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**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”**

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## ABSTRACT

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

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

## 1. Introduction

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

## 2. Experimental

### 2.1. Samples

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

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

### 2.2. Extraction and fractionation

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

#### 2.2.1. Extraction using protocol A

The samples (70 mg, 80 mg, 606 mg, for E21727, E21809 and E28010 respectively) were extracted by sonication with a mixture of  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (1:1, v/v). An aliquot of each crude

extract obtained by filtration on celite was successively acetylated (Ac<sub>2</sub>O/Pyridine 1:1 v/v, 60°C, 2 h) and, after removal of the solvent and excess reagent under reduced pressure, esterified using a solution of diazomethane in Et<sub>2</sub>O. The derivatized extracts were fractionated by liquid chromatography (LC) on silica gel eluting successively with a mixture of CH<sub>2</sub>Cl<sub>2</sub>/EtOAc (8:2 v/v) and CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (6:4 v/v), leading, respectively, to a moderately polar fraction **F1** analysed using GC-flame ionization detection (GC-FID) and GC-MS, and a polar fraction **F2** not being investigated further.

### 2.2.2. Extraction using protocol B

The samples (573 mg, 587 mg, 426 mg, for E21727, E21809 and E28010 respectively) were extracted by sonication with CH<sub>2</sub>Cl<sub>2</sub>. The crude extracts obtained by filtration on celite were concentrated under reduced pressure, avoiding complete solvent removal to preserve the volatile compounds. The extract was chromatographed over silica gel using CH<sub>2</sub>Cl<sub>2</sub> as eluent in order to recover an apolar fraction (**F1<sub>volatile</sub>**) and remove the polar constituents of the extract, and **F1<sub>volatile</sub>** was concentrated under reduced pressure and analysed by GC-FID and GC-MS.

### 2.3. Identification of key components

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

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

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

compounds were extracted with pentane (400  $\mu$ l) and analyzed by GC-MS without further purification.

## *2.5. Analytical methods*

### *2.5.1. GC-FID*

GC-FID was carried out with a Hewlett Packard 6890 gas chromatograph equipped with an on-column injector, FID and a HP-5 fused silica capillary column (30 m x 0.32 mm; 0.25  $\mu$ m film thickness). H<sub>2</sub> was used as carrier gas (constant flow mode, 2.5 ml/min), and the oven was programmed as follows: Temperature program: 40 °C (10 min<sup>-1</sup>), 40 °C-300 °C (5 °C min<sup>-1</sup>), isothermal at 300 °C for 30 min.

### *2.5.2. GC-MS*

GC-MS was carried out with a Thermo Scientific Trace Ultra gas chromatograph 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. Gas chromatographic separations were performed on a HP5-MS column (30 m x 0.25 mm; 0.1  $\mu$ m film thickness) using He as carrier gas. Temperature program: 40 °C (10 min<sup>-1</sup>), 40 °C-300 °C (5 °C min<sup>-1</sup>), isothermal at 300 °C for 30 min. The data were investigated using the Xcalibur Software.

### *2.5.3. Nuclear Magnetic Resonance (NMR)*

The NMR spectra were recorded on a Bruker Avance I 500 MHz spectrometer (500 MHz for <sup>1</sup>H; 125 MHz for <sup>13</sup>C). The chemical shifts are reported in ppm relative to tetramethylsilane with the solvent used as internal standard (CHDCl<sub>2</sub>:  $\delta^1$ H 5.32 ppm; CD<sub>2</sub>Cl<sub>2</sub>:  $\delta^{13}$ C 53.8 ppm) and the coupling constants are expressed in Hz.

### 3. Results and discussion

#### 3.1. GC-MS investigation of the lipid extracts

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

##### 3.1.1. Sesquiterpenic structures

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

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

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



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

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

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

### 3.1.2. Diterpenoids

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

### 3.1.3. *Benzene polycarboxylic acids and acetophenone derivatives*

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

### 3.1.4. *Fatty acids*

Fatty acids (FA) were minor constituents of the samples (cf Fig. 2a; Fig. S1). Their distribution was dominated by palmitic (C<sub>16</sub>) and stearic (C<sub>18</sub>) acid. Longer chain FA (C<sub>22</sub>-C<sub>28</sub>) indicating a contribution from plants appear in trace amounts. No typical oxidation products of unsaturated FA such as carboxylic acid diols or diacids (Colombini et al., 2005; Hansel and Evershed, 2009) were detected. The relatively low proportion of these compounds as compared to the sesquiterpenic structures and to the aromatic (poly)carboxylic acids suggests that oil or fat are not part of the ingredients used to prepare the substances from the jars.

## 3.2 *Oxidation processes of terpenoids*

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

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

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

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

### 3.3 Botanical and geographical origin of the substances from the jars

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

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

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

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

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

### 3.4. Implications on the constitution of “cedrium”

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

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

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



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

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

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

### 3.5. Use of the organic material from the jars

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

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

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

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

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

#### 4. Conclusions

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

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

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

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

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 673

## Figure captions

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

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

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

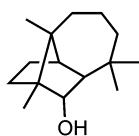
**Fig. 4.** Possible oxidative pathway leading from terpenic structures to the aromatic (poly)carboxylic acids **22-27** found in jars E21727 and E21809.

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

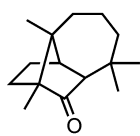
**Fig. 6.** Gas chromatograms of (a) *C. atlantica* essential oil, (b) *C. atlantica* essential oil after oxidation with O<sub>2</sub> catalyzed by *N*-hydroxyphthalimide and cobalt(II) acetylacetonate.

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

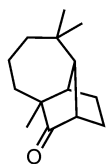
# Appendix



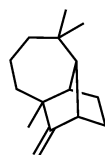
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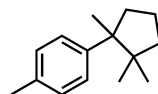
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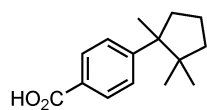
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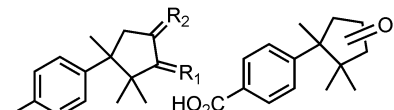
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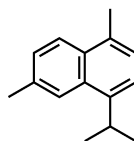


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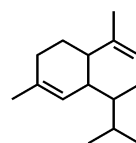


7 7a : R<sub>1</sub>=O; R<sub>2</sub>=H,H  
7b : R<sub>1</sub>=H,H; R<sub>2</sub>=O

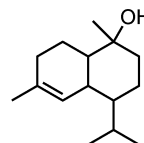
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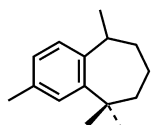
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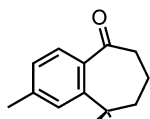
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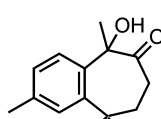
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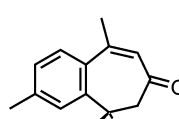
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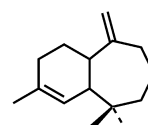
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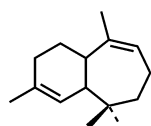
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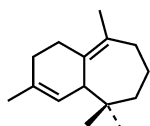
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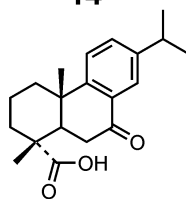
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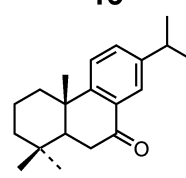
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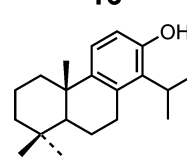
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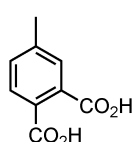
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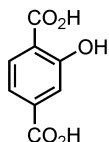
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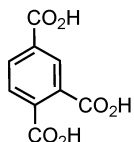
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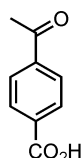
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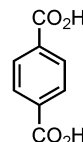
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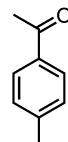
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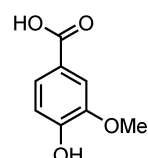
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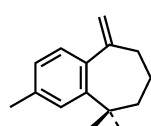
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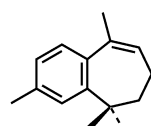
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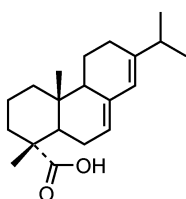
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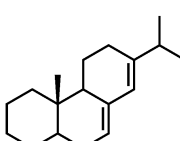
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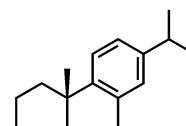
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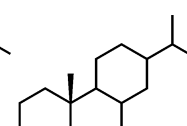
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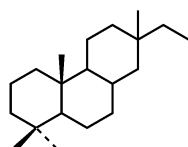
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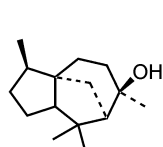
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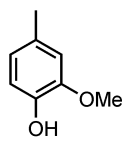
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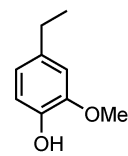
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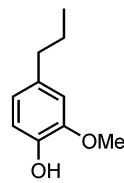
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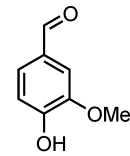
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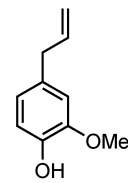
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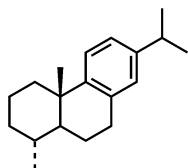
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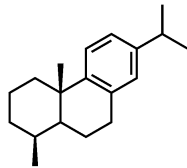
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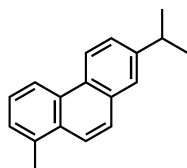
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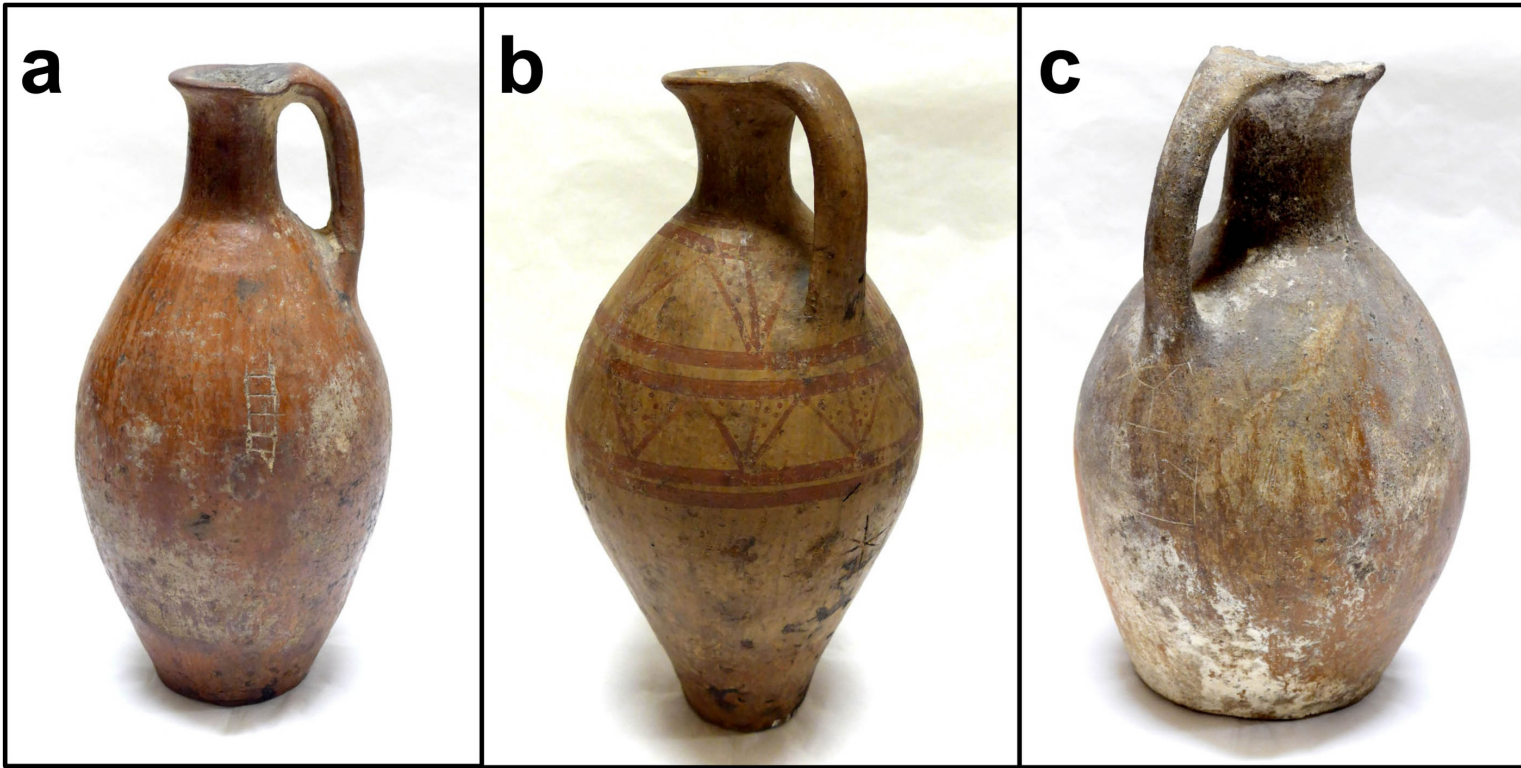
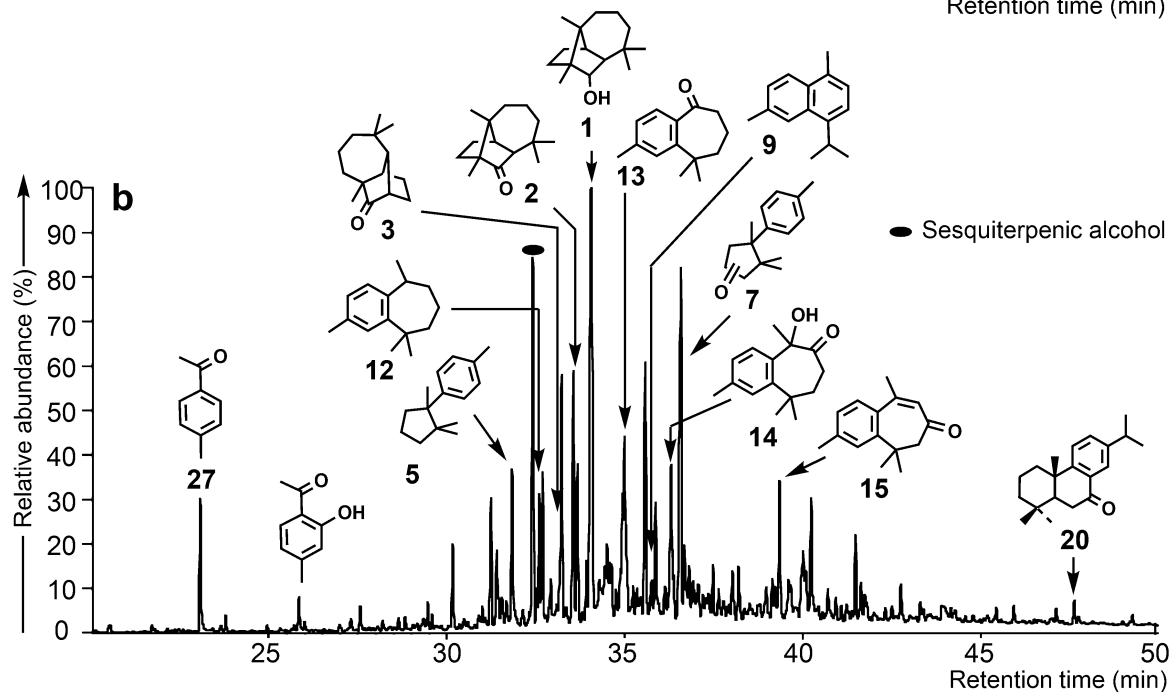
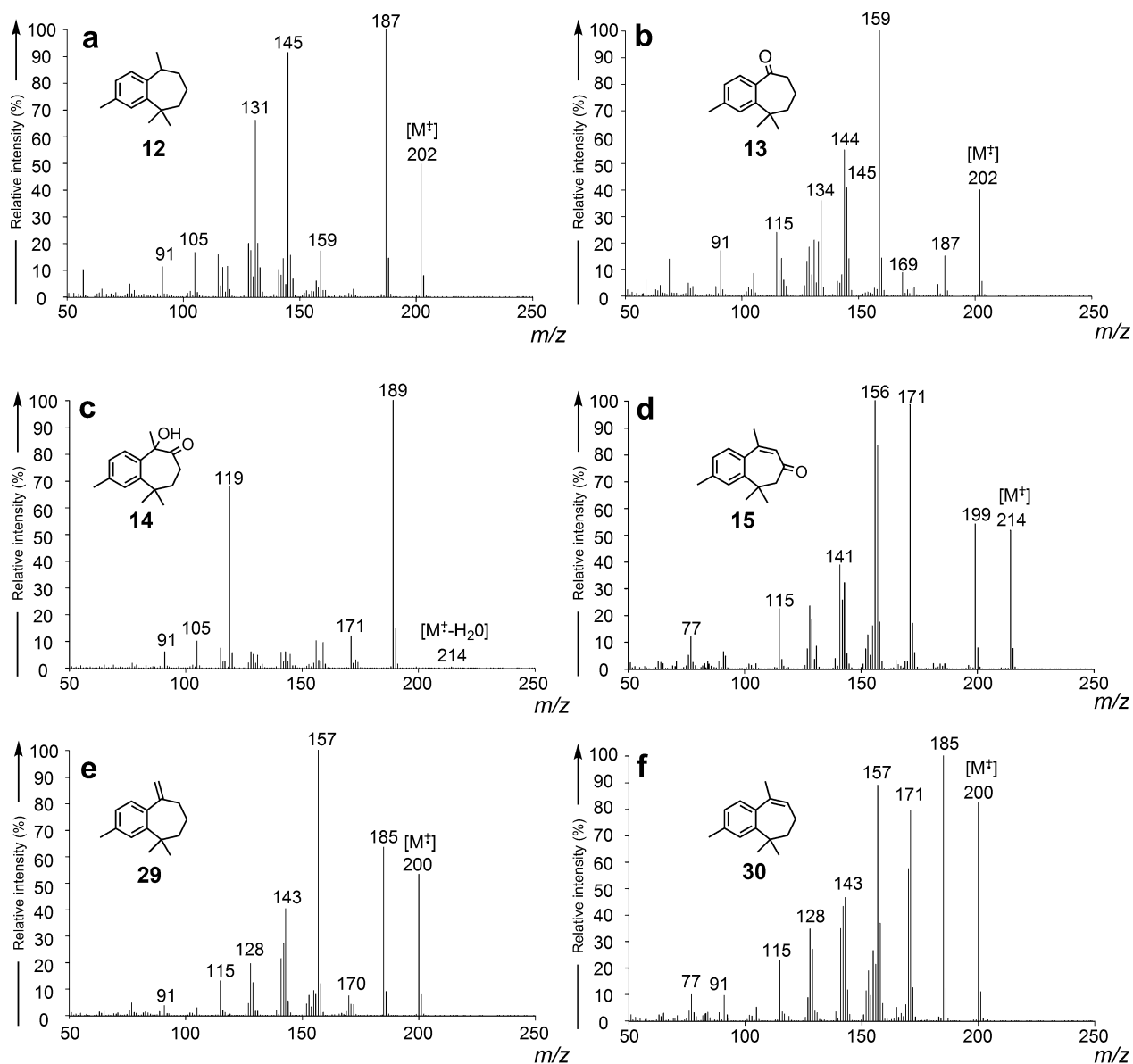


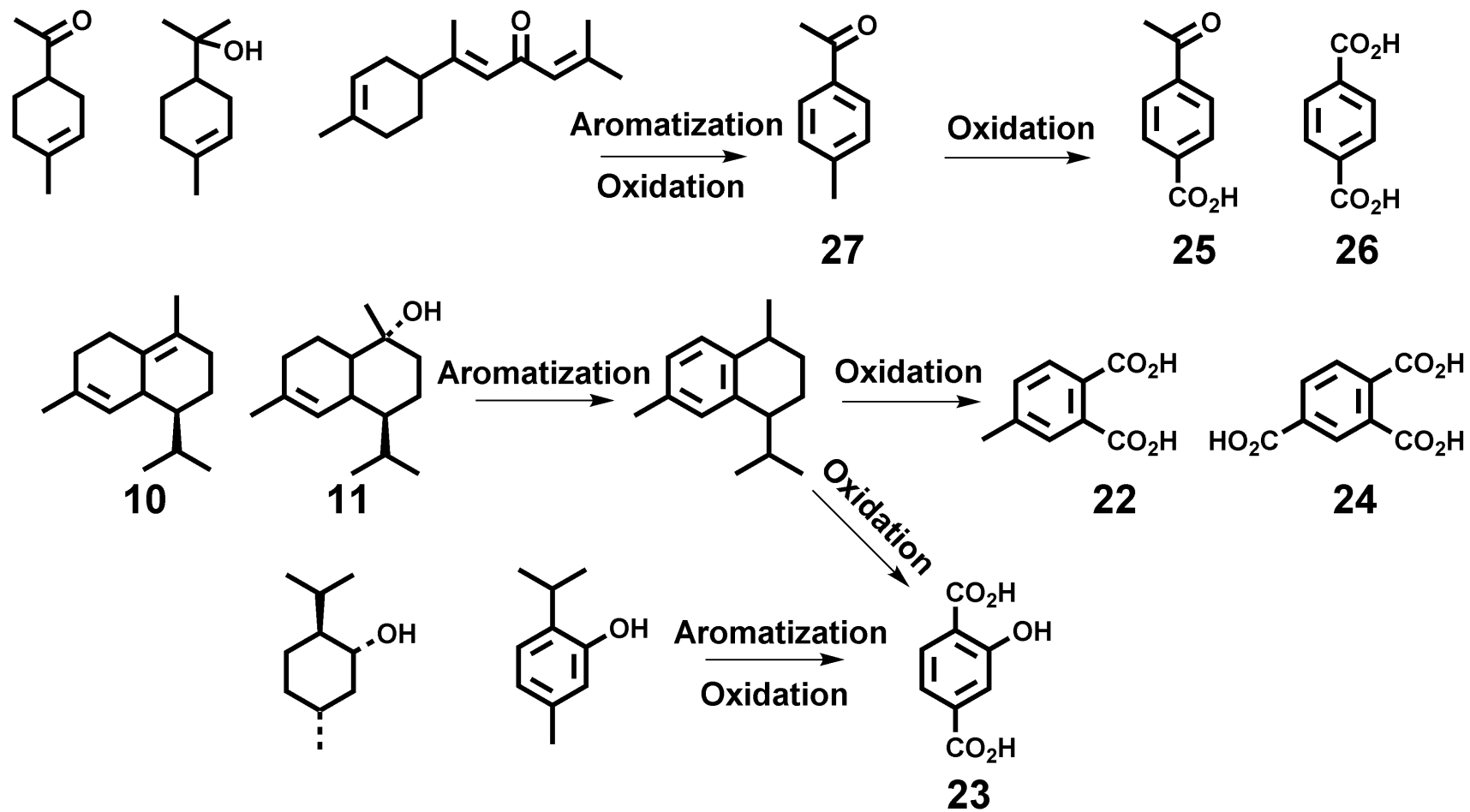
Fig. 1



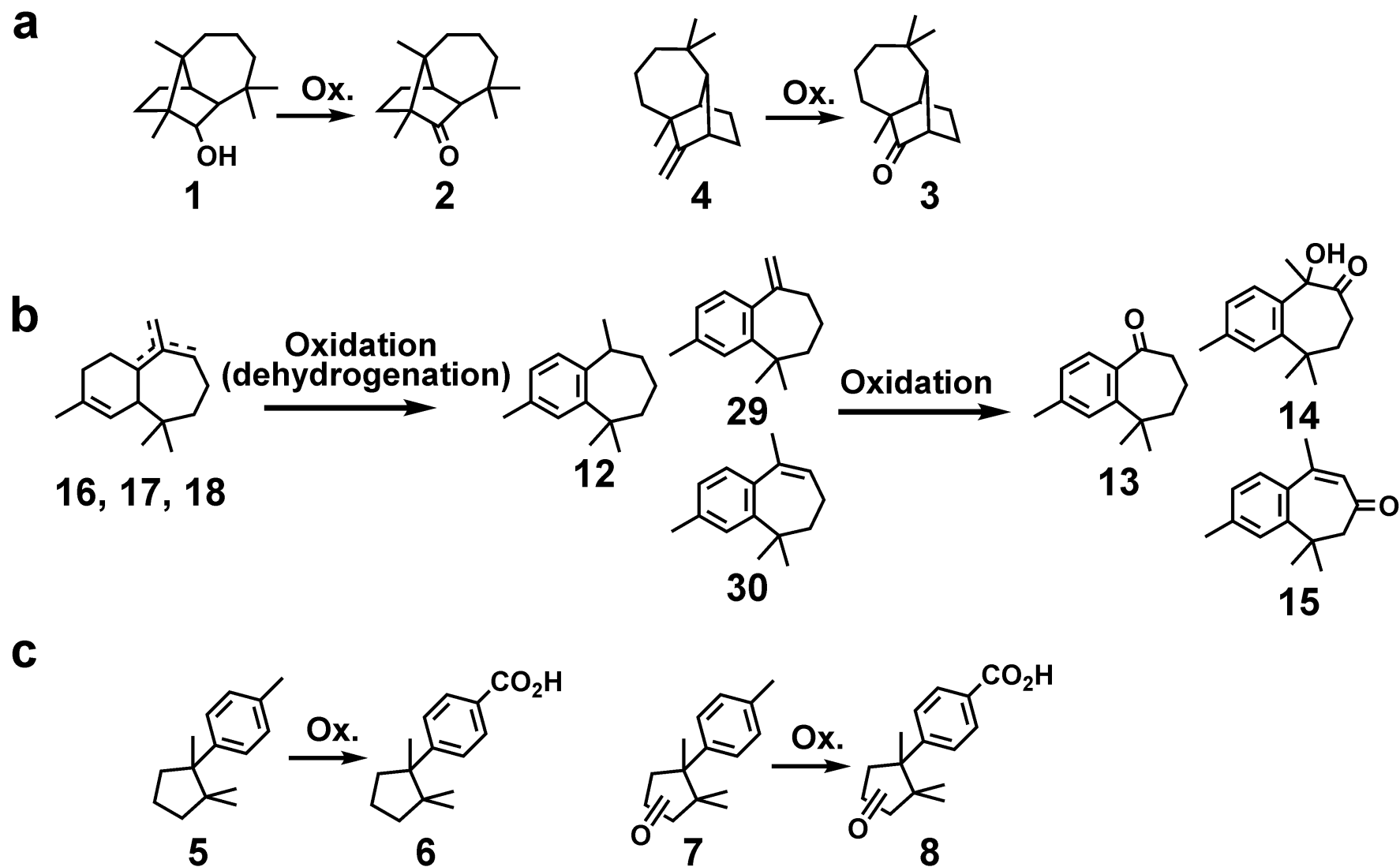
**Fig. 2**



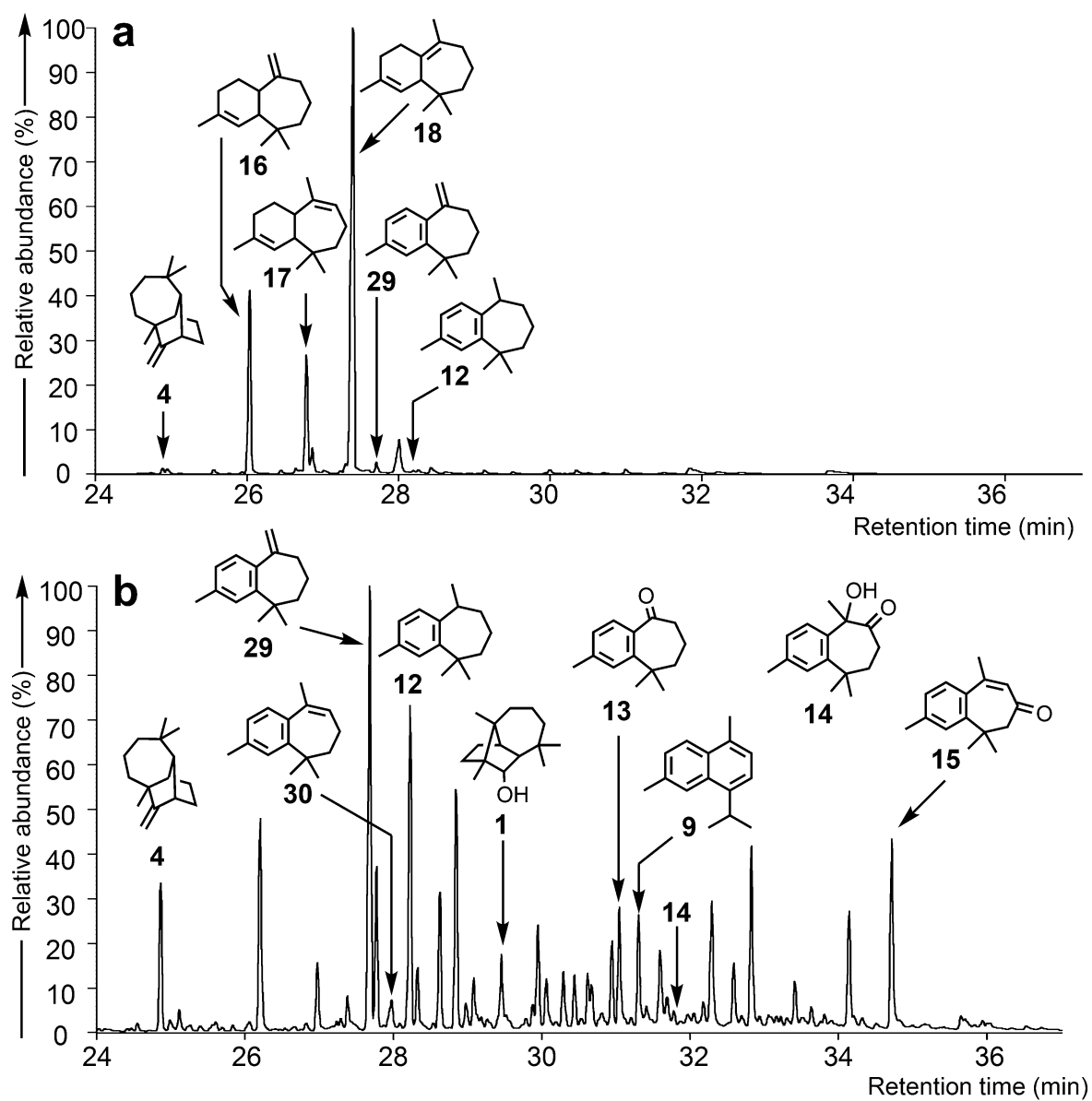
**Fig. 3**



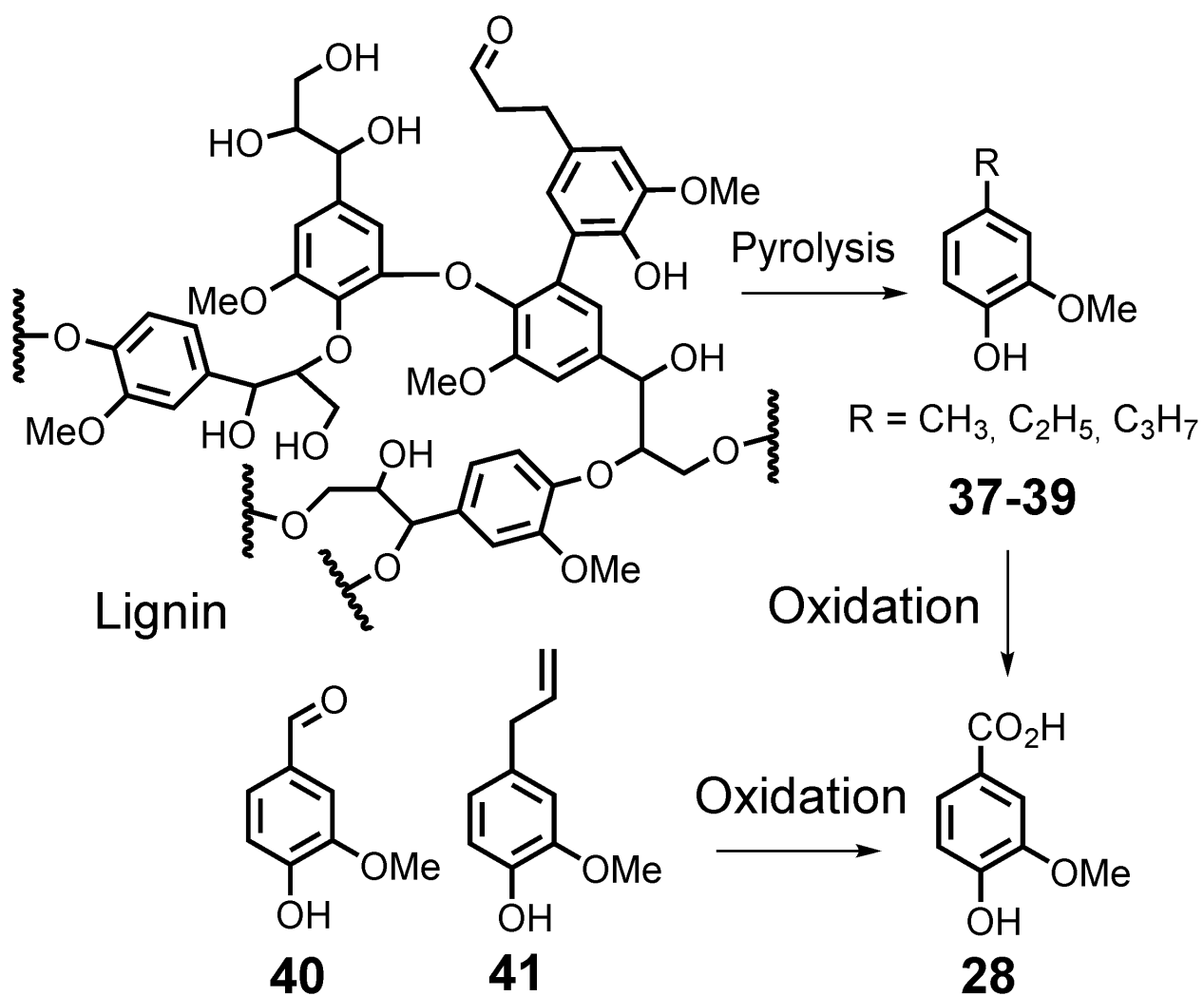
**Fig. 4**



**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”**

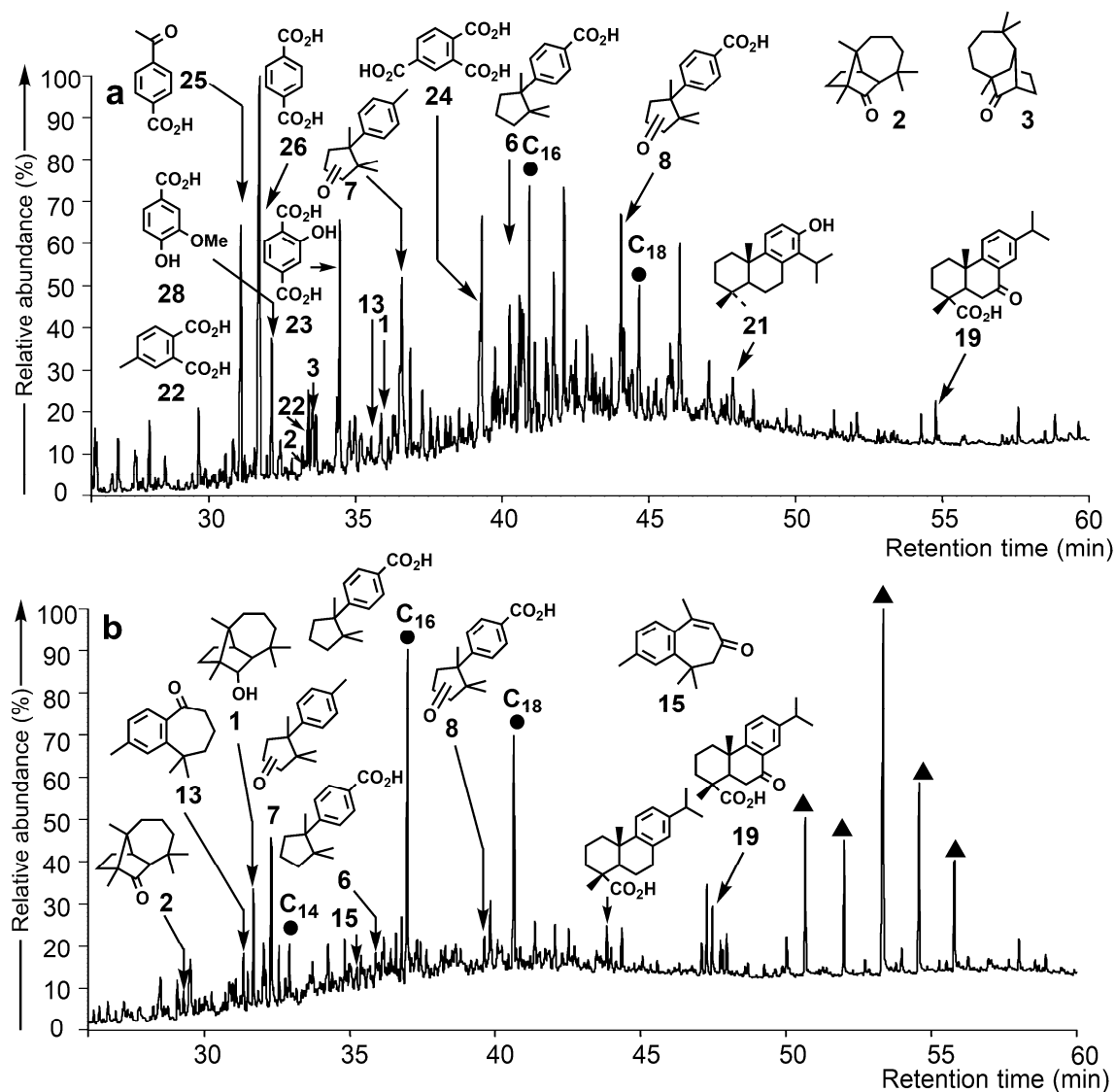
Mathilde Sarret,<sup>a</sup> Pierre Adam,<sup>a\*</sup> Philippe Schaeffer,<sup>a</sup> Quentin Ebert,<sup>a</sup> Julien  
Perthuisson,<sup>a</sup> Geneviève Pierrat-Bonnefois<sup>b</sup>

<sup>a</sup> *Université de Strasbourg, CNRS, Institut de Chimie de Strasbourg UMR 7177, F-67000 Strasbourg, France*

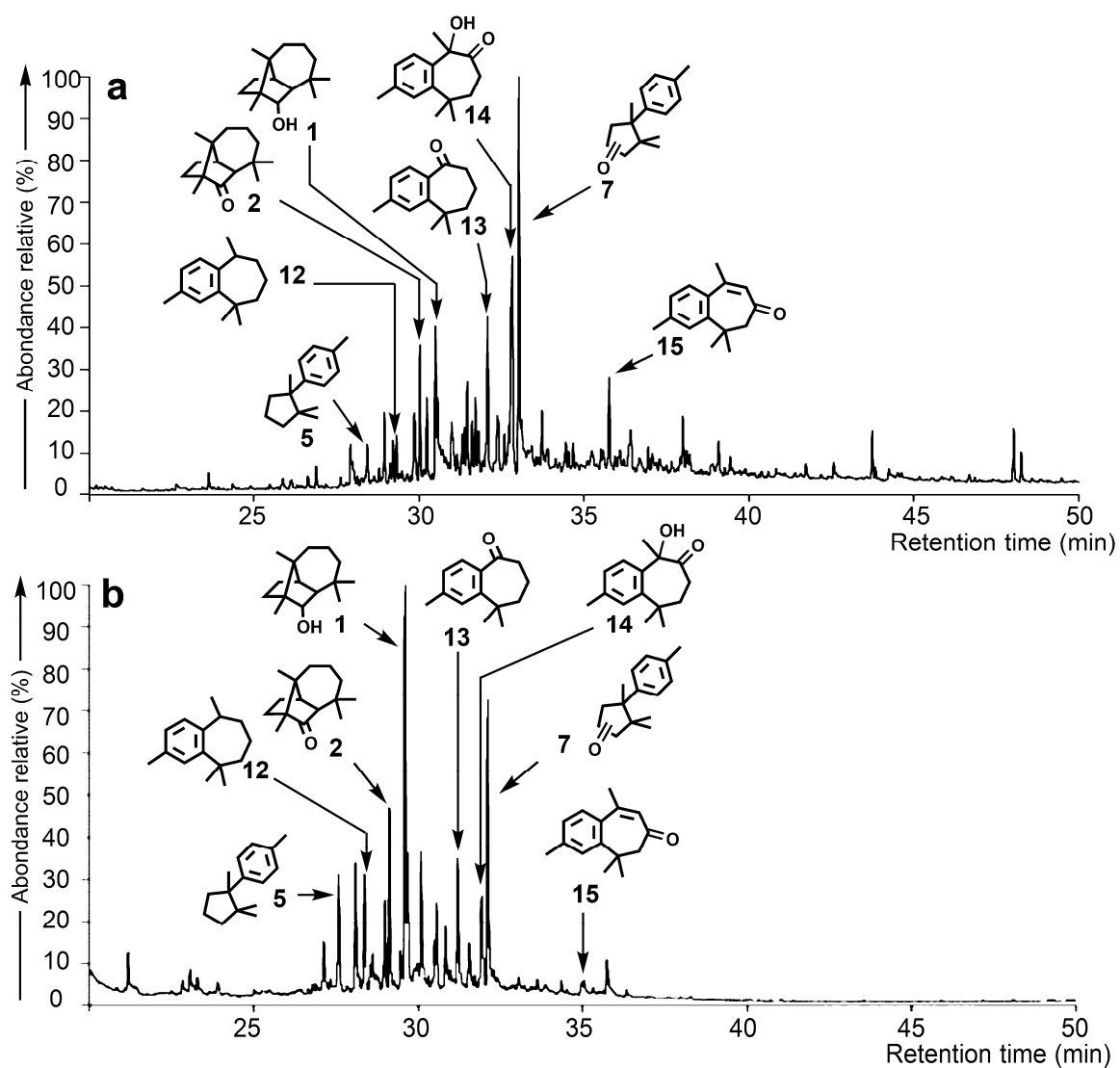
<sup>b</sup> *Département des Antiquités Égyptiennes, Musée du Louvre, F-75058 Paris, France*



# 1. Gas chromatograms of F1 and F1<sub>volatile</sub> 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.

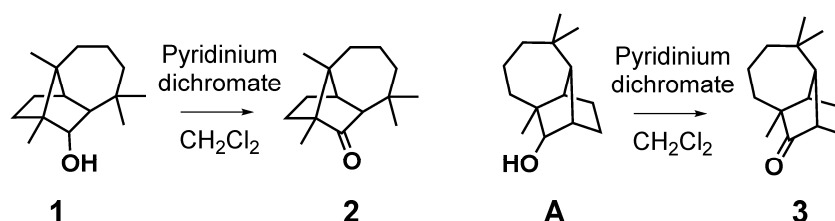


**Figure S2** : Gas chromatograms of the fractions  $F1_{\text{volatile}}$  from the organic material found in jars (a) E21809 and (b) E28010 (numbers refer to structures in the Appendix).

## 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 longicamphenylol **A**, respectively.

Longiborneol **1** (300  $\mu\text{g}$ ) (respectively longicamphenylol **A**, 340  $\mu\text{g}$ ) was reacted with pyridinium dichromate (20 mg) in dry  $\text{CH}_2\text{Cl}_2$  (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.

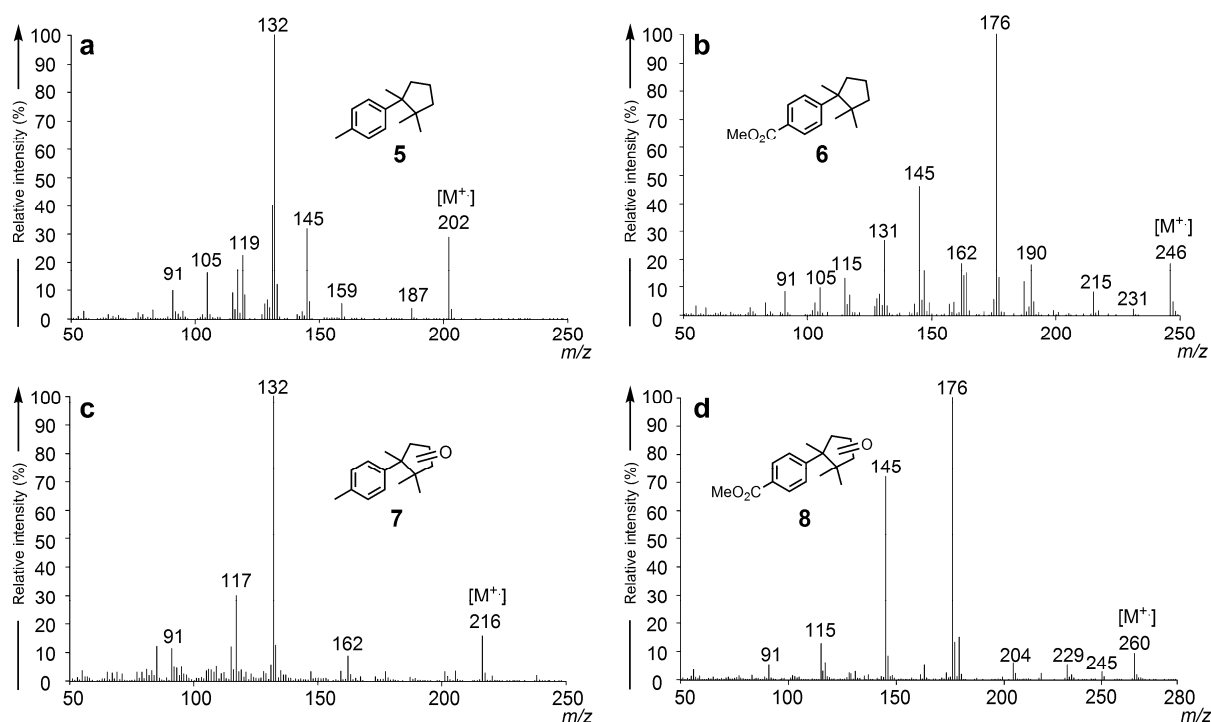
Longicamphor **2**: GC-MS (EI, 70eV);  $m/z$  (relative intensity) 220 ( $\text{M}^+$ , 92%), 177 (91), 137 (45), 136 (44), 124 (52), 121 (72), 107 (64), 95 (100)

Longicamphenilone **3**: GC-MS (EI, 70eV);  $m/z$  (relative intensity) 206 ( $\text{M}^+$ , 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

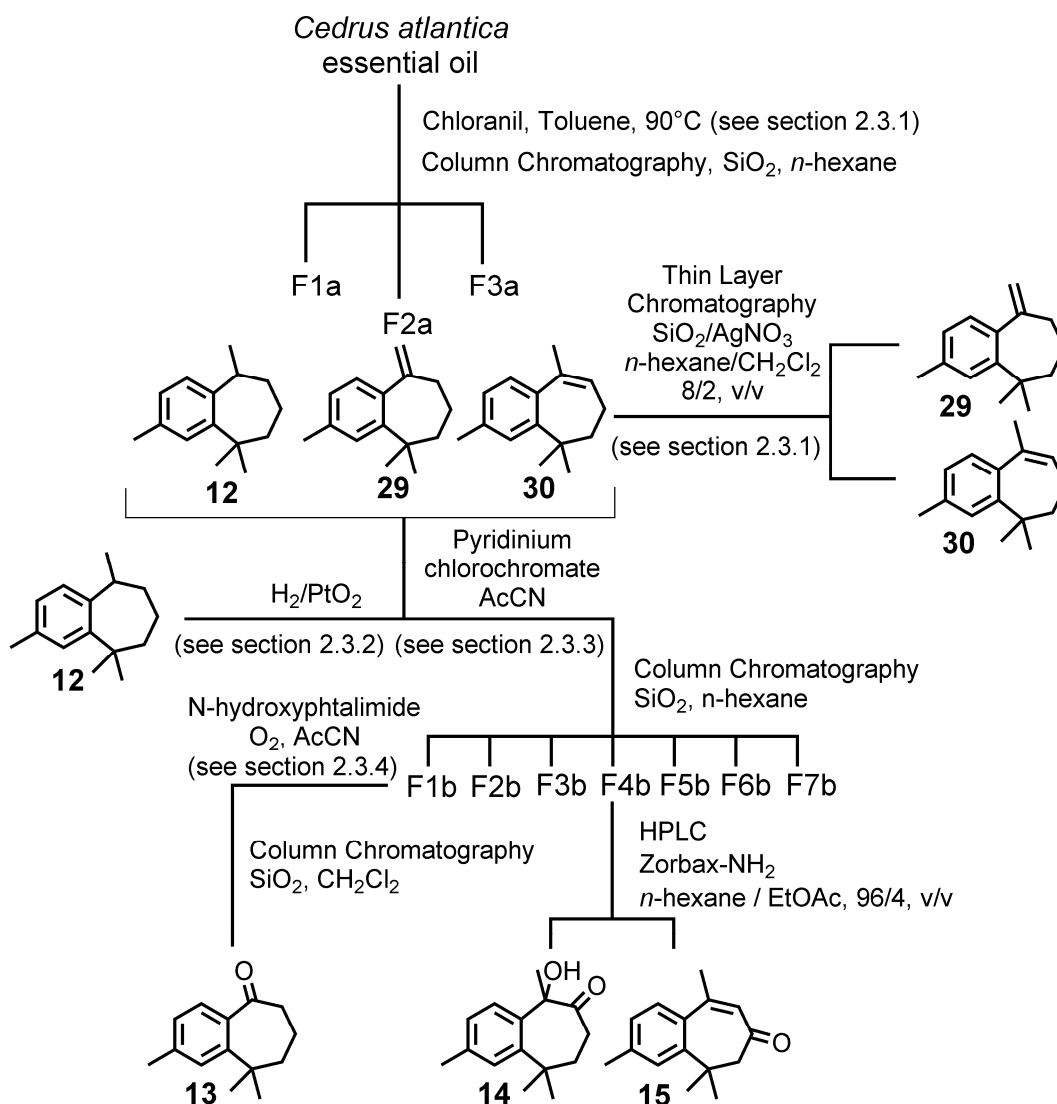
Cuparene **5** was identified in the archaeological samples by comparison with the mass spectrum and retention time on GC with that of cuparene **5** from the essential oil of Texas cedar (*Juniperus mexicana*) (Sigma-Aldrich). The methyl ester of cuparenic acid **6** was identified based on the comparison 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  $\alpha$ -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

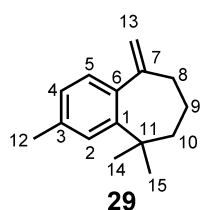
Compounds **12-15**, **29** and **30** were prepared from the essential oil of *Cedrus atlantica* (Laboratoire Sirius), the terpenoid distribution of which being dominated by  $\beta$ -,  $\alpha$ - and  $\gamma$ -himachalene **16-18** (Fig. 6a). The experimental protocol used is shown in Fig. S5.



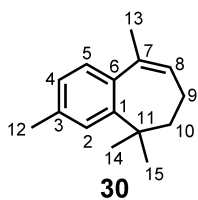
**Figure S5:** Preparation and isolation of himachalene-related sesquiterpenoids **12-15**, **29** and **30** from *Cedrus atlantica* essential oil. AcCN: acetonitrile; HPLC: high performance liquid chromatography.

### 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 N<sub>2</sub> 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/CH<sub>2</sub>Cl<sub>2</sub> (8/2, v/v) as developer (Fig. S5).



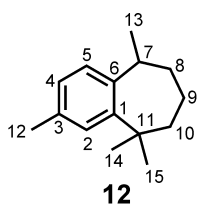
**Compound 29.** GC-MS (EI, 70 eV); *m/z* (relative intensity) 200 (M<sup>+</sup>, 55%), 185 (69), 157 (100), 143 (43), 128 (13), 115 (12). <sup>1</sup>H-NMR (500 MHz; CD<sub>2</sub>Cl<sub>2</sub>): 1.315 (6H, s, H-14 and H-15), 1.69 (2H, m), 1.86-1.98 (2H, m), 2.31 (3H, s, H-12), 2.33 (2H, m), 4.92 (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). <sup>13</sup>C-NMR (125 MHz; CD<sub>2</sub>Cl<sub>2</sub>): 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;  $\text{CD}_2\text{Cl}_2$ ): 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;  $\text{CD}_2\text{Cl}_2$ ): 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).  $\text{PtO}_2$  (ca. 10 mg) was added and the mixture stirred under a  $\text{H}_2$  atmosphere. After 2 h, the mixture was put under  $\text{N}_2$  atmosphere and the solid catalyst was filtered off and washed with  $\text{CH}_2\text{Cl}_2$ . *ar*-Himachalene **12** was obtained in almost quantitative yield.

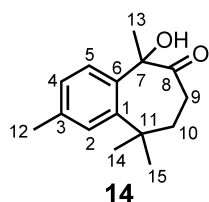


**Compound 12.** GC-MS (EI, 70 eV);  $m/z$  (relative intensity) 202 ( $M^+$ , 50%), 187 (100), 159 (16), 145 (92), 131 (67), 128 (21).  $^1\text{H-NMR}$  (500 MHz;  $\text{CD}_2\text{Cl}_2$ ): 1.21 (1H, m), 1.312 (3H, s, H-14 or H-15), 1.319 (3H, d,  $J = 6.5$  Hz, H-13), 1.41 (3H, s, H-14 or H-15), 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;  $\text{CD}_2\text{Cl}_2$ ): 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:

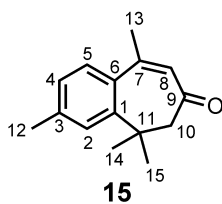
#### Preparation of **14** and **15**

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 CH<sub>2</sub>Cl<sub>2</sub> (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 CH<sub>2</sub>Cl<sub>2</sub>/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-NH<sub>2</sub> 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*<sup>+</sup> - H<sub>2</sub>O, 3%), 199 (3), 189 (100), 171 (15), 119 (72), 105 (11), 91 (7). GC-MS (CI, isobutane) *m/z* (relative intensity) 233 ([*M*+*H*]<sup>+</sup>, <1%), 215 ([*M*+*H*-H<sub>2</sub>O]<sup>+</sup>, 100). <sup>1</sup>H-NMR (500 MHz; CD<sub>2</sub>Cl<sub>2</sub>) : 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). <sup>13</sup>C-NMR (125 MHz; CD<sub>2</sub>Cl<sub>2</sub>): 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.

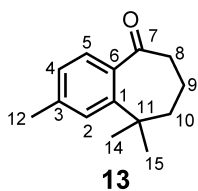




**Compound 15.** GC-MS (EI, 70 eV);  $m/z$  (relative intensity) 214 ( $M^+$ , 42%), 199 (47), 171 (100), 157 (77), 156 (98), 141 (47), 128 (33), 115 (33).  $^1\text{H-NMR}$  (500 MHz;  $\text{CD}_2\text{Cl}_2$ ): 1.41 (6H, s, H-14 and H-15), 2.37 (6H, s, H-12 and H-13), 2.66 (2H, s, H-10), 6.24 (1H, 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;  $\text{CD}_2\text{Cl}_2$ ): 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 $\text{O}_2$ and *N*-hydroxyphthalimide (NHPI): Preparation of **13** (adapted from Lin et al., 2012)

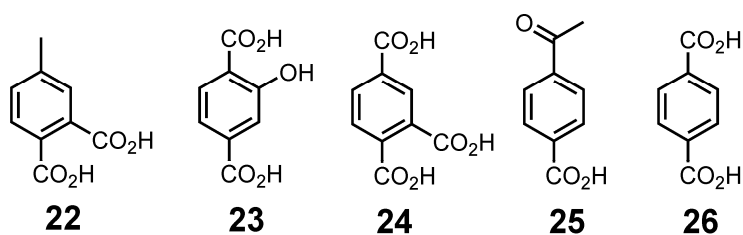
Fraction F1b (cf. section 2.3.3, **Fig. S5**) was shown by GC-MS to be mainly constituted by the unsaturated ar-himachalene derivative **29** and minor amounts of compounds **12**, **30**. To an aliquot of this fraction (20 mg), NHPI (4.5 mg),  $\text{H}_2\text{O}$  (2  $\mu\text{l}$ ) and AcCN (800  $\mu\text{l}$ ) were added and the mixture heated at 80  $^\circ\text{C}$  under stirring in a round-bottomed flask under  $\text{O}_2$  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  $\text{O}_2$  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  $\text{O}_2$  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/ $\text{CH}_2\text{Cl}_2$  (8/2, v/v) and  $\text{CH}_2\text{Cl}_2$ , successively, yielding 0.9 mg of ketone **13** ( $\text{CH}_2\text{Cl}_2$  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;  $\text{CD}_2\text{Cl}_2$ ): 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;  $\text{CD}_2\text{Cl}_2$ ): 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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