cadinatriene or
382 acetylmethoxynaphthalene. The argument raised by the authors was, however, not completely
383 convincing for the following reasons: (1) cuparene **5** and related compounds most likely
384 originate from Cupressaceae (e.g. Otto and Wilde and references cited therein) rather than from
385 real cedar. As discussed above, the relative low proportions of cuparene **5** in essential oils of *C.*

386 *libani* (a single report, by Baser and Demirçakmak, 1995) can probably not explain the
387 importance of these compounds in our extracts, unless an extreme and unrealistic enrichment
388 relative to other sesquiterpenoid series due to an enhanced resistance of cuparene **5** towards
389 oxidative alteration is envisaged; (2) cadalene **9** and cadinatriene may originate from the
390 aromatization/dehydrogenation of sesquiterpenoids from the cadinene series (e.g. **10**, **11**), the
391 latter being non-specific of real cedars and being also found in junipers (Seca and Silva, 2005);
392 (3) acetylmethoxynaphthalene has not been reported from real cedars. However, it appears
393 following the present identification work - cf. discussion above - that some of these structures
394 were erroneously attributed by the authors with cadinatriene and acetylmethoxynaphthalene,
395 for instance, corresponding in fact to the himachalene derivatives (**12**, **29** or **30**) identified in
396 the present study, which indeed supports the hypothesis of a significant contribution of material
397 originating from *C. libani*.

398 To conclude, comparison of the results of the present molecular study with previous studies
399 on embalming material from more recent historical periods leads to the conclusion that the
400 material called “cedrium” used for embalming originates from true cedar (most likely *C. libani*)
401 mixed with a second botanical source belonging to the Cupressaceae family, possibly cypresses
402 and/or junipers.

403 *3.5. Use of the organic material from the jars*

404 Given the molecular analogies of the organic substances in the jars with “cedrium”, one
405 might envisage that they were used as an ingredient for embalming as reported by several
406 authors (Buckley et al., 2004; Koller et al., 2003, 2005, Łucejko et al., 2012; Scholz-Böttcher
407 et al., 2013; Brettel et al., 2015). However, true embalming only began in Egypt after 2500 BC,
408 although recent work indicates some earlier examples of mummification, before 2900 BC
409 (Jones et al., 2014). Therefore, the use of the odoriferous substances from the jars dating to the

410 Early Dynastic period for embalming remains rather uncertain, and alternate uses, for
411 cosmetology or medical and cultural purposes have to be considered.

412

413 *3.6. Mode of preparation of the substances identified in the jars*

414 The predominance of low molecular constituents (e.g. sesquiterpenoids) suggests that the
415 preparation of the organic material from vegetal sources most likely involves a concentration
416 step of volatile compounds, which could be achieved by distillation. Consequently, one might
417 first envisage that the substances from the jars correspond to wood tar, similar to cade oil, made
418 by dry distillation. Although unambiguous markers for wood tar such as methyl, ethyl or propyl
419 guaiacols **37-39** formed by pyrolysis of wood lignin (e.g. Faix et al., 1990; Bailly et al., 2016)
420 were not detected, it cannot be completely excluded that vanillic acid **28** identified in the
421 samples (Fig. 2a and Fig. S1 in Supplementary data) corresponds to an oxidation product of
422 alkylguaiacols **37-39** (Fig. 7) given the severe oxidative alteration undergone by the substances.
423 Alternatively, vanillic acid may result from the oxidation of low molecular weight
424 methoxyphenols such as, e.g., vanillin **40** or eugenol **41** (Fig. 7) from the plants used as
425 ingredients. In addition, the aromatic diterpenoids frequently encountered in conifer wood tar
426 such as 18- and 19- nor abietatrienes **42, 43**, and retene **44** related to abietic acid **31** and resulting
427 from the aromatization of resin acids (e.g. Evershed et al., 1985; Beck and Borromeo, 1990;
428 Egenberg et al., 2002; Connan and Nissenbaum, 2003; Hjulström et al., 2006; Connan and
429 Charrié-Duhaut, 2010) could not be detected in the samples. Therefore, we propose that the
430 substances from the jars correspond to essential oils obtained by steam distillation of plant
431 material comprising wood, twigs, cones or berries from a plant mixture comprising cedar of
432 Lebanon and a member of the Cupressaceae family (juniper, cypress) to account for the
433 occurrence of himachalene and cuparene derivatives.

434 There is, to our knowledge, no historical evidence that steam distillation was a process
435 known in Egypt at the beginning of the 3rd millennium B.C. However, Levey et al. (1955)
436 proposed that this technology was known in the Levant as early as the middle of the 4th
437 millennium B.C. This hypothesis relies on the discovery of a double-rimmed pot in Tepe Gawra
438 in Iraq (Levey, 1955) postulated to correspond to the oldest known part of a steam distillation
439 apparatus. It can therefore be envisaged that preparation of the material in the jars, which most
440 likely occurred in the Levant prior to exportation to Egypt, has been achieved using steam
441 distillation. In such a process, the plant material mixed with water mixture is brought to boiling,
442 and the resulting vapors comprising water and volatile organic constituents from the
443 odoriferous plants are condensed and gathered in the trough of the double-rimmed pot, the
444 organic phase then being separated from water and recovered.

445 **4. Conclusions**

446 GC-MS investigation of the organic materials from three jars found in Abydos and dating
447 back to the two first Egyptian dynasties (3100-2700 B.C.) resulted in the identification of
448 oxidized terpenic structures of the himachalene and cuparene series and totarol, which led us to
449 propose that the organic substances were most likely prepared from at least two distinct conifer
450 species comprising cedar of Lebanon and a member of the Cupressacea family (*Cupressus*
451 *sempervirens* and/or one of the various juniper species growing in the Levant). The
452 identification of novel oxidized sesquiterpenoids from the himachalene family, predominating
453 in the severely oxidized samples, was carried out with prepared reference molecules. The latter
454 constitute the first unambiguous biomarkers related to himachalenes which can be used to
455 identify oil from real cedar (*Cedrus* sp.) in organic material from ancient Egypt.

456 The predominance of low molecular weight constituents in the extracts, together with the
457 absence of biomarkers indicative of lignin pyrolysis suggests that the organic substances were

458 prepared by steam distillation of conifer material rather than by dry pyrolysis of wood.
459 However, it cannot be completely excluded that the typical markers for tar, in particular alkyl
460 guaiacols, were lost by evaporation or upon intense oxidative alteration during ageing.

461 Although there are obvious molecular analogies between the investigated material and
462 "cedrium", we propose that the organic substances have been used as cosmetics, medicinal
463 ingredients or for cultural purposes rather than for embalming, which is a practice that developed
464 during later historical periods. Reinvestigation of the molecular composition of "cedrium"
465 reported in the literature in the light of our results shows that oxidized cuparene and
466 himachalene-related sesquiterpenoids co-occur in "cedrium", giving clues to the controversy
467 concerning its ingredients and indicating that "cedrium" was most likely prepared using both
468 true cedar (*C. libani*) and juniper or cypress.

469 **Acknowledgements**

470 E. Motsch (Université de Strasbourg) is thanked for the GC-MS analyses and B. Vincent
471 (Université de Strasbourg) for NMR measurements. J. Connan is thanked for facilitating the
472 access to the samples. A. Charrié-Duhaut and C. Bastien (Université de Strasbourg) are thanked
473 for the sampling of the organic substances in jars E21727 and E21809. The "Centre National
474 de la Recherche Scientifique" and "Université de Strasbourg" are thanked for financial support.

475

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674

675 **Figure captions**

676 **Fig. 1.** Jars (a) E21727, (b) E21809, (c) E28010 (©Musée du Louvre /Geneviève Pierrat-
677 Bonnefois).

678 **Fig. 2.** Gas chromatograms of (a) the moderately polar fraction **F1**; (b) the fraction of the
679 volatile compounds (**F1_{volatile}**) isolated from the organic material Ffrom jar E21727. Alcohols
680 are analyzed as acetates and carboxylic acids as methylesters in fraction **F1**. Fraction **F1_{volatile}**
681 was not derivatized. Filled circles: linear carboxylic acids, numerals refer to the carbon number
682 of the molecules.

683 **Fig. 3.** Mass spectra (electron impact, 70 eV) of the himachalene-related sesquiterpenoids **12-**
684 **15** identified in the organic material from jars E21727, E21809 and E28010 (cf. Fig. 2, Fig. S1,
685 Fig. S2) and of the himachalene derivatives **29-30** formed upon autoxidation of *C. atlantica*
686 essential oil (cf. Fig. 6b).

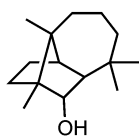
687 **Fig. 4.** Possible oxidative pathway leading from terpenic structures to the aromatic
688 (poly)carboxylic acids **22-27** found in jars E21727 and E21809.

689 **Fig. 5.** Genetic relationship between dehydrogenated and oxidized sesquiterpenoids found in in
690 jars E21727, E21809 and E28010 and their postulated precursor molecules in the case of (a)
691 longiborneol **1** and longifolene **4** derivatives, (b) himachalene (**16-18**) derivatives, (c) cuparene
692 **5** derivatives.

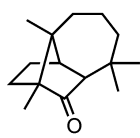
693 **Fig. 6.** Gas chromatograms of (a) *C. atlantica* essential oil, (b) *C. atlantica* essential oil after
694 oxidation with O₂ catalyzed by *N*-hydroxyphthalimide and cobalt(II) acetylacetonate.

695 **Fig. 7.** Proposed origins of vanillic acid **28** found in jars E21727, E21809 and E28010.

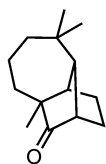
Appendix



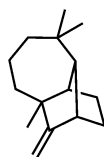
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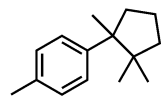
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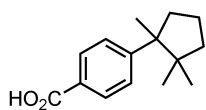
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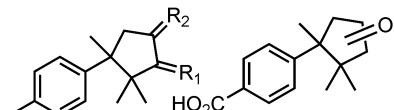
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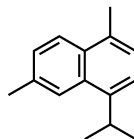


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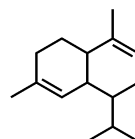


7 7a : R₁=O; R₂=H,H
7b : R₁=H,H; R₂=O

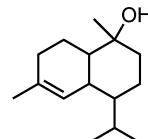
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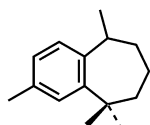
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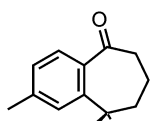
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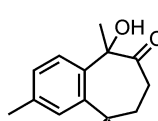
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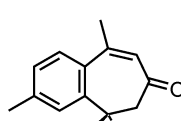
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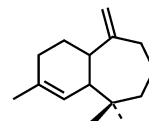
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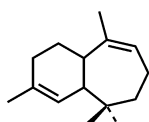
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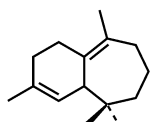
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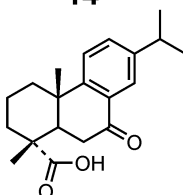
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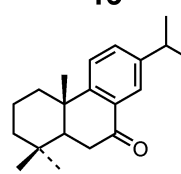
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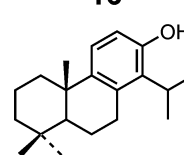
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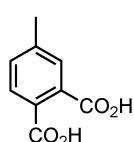
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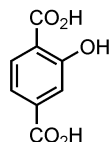
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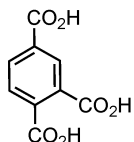
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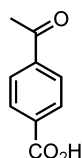
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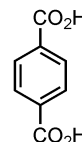
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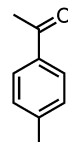
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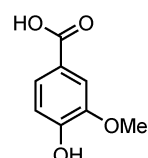
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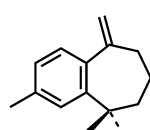
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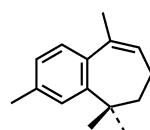
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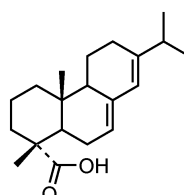
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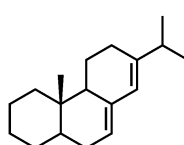
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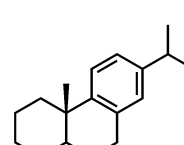
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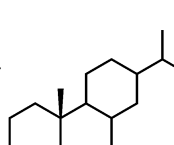
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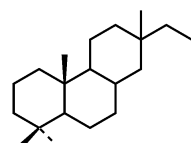
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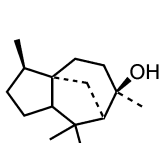
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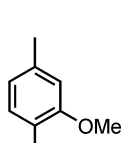
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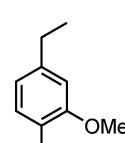
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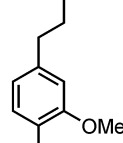
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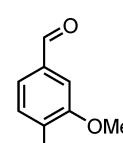
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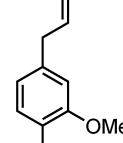
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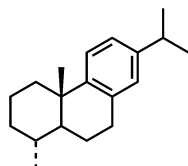
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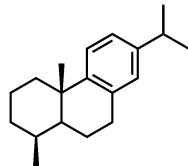
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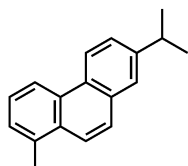
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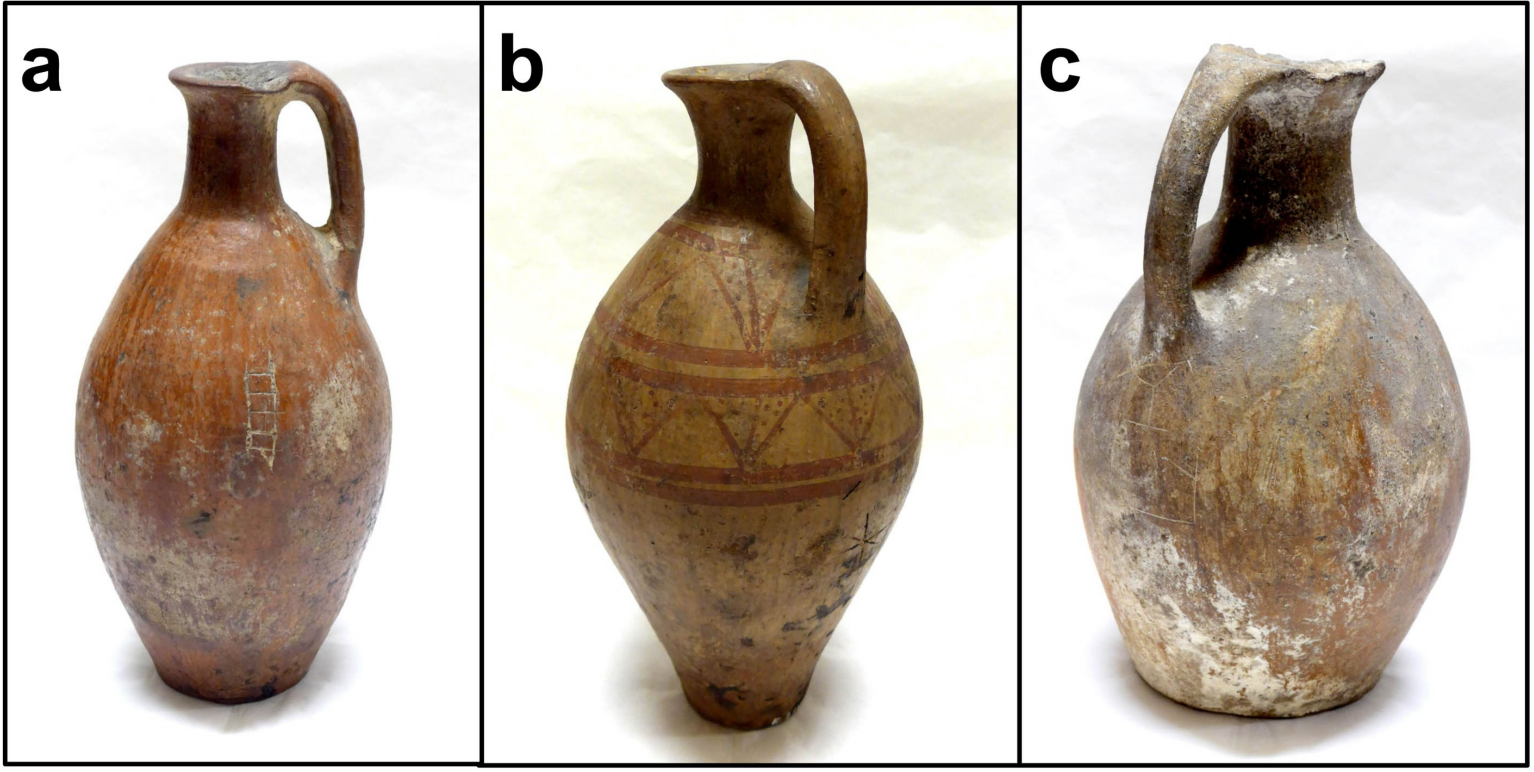


Fig. 1

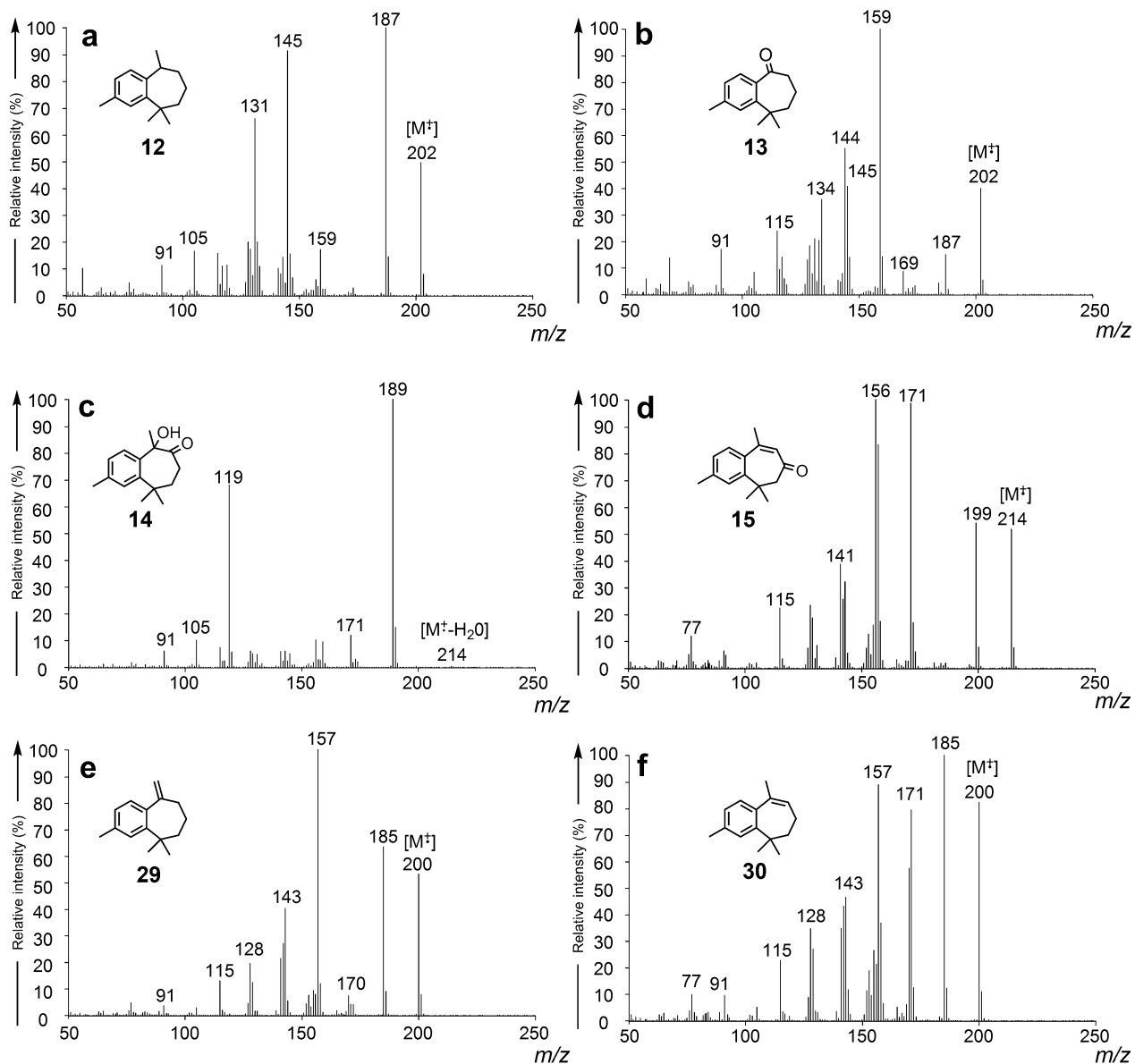


Fig. 3

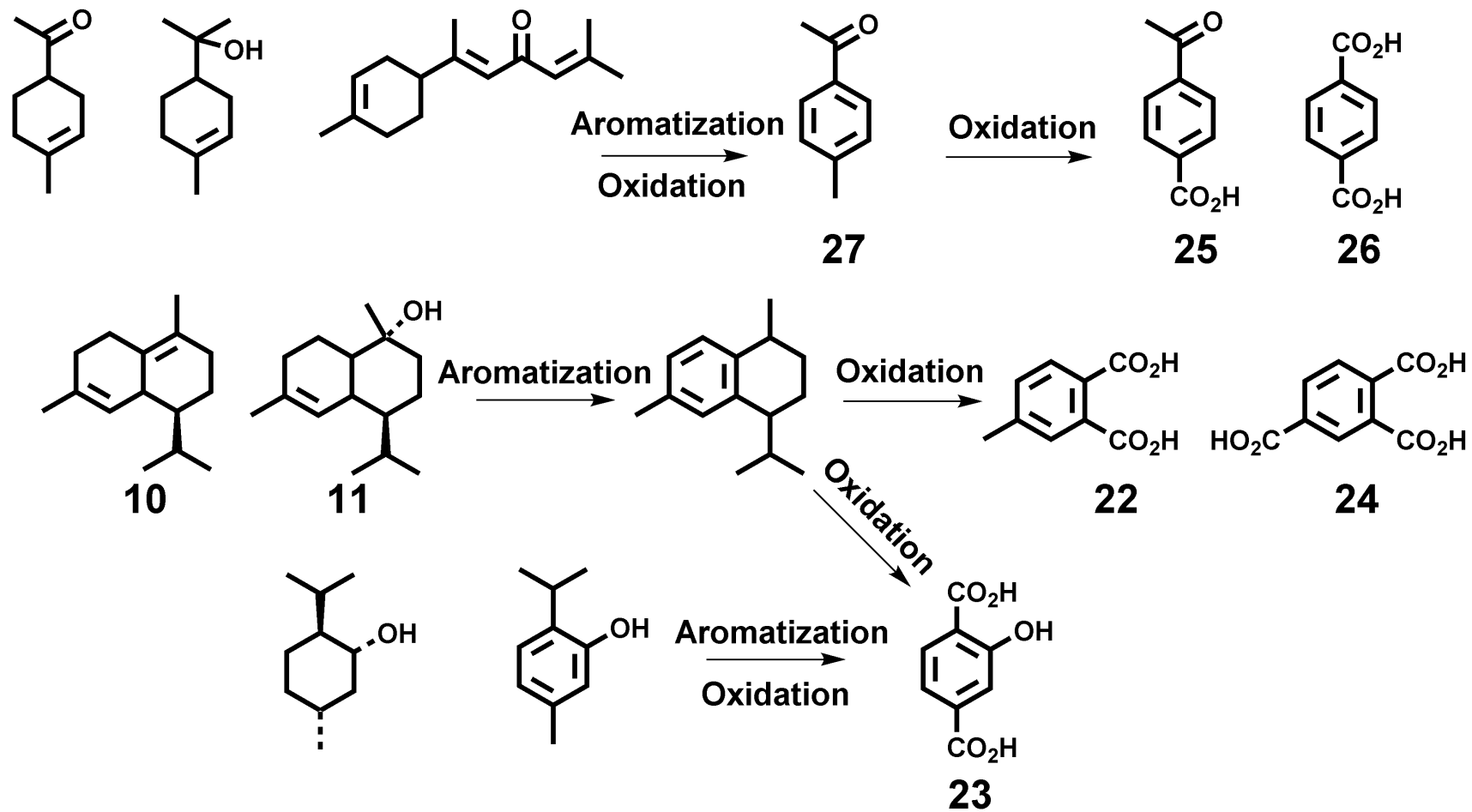


Fig. 4

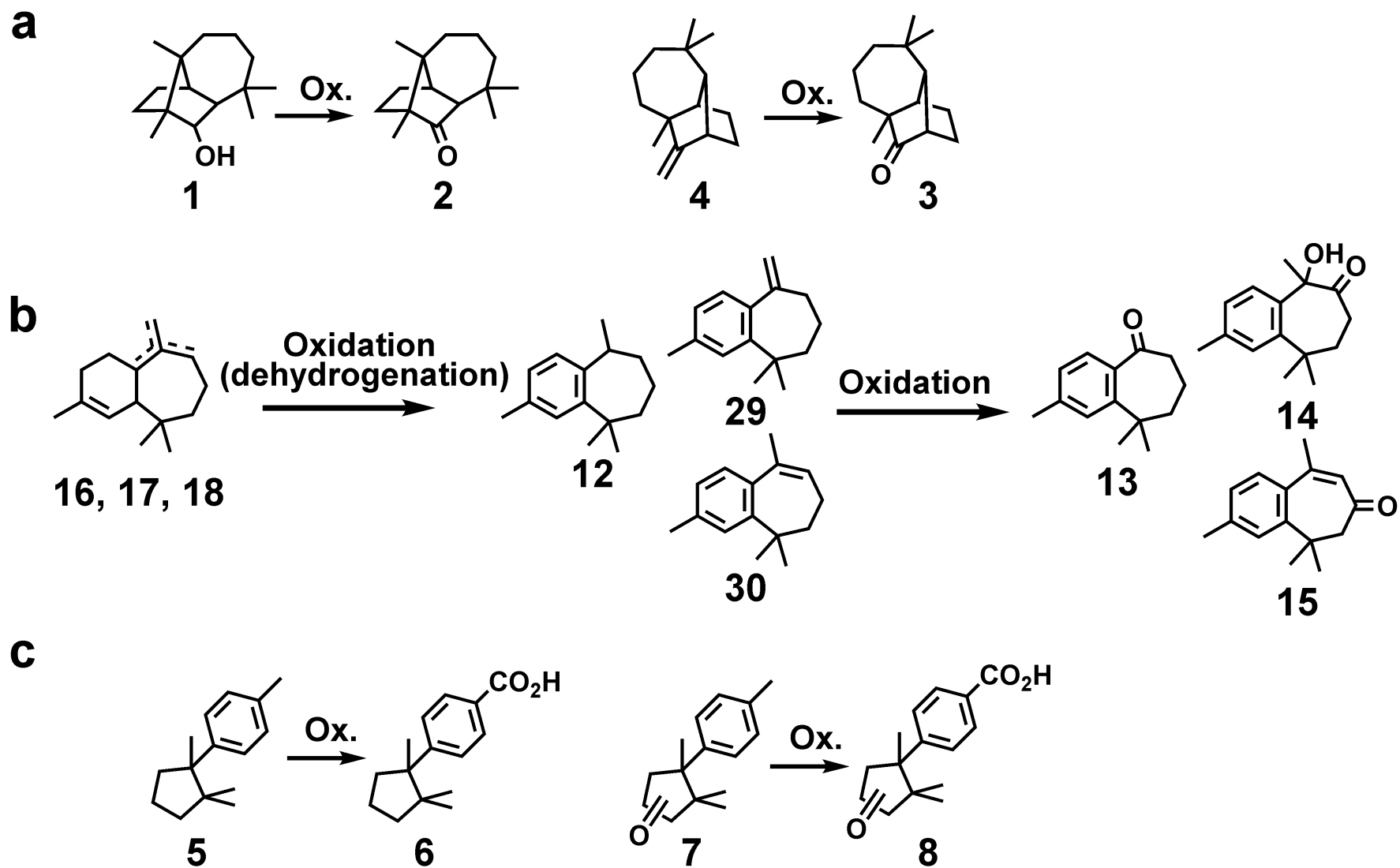


Fig. 5

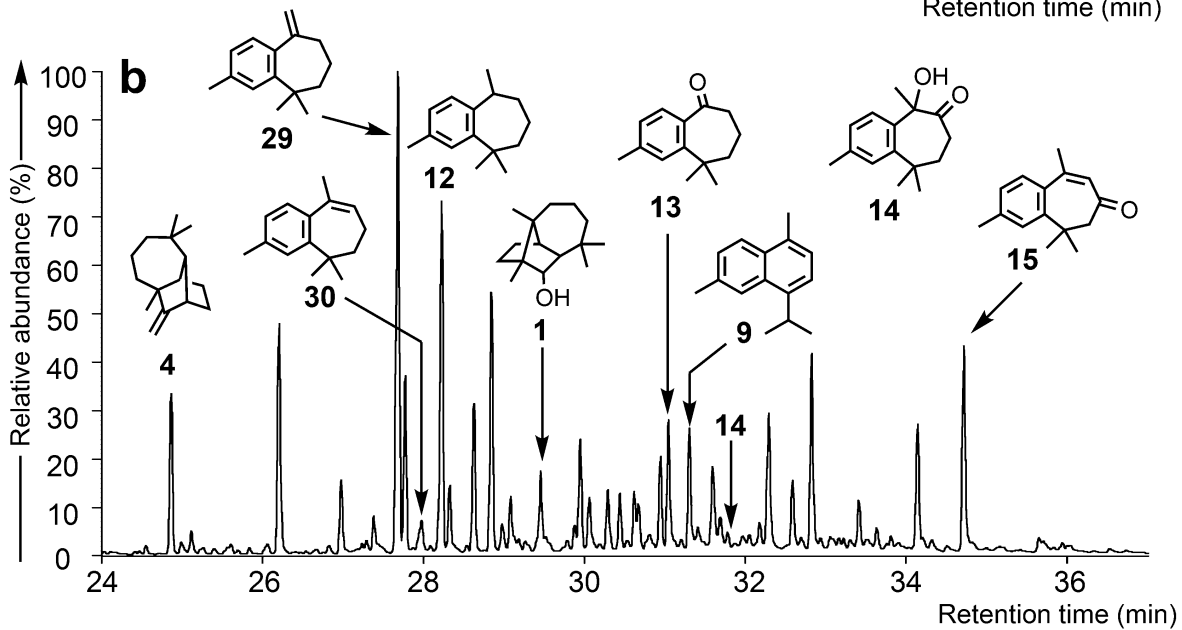
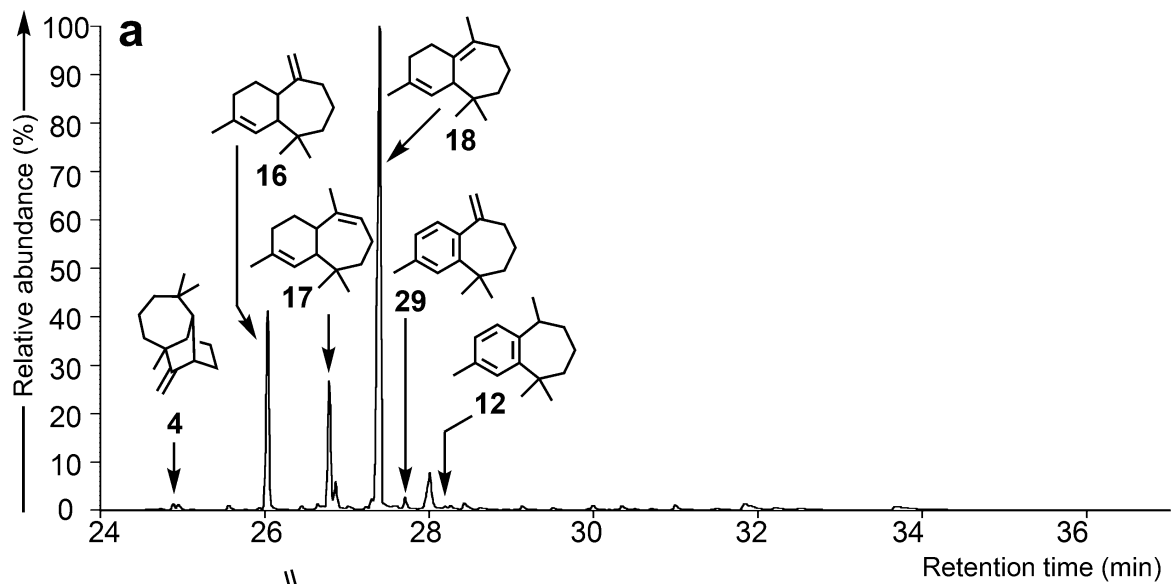


Fig. 6

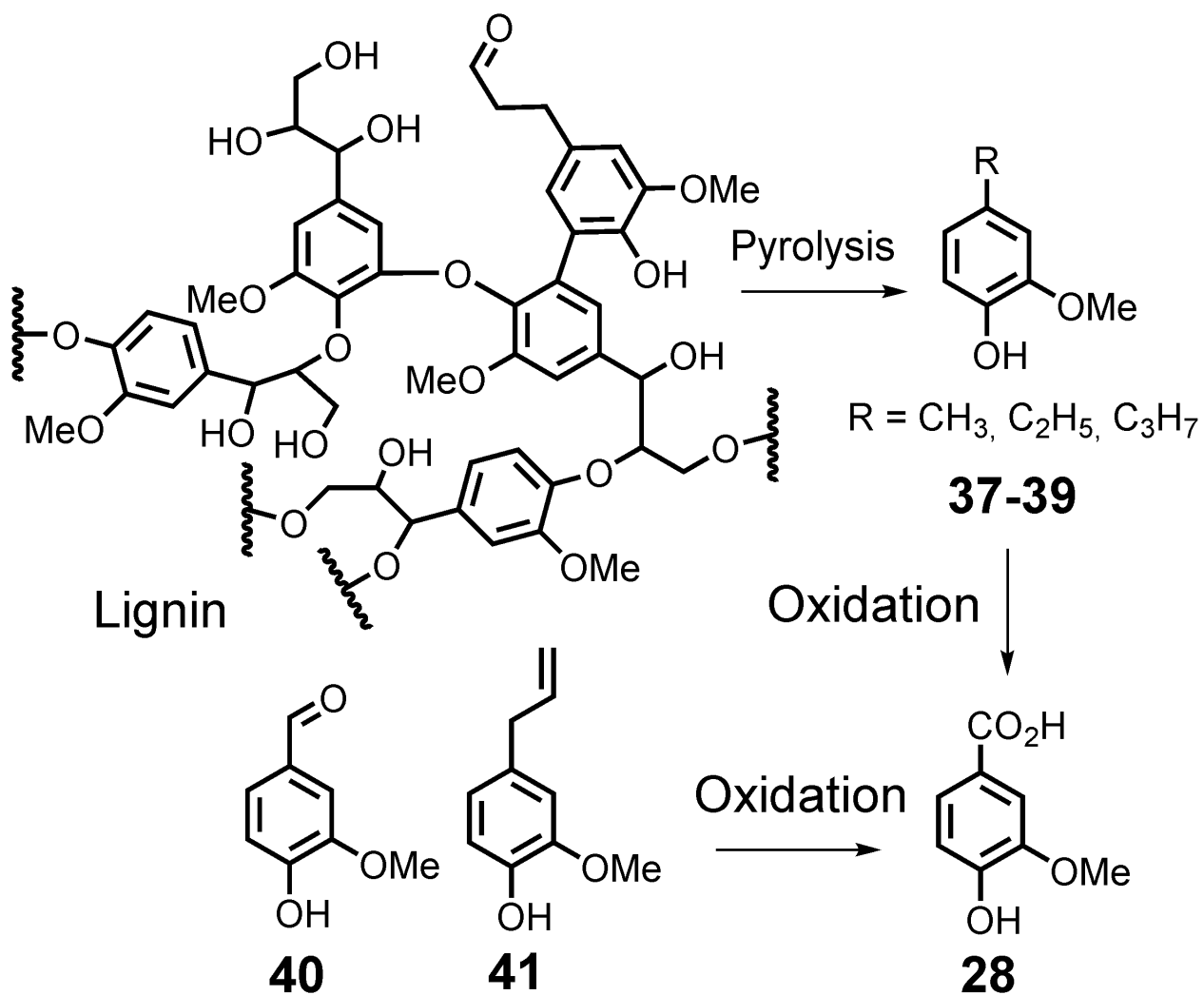


Fig. 7

Supplementary Data

Organic substances from Egyptian jars of the Early Dynastic period (3100-2700 B.C.): mode of preparation, alteration processes and botanical (re)assessment of “cedrium”

Mathilde Sarret,^a Pierre Adam,^{a*} Philippe Schaeffer,^a Quentin Ebert,^a Julien Perthuisson,^a Geneviève Pierrat-Bonnefois^b

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1. Gas chromatograms of F1 and F1_{volatile} fractions from samples E21809 and 28010

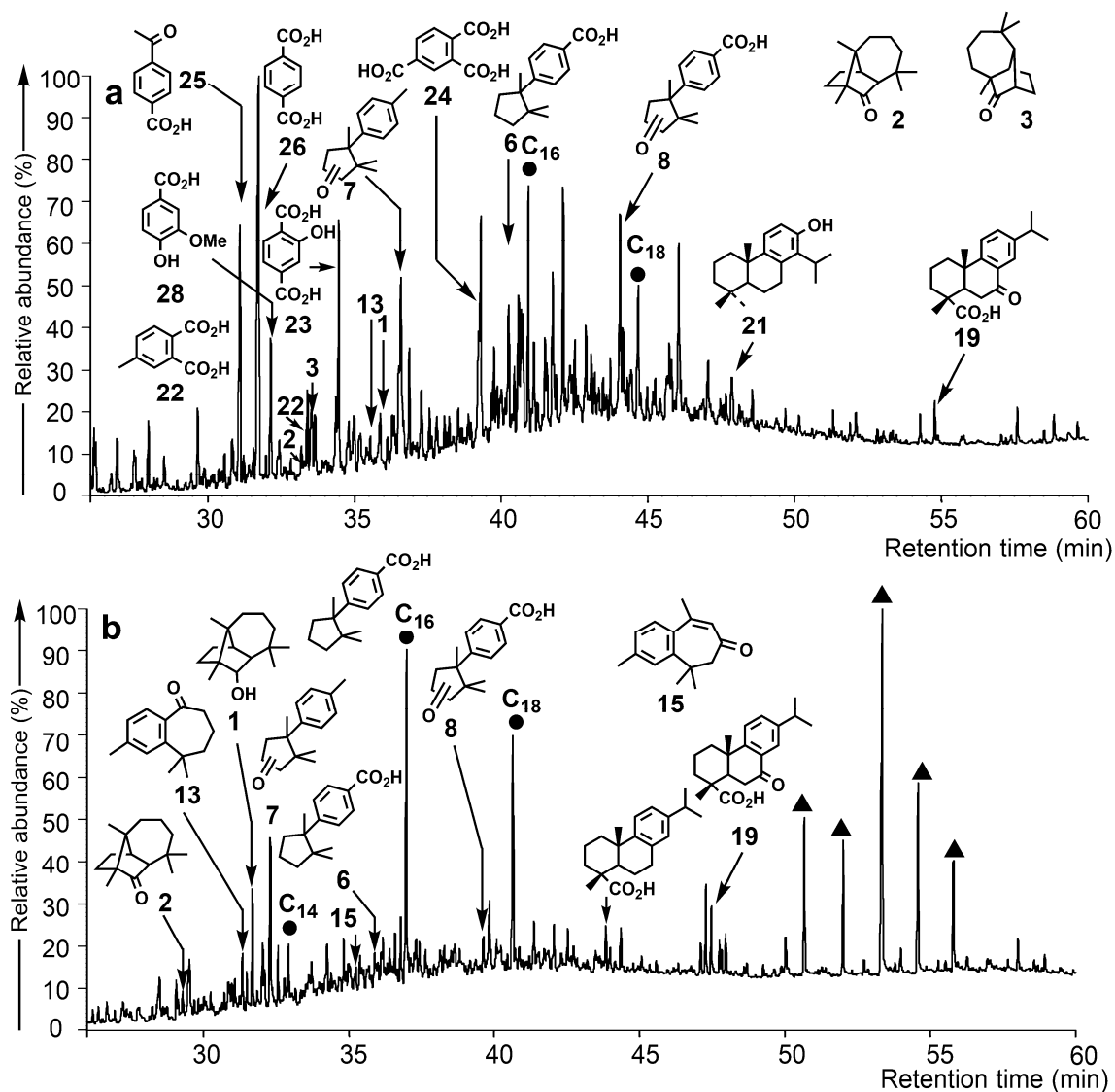


Figure S1 : Gas chromatograms of the fractions **F1** isolated from the organic material found in jars (a) E21809 and (b) E28010 (numbers refer to structure in the Appendix). Filled circles: fatty acids; filled triangles: 2-hydroxy acids. Alcohols were analyzed as acetates and carboxylic acids as methyl esters.

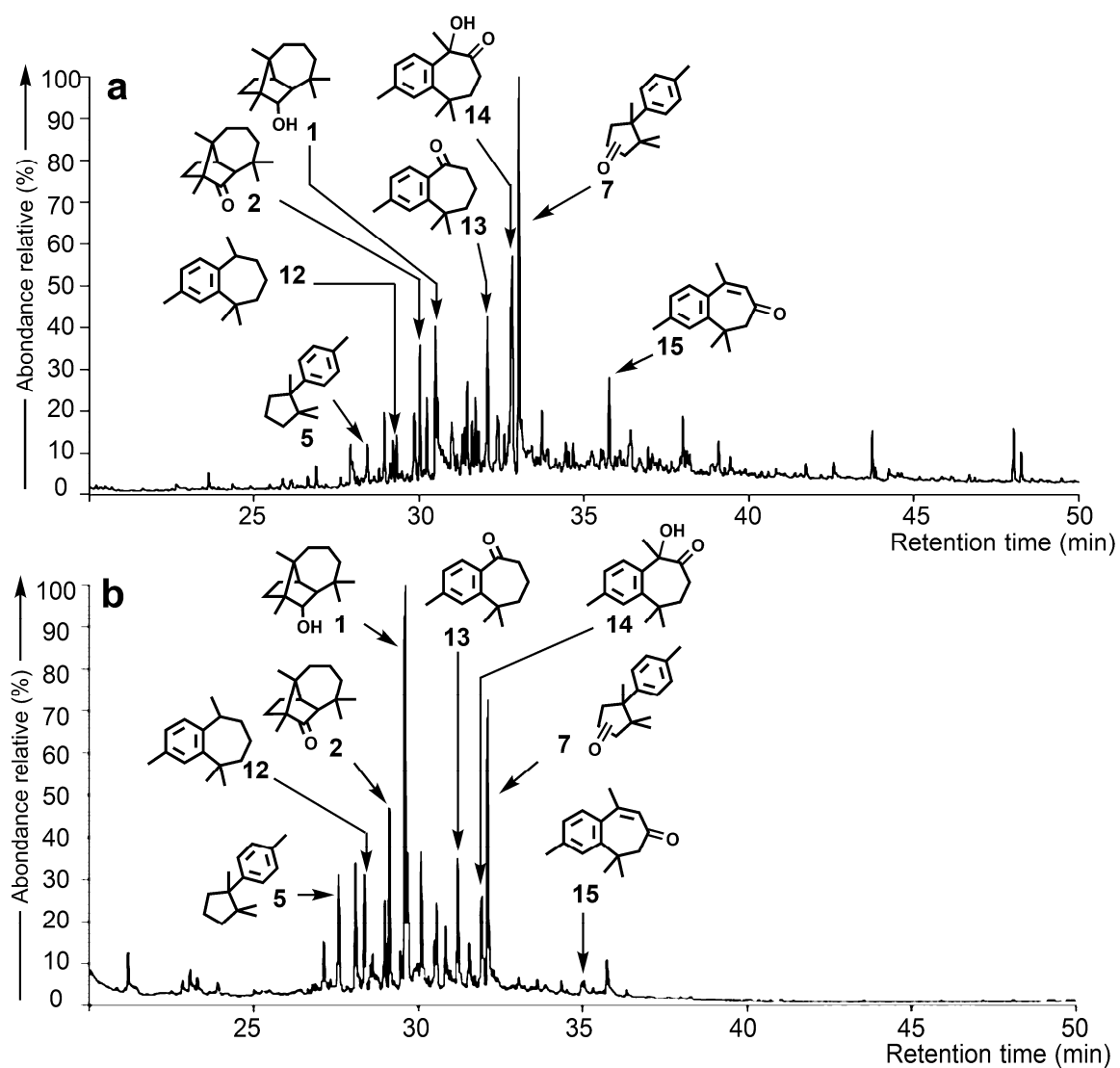


Figure S2 : Gas chromatograms of the fractions $F1_{\text{volatile}}$ from the organic material found in jars (a) E21809 and (b) E28010 (numbers refer to structures in the Appendix).

2. Identification of sesquiterpenoids and aromatic carboxylic acids in samples E21727, E21809 and 28010

2.1 Identification of longiborneol **1**, longicamphor **2** and longicamphenilone **3**

Preparation of longicamphor **2** and longicamphenilone **3** (after Schnell et al., 2014)

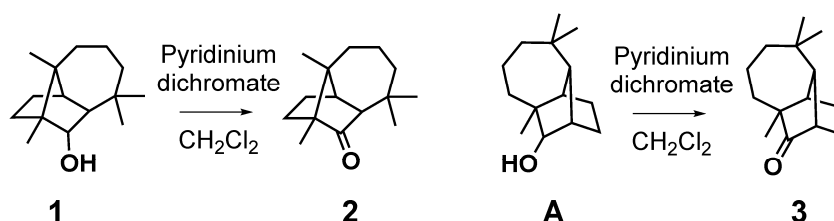


Figure S3 : Preparation of longicamphor **2** and longicamphenilone **3** by oxidation of longiborneol **1** and longicamphenilol **A**, respectively.

Longiborneol **1** (300 µg) (respectively longicamphenilol **A**, 340 µg) was reacted with pyridinium dichromate (20 mg) in dry CH₂Cl₂ (1 ml) for 2 hours at room temperature. Filtration on a short silica gel column followed by elimination of the solvent under reduced pressure yielded longicamphor **2** (respectively, longicamphenilone **3**) in almost quantitative yield.

Longicamphor **2**: GC-MS (EI, 70eV); *m/z* (relative intensity) 220 (M⁺, 92%), 177 (91), 137 (45), 136 (44), 124 (52), 121 (72), 107 (64), 95 (100)

Longicamphenilone **3**: GC-MS (EI, 70eV); *m/z* (relative intensity) 206 (M⁺, 100%), 173 (84), 147 (62), 145 (95), 109 (61), 107 (84), 93 (71), 81 (58)

The identification of compounds **1-3** in samples E21727, E21809 and 28010 was based on the comparison of mass spectra and of retention times in GC of the synthetic compounds with those of the compounds from the archaeological samples.

2.2 Identification of cuparene derivatives 5-8

Cuparene **5** was identified in the archaeological samples by comparison with the mass spectrum and retention time on GC with that of cuparene **5** from the essential oil of Texas cedar (*Juniperus mexicana*) (Sigma-Aldrich). The methyl ester of cuparenic acid **6** was identified based on the comparison with the mass spectrum published by Grantham and Douglas (1980). Compounds **7** and **8** were identified based on the interpretation of their mass spectra as the analogues of compounds **5** and **6** bearing a carbonyl group on the cyclopentane ring. The mass spectra of **7** and **8** are indeed very similar to those of cuparene **5** and cuparenic acid **6** (as methyl ester), respectively, with a molecular ion shifted upwards by 14 Da and even fragments typical for cuparene-related compounds (m/z 132 and m/z 176, respectively, **Fig S4**). In addition, the mass spectrum of **7** is almost superimposable with that of α -cuparenone **7a** from the NIST library. The position of the carbonyl group on the archaeological compounds could, however, not be determined based on the interpretation of mass spectra.

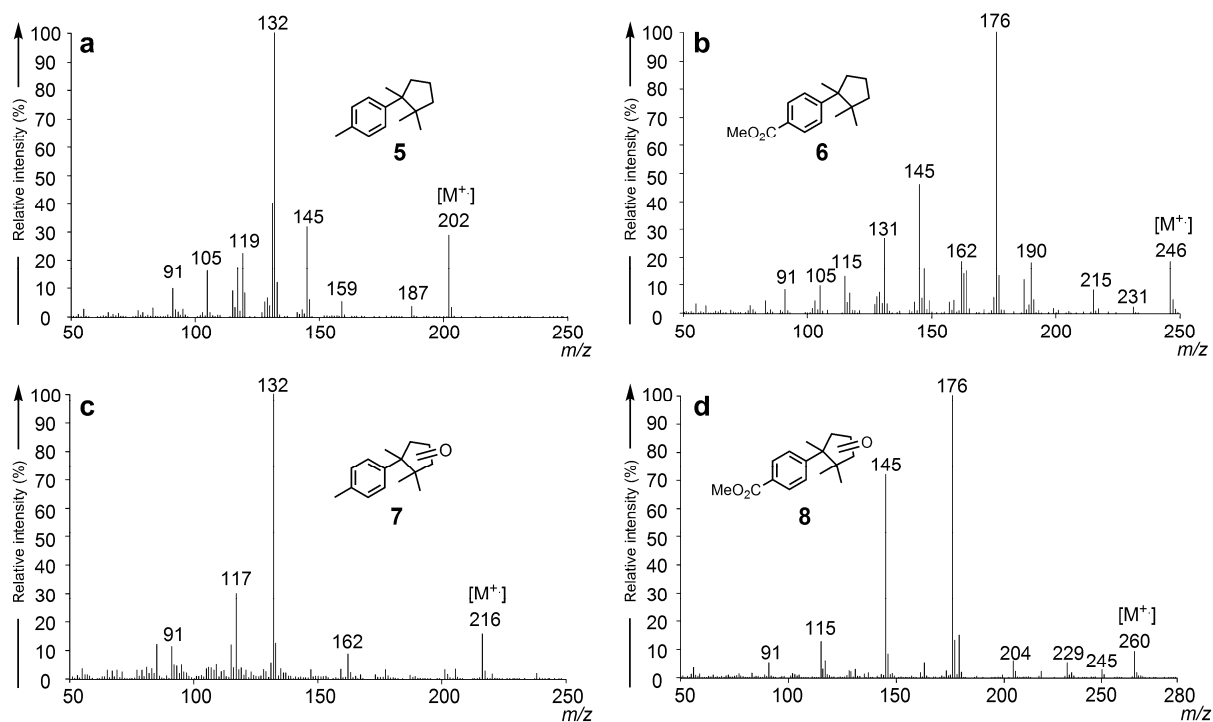


Figure S4: Mass spectra (electron impact, 70 eV) of cuparene-related sesquiterpenoids **5-8** detected in the organic material from jars E21727, E21809 and E28010

2.3 Preparation and identification of himachalene-related sesquiterpenoids

Compounds **12-15**, **29** and **30** were prepared from the essential oil of *Cedrus atlantica* (Laboratoire Sirius), the terpenoid distribution of which being dominated by β -, α - and γ -himachalene **16-18** (Fig. 6a). The experimental protocol used is shown in Fig. S5.

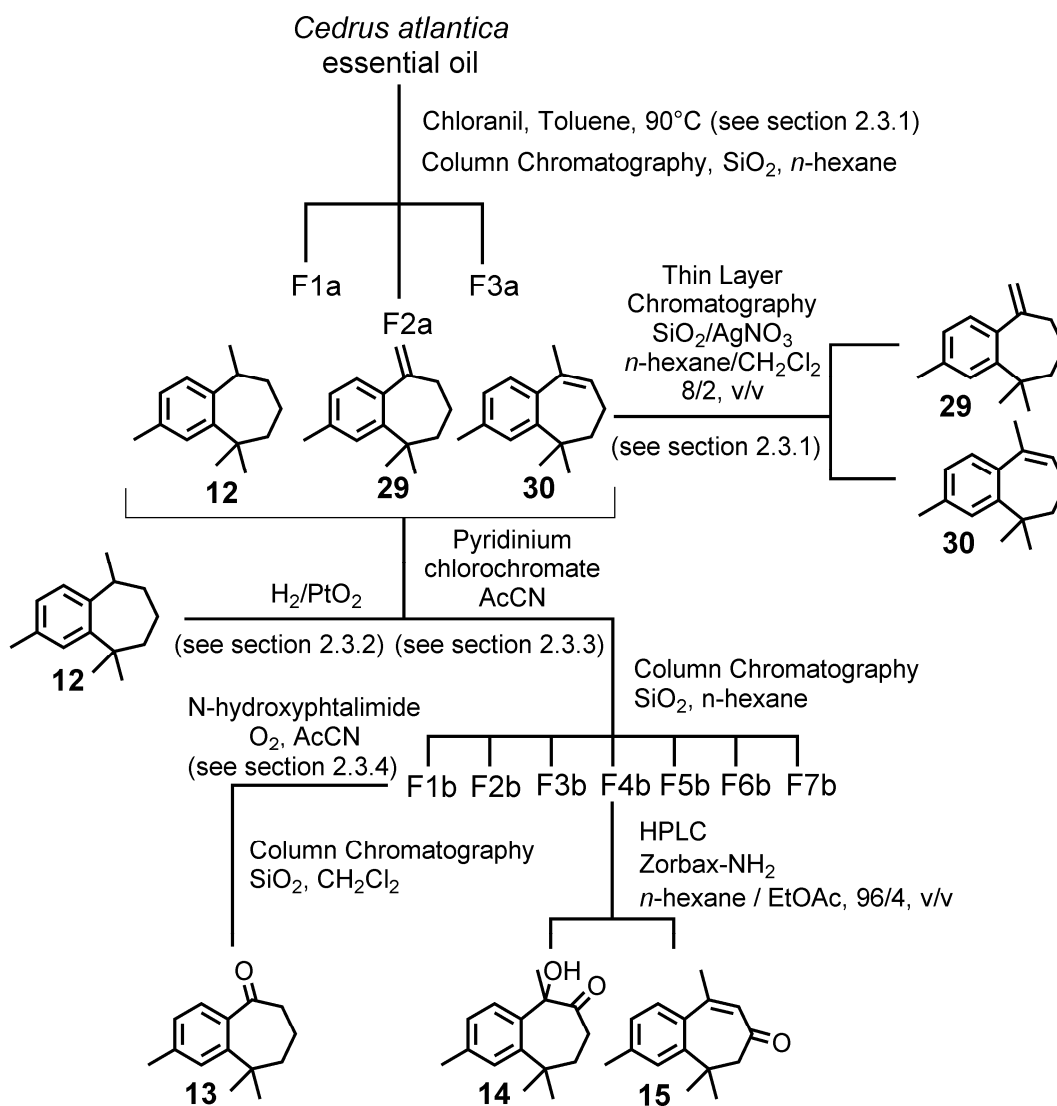
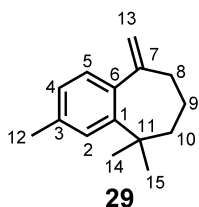


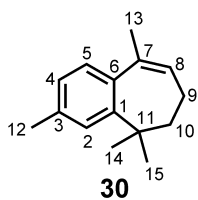
Figure S5: Preparation and isolation of himachalene-related sesquiterpenoids **12-15**, **29** and **30** from *Cedrus atlantica* essential oil. AcCN: acetonitrile; HPLC: high performance liquid chromatography.

2.3.1 Dehydrogenation of essential oil of *Cedrus atlantica* with chloranil (adapted from Panday and Dev, 1968)

Essential oil of *Cedrus atlantica* (502 mg) and chloranil (886 mg) were dissolved in toluene (10 ml). The mixture was heated at 90 °C for 4 h under an atmosphere of N₂ in a tube closed with a screwcap. The solid was filtered off and washed with hexane. The crude organic mixture after filtration was fractionated by column chromatography on a silica gel column using *n*-hexane as eluent. Three fractions were recovered: F1a (124 mg), F2a (277 mg), F3a (24mg). F2a comprised the two unsaturated analogues of *ar*-himachalene **29**, **30** as predominant compounds and small amounts of *ar*-himachalene **12**. Compounds **29**, **30** were isolated for analytical purposes (4 mg, 3 mg, respectively) from 8 mg of F2a fraction by thin layer chromatography silica gel impregnated with silver nitrate using *n*-hexane/CH₂Cl₂ (8/2, v/v) as developer (Fig. S5).



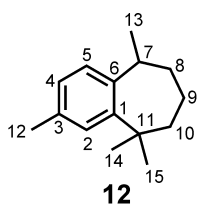
Compound 29. GC-MS (EI, 70 eV); *m/z* (relative intensity) 200 (M⁺, 55%), 185 (69), 157 (100), 143 (43), 128 (13), 115 (12). ¹H-NMR (500 MHz; CD₂Cl₂): 1.315 (6H, s, H-14 and H-15), 1.69 (2H, m), 1.86-1.98 (2H, m), 2.31 (3H, s, H-12), 2.33 (2H, m), 4.92 (1H, d, *J* = 2.5 Hz, H-13), 5.03 (1H, m, H-13), 6.93 (1H, bd, *J* = 7.5 Hz, H-4 or H-5), 7.01 (1H, d, *J* = 7.5 Hz, H-4 or H-5), 7.17 (3H, bs, H-2). ¹³C-NMR (125 MHz; CD₂Cl₂): 21.4, 26.5, 31.4, 31.4, 37.8, 39.1, 41.0, 113.2, 126.7, 126.9, 130.2, 136.7, 141.2, 146.9, 154.5.



Compound 30 GC-MS (EI, 70 eV); m/z (relative intensity) 200 (M^+ , 86%), 185 (100), 170 (60), 159 (80), 157 (85), 143 (45), 128 (30), 115 (18). $^1\text{H-NMR}$ (500 MHz; CD_2Cl_2): 1.300 (6H, s, H-14 and H-15), 1.88-1.96 (4H, m, H-9 and H-10), 2.09(3H, d, $J = 1.5$ Hz, H-13), 2.33 (3H, s, H-12), 5.86 (1H, ddd, $J = 6.5$ Hz, $J = 6.5$ Hz, $J = 1.5$ Hz, H-8), 7.03 (1H, bd, $J = 7.5$ Hz, H-4 or H-5), 7.18 (1H, d, $J = 7.5$ Hz, H-4 or H-5), 7.20 (3H, bs, H-2). $^{13}\text{C-NMR}$ (125 MHz; CD_2Cl_2): 21.5, 23.7, 26.6, 31.4, 31.4, 38.3, 48.1, 126.4, 126.6, 126.7, 127.8, 136.2, 137.6, 138.3, 147.8.

2.3.2 Hydrogenation of unsaturated analogues (**29**, **30**) of *ar*-himachalene **12**

An aliquot (10 mg) of fraction F2a (cf. section 2.3.1; **Fig. S5**) was dissolved in EtOAc (10 ml). PtO_2 (ca. 10 mg) was added and the mixture stirred under a H_2 atmosphere. After 2 h, the mixture was put under N_2 atmosphere and the solid catalyst was filtered off and washed with CH_2Cl_2 . *ar*-Himachalene **12** was obtained in almost quantitative yield.

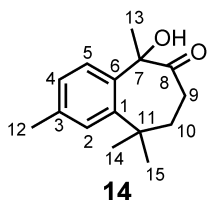


Compound 12. GC-MS (EI, 70 eV); m/z (relative intensity) 202 (M^+ , 50%), 187 (100), 159 (16), 145 (92), 131 (67), 128 (21). $^1\text{H-NMR}$ (500 MHz; CD_2Cl_2): 1.21 (1H, m), 1.312 (3H, s, H-14 or H-15), 1.319 (3H, d, $J = 6.5$ Hz, H-13), 1.41 (3H, s, H-14 or H-15), 1.47 (1H, m), 1.61 (1H, m), 1.72 - 1.84 (3H, m), 2.28 (3H, s, H-12), 3.28 (1H, m, H-7), 6.95 (1H, d, $J = 8.0$ Hz, H-4 or H-5), 7.09 (1H, d, $J = 8.0$ Hz, H-4 or H-5), 7.17 (3H, s, H-2). $^{13}\text{C-NMR}$ (125 MHz; CD_2Cl_2): 20.8, 20.9, 24.0, 29.5, 33.7, 34.3, 36.6, 39.4, 41.1, 125.3, 126.3, 127.5, 134.9, 141.2, 147.7.

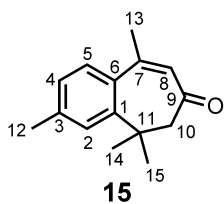
2.3.3 Oxidation of unsaturated analogues of ar-himachalene with pyridinium chlorochromate:

Preparation of **14** and **15**

An aliquot (130 mg) of fraction F2a (cf. section 2.3.1; **Fig. S5**) was reacted with pyridinium chlorochromate (531mg) and 5Å molecular sieves (60 beads) in dry CH₂Cl₂ (15 ml) under reflux. After 4 h, the reaction mixture was filtered on a small silica gel column to remove the reagents. The fraction obtained by elution with EtOAc was evaporated under reduced pressure. The crude mixture was chromatographed on a silica gel column using CH₂Cl₂/EtOAc, (97/3, v/v) as eluent. Seven fractions (**Fig. S5**) were obtained: F1b (65mg), F2b, (12mg), F3b (11 mg) F4b (6.5 mg), F5b, (11 mg), F6b (<2 mg), F7b (7 mg). GC-MS analysis showed that fraction F4b contained compounds **14** and **15**. Fractionation by high performance liquid chromatography (HPLC) using a Waters model 590 HPLC pump (mobile phase: *n*-hexane/EtOAc 96/4, flow rate: 1 ml/min), connected to an Agilent ZORBAX-NH₂ column (25 cm x 2.1 mm i.d., 5 μm) and associated to a differential refractometer detector R401 (Waters Associates) yielded compound **14** (1.8 mg) and **15** (1.1 mg).



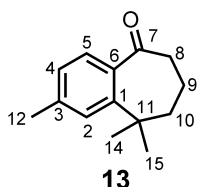
Compound 14. GC-MS (EI, 70 eV); *m/z* (relative intensity) 214 (M⁺ - H₂O, 3%), 199 (3), 189 (100), 171 (15), 119 (72), 105 (11), 91 (7). GC-MS (CI, isobutane) *m/z* (relative intensity) 233 ([M+H]⁺, <1%), 215 ([M+H-H₂O]⁺, 100). ¹H-NMR (500 MHz; CD₂Cl₂) : 1.29 (3H, s, H-14 or H-15), 1.37 (3H, s, H-14 or H-15), 1.68 (2H, m, H-10a, H-10b), 2.02 (1H, m, H-9b), 2.04 (3H, s, H-13), 2.24 (1H, m, H-9b), 2.32 (3H, s, H-12), 4.36 (1H, s, OH), 6.68 (1H, d, *J* = 8.0 Hz, H-4 or H-5), 6.96 (1H, d, *J* = 8.0 Hz, H-4 or H-5), 7.24 (3H, s, H-2). ¹³C-NMR (125 MHz; CD₂Cl₂): 21.1, 24.5, 30.1, 30.4, 30.9, 33.5, 33.7, 78.8, 127.2, 127.2, 127.7, 131.3, 138.4, 146.2, 211.1.



Compound 15. GC-MS (EI, 70 eV); m/z (relative intensity) 214 (M^+ , 42%), 199 (47), 171 (100), 157 (77), 156 (98), 141 (47), 128 (33), 115 (33). $^1\text{H-NMR}$ (500 MHz; CD_2Cl_2): 1.41 (6H, s, H-14 and H-15), 2.37 (6H, s, H-12 and H-13), 2.66 (2H, s, H-10), 6.24 (1H, s, H-8), 7.13 (1H, d, $J = 8.0$ Hz, H-4 or H-5), 7.27 (1H, s, H-2), 7.49 (1H, d, $J = 8.0$ Hz, H-4 or H-5). $^{13}\text{C-NMR}$ (125 MHz; CD_2Cl_2): 21.3, 21.3, 27.2, 27.2, 34.3, 56.8, 126.0, 127.0, 129.9, 130.0, 133.4, 139.8, 148.2, 150.2, 200.1.

2.3.4 Oxidation of compound 2 with O_2 and *N*-hydroxyphthalimide (NHPI): Preparation of **13** (adapted from Lin et al., 2012)

Fraction F1b (cf. section 2.3.3, **Fig. S5**) was shown by GC-MS to be mainly constituted by the unsaturated ar-himachalene derivative **29** and minor amounts of compounds **12**, **30**. To an aliquot of this fraction (20 mg), NHPI (4.5 mg), H_2O (2 μl) and AcCN (800 μl) were added and the mixture heated at 80 $^\circ\text{C}$ under stirring in a round-bottomed flask under O_2 atmosphere for 6 hours. Because of the difficulty to handle small volumes of solvent in a round-bottom flask and to regulate the temperature, the reaction mixture was transferred into a glass tube closed with a screwcap and pursued after addition of NHPI (4.5 mg) and AcCN (1 ml) at 80 $^\circ\text{C}$ under O_2 atmosphere. After 20 h, NHPI (4.5 mg) was added and the mixture heated for a further 8 hour time period at 80 $^\circ\text{C}$ under O_2 atmosphere. The AcCN solution was directly extracted with pentane (4 ml, 3 x), the solvent was removed under reduced pressure and the crude extract chromatographed over a silica gel column eluting with pentane/ CH_2Cl_2 (8/2, v/v) and CH_2Cl_2 , successively, yielding 0.9 mg of ketone **13** (CH_2Cl_2 eluted-fraction; Fig. S5).



Compound 13. GC-MS (EI, 70 eV); m/z (relative intensity) 202 (M^+ , 40%), 187 (15), 159 (100), 145 (41), 144 (55), 134 (36), 117 (14), 115 (24), 91 (17), 69 (14). $^1\text{H-NMR}$ (500 MHz; CD_2Cl_2): 1.33 (6H, s, H-14 and H-15), 1.83 (2H, m, H-9 or H-10), 1.90 (2H, m, H-9 or H-10), 2.36 (3H, s, H-12), 2.68 (2H, t, $J = 8.0$ Hz, $J = 8.0$ Hz, H-8), 7.05 (1H, d, $J = 7.5$ Hz, H-4 or H-5), 7.237 (1H, d, $J = 7.5$ Hz, H-4 or H-5), 7.239 (1H, s, H-2). $^{13}\text{C-NMR}$ (125 MHz; CD_2Cl_2): 21.6, 21.7, 31.9, 31.9, 39.1, 40.7, 43.1, 127.1, 127.5, 128.6, 138.7, 141.2, 147.7, 208.6.

2.4 Identification of aromatic carboxylic acids

Compounds **22-26** were identified in fraction **F1** (cf. Section 2.3 Extraction and fractionation of the samples) from samples E21727, E21809, and E28010 by comparison of the mass spectra and retention of the related methyl ester and acetate derivatives with those of the corresponding commercial references (**Fig. S6**).

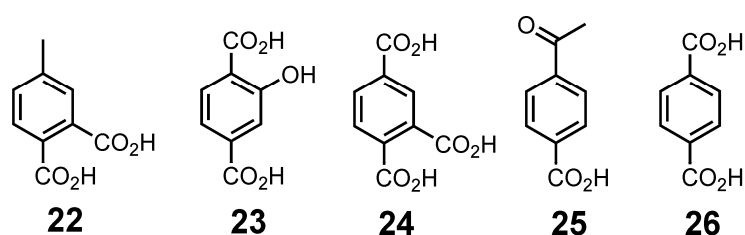


Figure S6: Structures of aromatic carboxylic acids **22-26** identified in samples E21727, E21809 and E28010.

Compound **22** (as dimethyl ester): GC-MS (EI, 70eV); m/z (relative intensity) 208 (M^+ , 13%), 178 (11), 177 (100), 149 (8), 147 (11), 105 (3), 91 (12), 89 (5)

Compound **23** (as dimethyl ester and acetate): GC-MS (EI, 70eV); m/z (relative intensity) 252 (M^+ , 1%), 221 (8), 211 (10), 210 (84), 179 (35), 178 (100), 150 (11), 119 (31)

Compound **24** (as trimethyl ester): GC-MS (EI, 70eV); m/z (relative intensity) 252 (M^+ , 7%), 222 (24), 221 (100), 193 (12), 191 (14), 162 (9), 161 (10), 103 (8)

Compound **25** (as methylester): GC-MS (EI, 70eV); m/z (relative intensity) 178 (M^+ , 16%), 163 (100), 147 (18), 135 (27), 119 (9), 103 (10), 91 (6), 76 (6)

Compound **26** (as dimethylester): GC-MS (EI, 70eV); m/z (relative intensity) 194 (M^+ , 26%), 179 (7), 164 (10), 163 (100), 135 (27), 120 (5), 103 (11), 76 (6)

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