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ABSTRACT

In the frame of a study aiming at defining new soil molecular biomarkers that could attest to former land use and that could also be screened in lake sediments for paleoenvironmental reconstruction, we analysed the 2-4 cm depth layer of a soil developed under a conifer forest in the Lake Aydat catchment (French Massif Central). The lipid fraction of this sample contained 12 serratenoids bearing at least a methoxyl (OMe) group at C-3 or C-21 and various additional functional groups (alcohol, ketone or acetate). A survey of the literature provides indubitable evidence that these compounds are typical for *Pinaceae* species, in agreement with the surrounding vegetation at the sampling site. Due to the economic and ecological importance of these taxa, the presence of these highly specific compounds in soil, sediments or peat would thus help in unravelling the timing of forestry activity.

Keywords: pentacyclic triterpenes, serratane, soils, biomarkers, *Pinaceae*

1. Introduction

Reconstructing the evolution of continental landscapes through time in relation to the development of human societies and climate change constitutes a challenge for both

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archaeologists and paleoenvironmentalists. Among other disruptions that profoundly affect vegetation composition, anthropogenic deforestation (for cultivation, fuel and building) has strongly modified the shape of continental surfaces for millennia. The timing and extent of the impact of such unsustainable use of natural resources on the global carbon cycle remains under debate. For example, it has long been hypothesised that the collapse of the Maya civilization was linked to over exploitation of natural resources, among them intensive deforestation and soil depletion (Demarest, 2004). Although this hypothesis has recently been denied in the light of new arguments (McNeil, 2011), it raises the question of environmental management by past populations and the subsequent recovery of natural systems. In Europe, Büntgen et al. (2011) used ca. 7300 oak ring dendrochronology measurements to relate periods of intense deforestation (numerous felling dates) to the prosperity of society (need for construction wood) whereas periods of reduced felling coincided with historical crises, highlighting at least 2,500 years of anthropogenic impacts on the evolution of landscapes. Boucher et al. (2009) applied the same approach to unravel the impact of logging on the composition and structure of the sub-boreal forest over the last 200 yr. Palynology provides additional information on the evolution of forestry, afforestation, treeline dynamics, forest clearance and, more generally, the evolution of continental landscapes under anthropogenic or climatic control (e.g. Pisaric et al., 2003; Stebich et al., 2005).

Independent of and complementary to pollen, molecular biomarkers are tracers that, if detected in soil, can provide clues about past local vegetation, and thus about past land use. In addition, when transferred to sedimentary archives, either directly from plants or via soils, these biomarkers can be used to reconstruct the evolution of ecosystems through time. With the notable exception of hopanoids that are principally attributed to bacteria (e.g. Ourisson et al., 1979) and fernane/arborane derivatives, the origin of which is still debated (Hauke et al., 1995; Jaffé and Hausmann, 1995), most pentacyclic triterpenes are classically attributed to Angiosperms (Cranwell, 1984; Das and Mahato, 1983). Due to their wide structural diversity, function and configuration, and to the limited number of organisms able to synthesize them, these compounds constitute valuable chemo-taxonomic markers and are increasingly being used in environmental reconstruction. For example, pentacyclic triterpene methyl ethers can be related to *Poaceae* (Ohmoto et al., 1970; Jacob et al., 2005), some of them being constrained to more specific taxa (Jacob et al., 2008a; 2008b; Zocatelli et al., 2010). Similarly, several triterpenyl acetates are produced mainly by *Asteraceae* (Lavrieux et al., 2011). Other compounds, such as long chain *iso*- and *anteiso*-monomethyl alkanes that have recently been described in *Lamiaceae*, also enlarge the panel of biomarkers specific to herbs

of potential economic importance (Huang et al., 2011). However, from an environmental point of view, all these compounds attest to the development of open vegetation, either under the influence of natural conditions or due to human activity. In contrast, very few molecular biomarkers have been reported as specific for trees, allowing, for example, distinguishing between angiosperm and conifer trees. For example, the contribution of gymnosperms to the organic matter (OM) of soils or sediments is often estimated through the quantitation of lignin monomers, a low syringyl/vanillyl phenols ratio (S/V) being typical for these plants (e.g. Hedges and Mann, 1979). More specific biomarkers of gymnosperm input are diterpenes and sesquiterpenes that are largely used to identify a gymnosperm contribution to OM in amber and resin, soil, sediments, rocks (coals included) and even aerosols (e.g. Simoneit et al., 1985, 1993; Killops et al., 1995; Otto and Simoneit, 2002; Otto et al., 2005; Bechtel et al., 2005; Otto and Simpson, 2005). Nevertheless, few of these terpenoids are specific at the level of the genus or at least of the family (e.g. Aplin et al., 1963; Otto and Wilde, 2001).

Here we report on a series of oxygenated methoxy-triterpenoids having a basic serratane structure and a wide diversity of functional groups [methoxyl(s) as well as ketone, acetate and alcohol] and configurations that were detected in the lipid extracts of a soil developed under a conifer forest. After describing their mass spectral characteristics and assignment, we discuss their known sources and potential as chemotaxonomical markers and as potential tracers of plants designed for palaeoenvironmental studies.

2. Material and methods

2.1. Setting

As in many other parts of the world, French landscapes have been intensively transformed over the two last centuries. After centuries of extensive clearing and overexploitation the forest surface progressively redeveloped to be doubled until ca. 1830, mainly thanks to the combined effect of rural exodus, increased agricultural productivity and industrial revolution (Bazin et al., 1983; Cinotti, 1996). Representative of this situation is the Massif Central, a basement massif in the centre of France. Its mainly artificial refforestation dates back to the middle of the 19th century, to further national programs aiming at fighting against erosion and floods, and at developing industrial management of the forests in a context of a worldwide increase in demand for wood (Gadant, 1968; Cinotti, 1996). Rapid growth, hardiness and quality of the wood made resiniferous trees the preferred species: *Picea Abies* (Norway/common spruce; 50%), *Pseudotsuga menzeiesii* (Douglas fir; 15%), *Pinus sylvestris* (Scots pine; 15%), *Abies alba* (silver fir; 10%) and *Abies grandis* (grand fir; 10%),

plus *Picea sitchensis* (Sitka spruce), *Pinus nigra* (European black pine) and its *subsp. laricio var. corsicana* (Corsican pine) and *Pinus strobus* (Eastern white pine) were planted in the region (Gadant, 1968).

Located on the northern part of the Massif Central, on the northeastern side of the volcanic Sancy Massif (ca. 25 km SW of Clermont-Ferrand), Pessade wood is a mid-mountainous forest in the Lake Aydat catchment. Ranging from 1175 to 1300 m above sea level (m,a.s.l.), this artificial forest is mainly made up of ca. 60 yrs-old trees (*Pinus sylvestris*, *Abies alba*, *Picea abies* and, to a lesser extent, *Pseudotsuga menzeisii*). The management of the forest is shared between wood exploitation (that started at the beginning of the 20th century) and a ski resort.

2.2. Sampling, lipid extraction and separation

Several soil samples representatives of the different contexts encountered in Lake Aydat catchment were sampled in course of the ERODE project (EC2CO/INSU/CNRS) and depicted in previous papers (Lavrieux, 2011; Zocatelli et al., 2012; Lavrieux et al., 2012). Here we only report results from a soil sample collected between 2 and 4 cm depth from a core (22 cm long x 8 cm diameter) drilled in the Pessade Forest that exhibited the largest diversity of methoxy-serratenes. After drying in an oven (40 °C, 48 h), samples were crushed in a mortar and sieved at 2 mm. Free lipids were extracted from ca. 2 g dried and milled soil samples by using an automated solvent extractor (Dionex ASE 200®) with dichloromethane DCM:MeOH (9:1 v/v). After solvent evaporation under N_2, the total extract was separated into neutral, acidic and polar compounds using solid phase extraction with aminopropyl bonded silica. Neutral compounds were eluted with DCM:isopropanol (2:1 v/v) and acidic compounds with Et_2O after acidification of the medium with Et_2O:HCO_2H (9:1 v/v). Neutral lipids were fractionated by way of flash chromatography on silica activated (48h) at 110 °C and then deactivated with 5% water. After successive elution of aliphatic hydrocarbons, aromatics and ethers, the ketone fraction (F4-5) was eluted with hexane:EtOAc (19:1 and then 9:1). Two alcohol fractions F6 and F7 were then recovered with hexane:EtOAc (17:3 and then 4:1). The alcohol fractions were silylated using BSTFA and pyridine, and an internal standard [5α(H)-cholestane] was added in all fractions.

2.3. Quantification and identification

The ketone and silylated alcohol fractions were analysed using gas chromatography-mass spectrometry (GC-MS) with a TRACE-PolarisGCQ instrument equipped with an AS

3000 autosampler. The gas chromatograph was fitted with an Rtx-5Sil MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness) with 5 m of guard column. The GC operating conditions were: 40 °C (1 min) to 100 °C at 10 °C min^{-1}, then to 300 °C (held 30 min) at 4 °C min^{-1}. The sample in toluene (2μl) was injected splitless, at 280°C. The carrier gas was He and at 1 ml min^{-1}. The mass spectrometer was operated in the electron ionisation (EI) mode at 70eV and scanned from *m/z* 50 to 600. Assignment was performed using two authentic standards [3α-methoxy-serrat-14-en-21β-ol (PJ1) and 3β-methoxy-serrat-14-en-21β-ol (PJ2), provided by R. Tanaka, Osaka University of Pharmaceutical Sciences, Japan], and their silylated and acetylated counterparts, by comparison of mass spectra and retention times (RTs) with published data (Kutney et al., 1969a; Tanaka et al., 1994; 1997) and interpretation of mass spectra. Quantitation was performed by comparing the area of the peak of 5α(H)-cholestane with that of the compound of interest on the total ion chromatogram (TIC). In cases of coelution, the area was measured on specific ion chromatograms and then converted to the calculated TIC peak area by applying a correction factor.

3. Results

Twelve compounds were assigned as methoxy-serratenes: compounds **1** to **7** were present in the ketone fraction (F4-5) between 58 and 71 min and compounds **8** to **12** were in the alcohol fractions (F6 and F7) between 57 and 73 min RT (Fig. 1). Concentration ranged from ca. 0.01 to 20 (avg. 2) μg g^{-1} soil.

3.1. Common MS features

The mass spectra and structures of the compounds detected in the soil sample are illustrated in Fig. 3, their main characteristics and the proposed fragmentation mechanisms are reported in Table 1 and in Fig. 4, respectively. Carbon numbering, ring labelling and location of functional groups are displayed in Fig. 2. Only structures bearing two or three oxygenated functional groups at C3 (R1), at C21 (R2) and C15 (R3) were detected but other compounds discussed in Section 4.2 and cited in Table 2 bear functional groups R4 to R8 (Fig. 2). Rearranged serratane structures such as piceanane and jezanane (Fig. 2) are only briefly discussed in Section 4.2.1.

Serratane triterpenes contain seven tertiary methyls (instead of eight in common pentacyclic triterpenes), a central seven membered C ring and a double bond between C-14 and C-15 (Δ^{14}) or C-13 and C-14 (Δ^{13}; Fig. 1). Such a double bond location implies a fragmentation pattern quite similar to that of taraxerane-type compounds (Budzikiewicz et al.,

1963; Kutney et al., 1969a), i.e. retro Diels-Alder (RDA) rearrangements leading to the fragments f, f', g and h, and ring C cleavage leading to the fragments i, j, k, k' and l (Fig. 4).

All 12 compounds bear at least two oxygenated functional groups at C-3 (R1) and C-21 (R2), respectively (Fig. 2). Compound **12** supposedly bears an additional oxygenated group at C-15 (R3). The loss of a methoxy from C-3 or C-21 is evidenced by the mass spectra of all compounds via the presence of pairs of ions differing by 32 mass units (i.e. fragments a-b, a'-b', a'-c', c-d, f-g, f'-h, i-j and k'-l,Fig. 4). Considering these general characteristics, the assignment of individual methoxy-serratenes is now discussed as follows on the basis of different oxygenated functional groups, and their location and isomerism.

3.2. Dimethoxy-serratenes

The mass spectra of compounds **2** and **3** (Fig. 3), found in the ketone fraction, exhibit molecular ion $M^{+\cdot}$ (a; Fig. 4) at *m/z* 470, consistent with a $C_{32}H_{54}O_2$ formula (Table 1). The loss of a first methoxyl group (OMe) from the molecular ion is evidenced by ion $[M - 32]^{+\cdot}$ (*m/z* 438; fragments b and c) and is followed by elimination of another OMe as attested by a significant fragment $[M - 64]^{+\cdot}$ (*m/z* 406; d). This implies that compounds **2** and **3** are di-methoxylated and is further supported by the loss of OMe groups on both rings AB and DE after ring C opening (fragments i-j at *m/z* 221-189 and fragments k'-l at *m/z* 219-187, respectively). RDA rearrangements that affect ring D (due to the presence of Δ^{14} bond) lead to ring AB fragments pairs f-g and f'-h (*m/z* 316-284 and *m/z* 301-269, respectively). Accordingly, compounds **2** and **3** are assigned as 3α,21α-dimethoxy-serrat-14-ene and 3β,21α-dimethoxy-serrat-14-ene, respectively, in agreement with Kutney et al. (1969a). The attribution of 3α,21α and 3β,21α configuration is based on the elution order of the standards PJ1 and PJ2, the 21β alcohol analogues of compounds **2** and **3** (Section 3.5).

3.3. Methoxy-acetoxy-serratene

The mass spectrum of compound **7** (Fig. 3) displays a $M^{+\cdot}$ ion at *m/z* 498 consistent with a $C_{33}H_{54}O_3$ formula. The presence of an OMe on rings A-B is evidenced by fragments f, f', g and h at *m/z* 316, 301, 284 and 269 (Fig, 4) that arise from RDA rearrangements from the Δ^{14} bond. Pairs of ions differing by 60 amu (a-b at *m/z* 498-438, a'-b' at *m/z* 483-423 and c-d at *m/z* 466-406; Fig. 4) reveal the presence of an acetate group. This group is located on rings D-E as attested by the 60 amu difference between ions k' (*m/z* 247) and l (*m/z* 187). Accordingly, compound **7** is interpreted as a 3-methoxy-serrat-14-en-21-yl acetate.

Comparison of the RT of **7** with those of the acetylated counterparts of 21β alcohols PJ1 and PJ2 allowed us to attribute a 21α configuration, with no further information on the configuration of the methoxy at C-3. Therefore, compound **7** is tentatively assigned as 3-methoxy-serrat-14-en-21α-yl acetate.

3.4. Methoxy-keto-serratenes

The mass spectra of compounds **1**, **4**, **5** and **6** (Fig. 3) are characterised by a $M^{+\cdot}$ (a; Fig. 4) at *m/z* 454 and a fragment at *m/z* 422 [$M^{+\cdot}$ - 32] (b or c; Fig. 4) that attest to the loss of an OMe. These fragments are accompanied by their demethylated counterparts at *m/z* 439 (a') and 407 (b' or c'; Fig. 4).

In the spectra of compounds **4** and **6**, fragments f (*m/z* 300) and f' (*m/z* 285) attest to RDA rearrangement from the Δ^{14} bond. For **5**, the rearrangement is evidenced by a weak fragment g at *m/z* 284, the corresponding f fragment at *m/z* 316 being very low in abundance. Compound **1** does not show any fragment that could be attributed to a RDA rearrangement and is therefore interpreted as a Δ^{13} structure, in agreement with observations by Kutney et al. (1969a) on such compounds. Therefore, compounds **2**, **5** and **6** are serrat-14-enoids whereas compound **1** is a serrat-13-enoid.

In the spectra of **4** and **6** (Fig. 3) the loss of an OMe from *m/z* 219 (k') is evidenced by an ion at *m/z* 187 (l). This ion further indicates that the OMe is located at C-21. In addition, fragments f (*m/z* 300) and f' (*m/z* 285) resulting from the RDA rearrangement in these Δ^{14} structures attest to the presence of a keto group at C-3. Hence, **4** and **6** are interpreted as C-3 keto, C-21 methoxy-serratenoids. The spectra of **1** and **5** do not show any of these fragments. They are thus interpreted as C-3 methoxy, C-21 keto-serratenes, consistent with the observation of Tanaka et al. (1994) who noticed that fragments *m/z* 454, 439, 422 and 407 are more abundant in C-3 methoxy, C-21 keto-serratenoids than in their C-3 methoxy, C-21 keto counterparts. Accordingly, **1** is interpreted as being a 3-methoxy-serrat-13-en-21-one. Because only a 3β epimer has been reported, **1** is likely to be 3β-methoxy-serrat-13-en-21-one. Compound **5** is interpreted as a 3-methoxy-serrat-14-en-21-one with no additional information on the configuration at C-3. Finally, **4** and **6** are assigned as 21-methoxy-serrat-14-en-3-ones. Tentative assignment of the C-21 OMe configuration can be based on the following considerations: (i) PJ1 (3α) elutes earlier than PJ2 (3β), consistent with the statement that 3α epimers of methoxy-triterpenes elute earlier than their 3β counterparts (Jacob et al., 2005); (ii) serratenes can be viewed as symmetrical structures (except for the

double bond location; Fig. 2). One can argue that the elution order would be the reverse for the α and β epimers at the C-21 (i.e. 21β elutes earlier than 21α), considering the presence of an identical group at C-3. Compounds **4** and **6** are thus tentatively attributed a 21β and a 21α configuration, respectively.

3.5. Methoxy- hydroxy-serratenes

The spectra of **8**, **9**, **10** and **11** (Fig. 3) display $M^{+\cdot}$ at *m/z* 528, consistent with silylated serratene structures bearing both a OMe and an OH group with a $C_{31}H_{52}O_2$ formula. Compounds **9** and **10** are assigned as 3α-methoxy-serrat-14-en-21β-ol (PJ1) and 3β-methoxy-serrat-14-en-21β-ol (PJ2), respectively, by comparison with authentic standards.
As observed for compound **1** in the ketone fraction, compound **8** elutes earlier than any other compound in the alcohol fraction. Because of a lack of any fragment attributable to a RDA rearrangement (fragment f at *m/z* 316 and g at *m/z* 284) in its spectrum, the double bond of **8** must be a Δ^{13} one. Compound **8** could be 3α-methoxy-serrat-13-en-21β-ol, the sole methoxy-serrat-13-en-ol reported in natural samples (Table 2). Conversely, significant fragments at *m/z* 316 (f) and 284 (g) in the spectrum of compound **11** which result from a RDA rearrangement, attest to a Δ^{14} structure with an OMe at C-3. The two 21β epimers being already assigned as **9** (PJ1), **10** (PJ2) and **11** must be a 21α epimer. No additional information on the configuration of the OMe group at C-3 can be provided.

3.6. *Methoxy-diketo-serratene*

The spectrum of **12** is very similar to that of 15-keto-21β-methoxy-serrat-13-en-3-one reported by Tanaka et al. (1997), with the tentative fragmentation scheme depicted in Fig. 4.

4. Discussion

Serratane-type compounds constitute an original group of naturally occurring pentacyclic triterpenes with an unusual 7 carbon C ring. These 14α-homo-27-norgammaceranes are biogenetically related to α-onocerin, i.e. synthesized through the cyclisation of bis-epoxy-squalene and not from epoxy-squalene, the precursor of more common pentacyclic triterpenes (oleanane, ursane, lupane…; Xu et al., 2004).

4.1. Known occurrences of serratenoids in plants and in geological archives

To our knowledge, the only descriptions of serratane derivatives in geological archives was reported by Tieguan et al. (1988) who reported on the occurrence of serratanes in oils and by Le Métayer et al. (2005) who found a related aromatic hydrocarbon in Oligocene sediments from the Rhine valley (France). The latter compound was interpreted as resulting from the microbial aromatisation of a triterpene related to serratenediol but with no clear relationship with a potential source.

More than 100 different serratenoids (serratenes, serratenones, serratene acids, serratene polyols and their acetates…) have been reported, most having been identified in club mosses such as *Lycopodiaceae* and *Huperziaceae*, in *Polypodiaceae*, in Conifers, and in few Angiosperms (*Primulaceae* and *Leguminosae*; see Supplementary Material**).**

4.2. Known occurrences of methoxy-serratenes in plants

As opposed to other serratenoids, methoxy-serratenes appear constrained to conifers, and more specifically to *Pinaceae* (Table 2). Four exceptions should be noted. Beneš et al. (1981) reported the occurrence of 2lα-methoxy-serrat-14-en-3-one (**6**) in the liverwort *Nardia scalaris* but indicated that the plant studied was sampled under common spruce and was probably contaminated by the latter species. Similarly, 3α-methoxy-serrat-14-en-21β-ol (**9** – PJ1), 3β-methoxy-serrat-14-en-21β-ol (**10** – PJ2) and 3β-methoxy-serrat-14-en-21-one (**5**) that were in *Homalia trichomanoides* (Bryophytes, *Neckeraceae*; Wang and Lou, 2005) might have originated from *Pinaceae* close to the sampling location. For the same reason, the detection of six methoxy-serratenes in the tree moss *Pseudevernia furfuracea* (Ascomycetes) that grows on *Pinus sylvestris* could be attributed to a contamination by the host tree (Joulain and Tabacchi, 2009). Finally, *Homogynes alpina* (*Asteraceae*) was cited as a potential source for 2lα-methoxy-serrat-14-en-3-one (**6**), allowing Beneš et al. (1981) to argue for a larger than expected distribution of methoxy-serratenes in plants. Due to the lack of precise information on this report, this citation must be taken with caution.

Except from these peculiar cases, most of the 40 methoxy-serratenes reported up to now (Table 2) were isolated from *Pinaceae*: *Picea* and *Pinus* genera and, to a lesser extent, plants of the *Abies* and *Cathaya* genera. It is worthwhile noting that these methoxy-serratenes appear tissue-specific since they were only reported in bark (Norin, 1972). The following discussion completes and updates a previous compilation by Otto and Wilde (2001).

4.2.1. Picea

With 23 structures reported, *Picea jezoensis* (Jezo or Yeddo spruce, *Picea ajanensis*) displays the greatest diversity of methoxy-serratenes of all plants studied (Chernenko et al., 1990; 1992a; Tanaka et al., 1994, 1995, 1996, 1997, 1998, 1999a, b; 2001, 2002; Doi et al., 2010). This species was reported to produce a large compound spectrum, from methoxy-serratenones (**1**, **4**, **5**), their 29-nor equivalents and methoxy-serratenedione (**12**), methoxy-serratenols (**9**, **10**) and methoxy-serratenediols, methoxy-hydroxy-serratenone, methoxy-hydroxy-serratenals, methoxy-epoxy-serratenones and methoxy-epoxy-serratenols. In addition, *P. jezonensis* is the sole known source of rearranged serratene structures such as piceanane and jezanane (Fig. 2; Tanaka et al., 1999a; 2002).

Eight serratene derivatives were extracted from the bark of *Picea sitchensis*, six being methoxy-serratenes (**5**, **10**, 3α,21β-dimethoxy-serrat-14-ene, 3β-methoxy-serrat-14-en-21-one, 3α-methoxy-serrat-13-en-21β-ol and 3α-methoxy-serrat-14-en-21α-ol) the two others being 21-episerratenediols (Kutney and Rogers, 1968; Kutney et al., 1969b; Rogers and Rozon, 1970). The bark of *Picea abies* was shown to contain 3α-methoxy-serrat-14-en-21β-ol (**9**), 21α-methoxy-serraten-14-one (**6**) and 3β-methoxy-serrat-14-en-21β-ol (**10**; Norin and Winell, 1972a). PJ1 (**9**) and PJ2 (**10**) were extracted from *Picea glehni* (Sakhalin spruce; Tanaka et al., 2000) and *Picea obovata* (Siberian spruce; Chernenko et al., 1992b). They were accompanied by 3α-methoxy-serrat-14-en-21β-yl formate and piceanonol A in the former and by compounds **1** and **5** (both isomers) in the latter.

4.2.2. Pinus

Eleven *Pinus* species have been reported to contain, in total, a series of 16 methoxy-serratenes. These species comprise *Pinus armandii* (Chinese white pine; Fang et al., 1991; Fang and Cheng, 1992), *Pinus banksiana* (Jack Pine; Rowe, 1964, Rowe, 1965; Rowe and Bower, 1965; Rowe et al., 1972), *Pinus contorta* (Rowe et al., 1972), *Pinus lambertiana* (Sugar Pine; Rowe, 1964; Rowe and Bower, 1965), *Pinus luchuensis* (Cheng et al., 1975; Wada et al., 2001), *Pinus palustris* (Longleaf Pine; Rowe 1964), *Pinus strobus* (Zinkel and Evans, 1972), *Pinus taeda* (Loblolly Pine; Rowe 1964), *Pinus radiata* (Weston, 1973) and *Pinus taiwanensis* (Cheng and Chao, 1979). Of importance is the detection of compounds **3**, **5**, **10**, **11**, 3β,21β-dimethoxy-serrat-14-ene, 21β-methoxy-serrat-14-en-3β-ol, 3β-methoxy-serrat-14-ene-21α,29-diol, 3β-methoxy-serrat-14-en-21β,29-diol, 3β-methoxy-serrat-14-ene-21α,30-diol and 29-nor-3β-methoxy-serrat-14-en-21-one in *Pinus monticola* Doug. (*Pinus albicaulis*, Western White Pine or Douglas Pine; Rowe et al., 1972; Conner et al., 1980, 1981;

1984) whereas **2**, **3** and **5** were found in the bark of *Pinus sylvestris* (Norin and Winnel, 1972b).

4.2.3. Abies, Cathaya and Cedrus genera

Except for the *Pinus* and *Picea* genera, reports on the occurrence of methoxy-serratenes in other *Pinaceae* are very restricted. Recently, Ou-Yang et al. (2011) found 3β-methoxy-serrat-14-en-21-one, 21α-methoxy-serrat-14-en-3-one and 3β-methoxy-serrat-14-en-21α-ol in *Abies nephrolepis*. He et al. (1981) and Ma et al. (1982) detected compound **5** in *Cathaya argyrophylla*. These are the only reports of methoxy-serratenes in these two genera. *Cedrus*, the last genus of the *Pinaceae* family, is not reported to contain methoxy-serratenes. As a matter of fact, Joulain and Tabacchi (2009) did not report any methoxy-serratene in lichens grown on a *Cedrus* sp., whereas they found some in lichens grown on pines (see above).

From the above evidences, and in agreement with a previous statement by Otto and Wilde (2001), it is clear that methoxy-serratenes are almost exclusive to *Pinaceae* bark, and more specifically to the *Pinus* and *Picea* genera.

4.3. Potential of methoxy-serratenes as chemotaxonomical markers of Pinaceae

The local conifer vegetation developed in the Pessade forest, mainly dedicated to forestry, is made up of *Picea abies*, *Pinus sylvestris*, *Abies alba* and *Pseudotsuga menziesii*. No *Pseudotsuga* species is known to produce methoxy-serratenes. *Abies alba* is not reported to produce these compounds but the occurrence of 3β-methoxy-serrat-14-en-21-one (**5**), 21α-methoxy-serrat-14-en-3-one (**6**) and 3β-methoxy-serrat-14-en-21α-ol (**11**) in *Abies nephrolepis* could preclude the discovery of such compounds in *Abies alba*. The bark of *Picea abies* was shown to contain compounds **6**, **9** and **10** (Norin et al., 1972a) and that of *Pinus sylvestris* is the source of **2**, **3** and **5**. Current knowledge on the distribution of methoxy-serratenes in *Pinaceae* thus only allows explaining the presence of compounds **2**, **3**, **5** (both isomers), and **6**, **9** and **10**. Among the compounds in the extract of the Pessade wood soil sample, only compound **7** (3-methoxy-serrat-14-en-21α-yl acetate) has never been reported in living plants. Compounds **1**, **4**, **8**, **11** and **12** are known in plants other than those presently growing in the Pessade wood.

As stated earlier (e.g. Jacob et al., 2005), the detection of peculiar biochemicals in soils and sediments allows anticipating phytochemical discoveries because they integrate the

chemical diversity of the local vegetation. Accordingly, the discrepancy between the diversity of methoxy-serratenes in our soil sample and that expected in the local vegetation on the basis of literature data could result from our incomplete phytochemical knowledge of the distribution of methoxy-serratenes in the plant kingdom.

Very few studies on methoxy-serratenes address the question of their variability in a single species depending on environmental conditions or simply to intra-specific variability. When compared to extracts directly obtained from *P. sylvestris* (Norin and Winell, 1972b), the greater diversity of methoxy-serratenes in lipid extracts of *Pseudevernia furfuracea* grown on this latter species (Joulain and Tabacchi, 2009) potentially illustrates this intra-specific variability.

New structures could also arise from transformation of existing methoxy-serratenes or other compounds during early diagenesis. Several studies exemplify the ability of early diagenetic transformations to increase chemodiversity through the transformation of biochemicals to geochemicals unknown in living organisms (see for example Jacob et al., 2005). Despite the presence of serratenediol or serratenetriol in numerous *Pinaceae* and especially *Picea* (Roger and Rozon, 1970) and *Pinus* (Rowe, 1970) species, no such compound was found in our soil sample. It can thus be hypothesized that some of our methoxy-serratenes could be derived from the transformation of precursor serratenediols or serratenetriols. Double bond migration or epimerisation of functional groups, known to occur in other pentacyclic triterpenes (Ageta et al., 1987; Rullkötter et al., 1994; Jacob et al., 2005), can also lead to compounds alien to the local vegetation.

From these evidences, methoxy-serratenes can be considered as biomarkers of *Pinaceae* but their use as biomarkers of more restricted taxa, or even at the species scale, must rely on additional data on their distribution in plants and on potential structural alterations they could suffer in soils.

5. Conclusions

The analysis of the lipid content of a soil from a conifer forest revealed a series of methoxy-serratenes bearing a central seven membered C ring and a wide diversity of functional groups. According to a literature survey, such compounds appeared to be mainly produced by *Pinaceae*. They thus constitute a new family of biomarkers specific for these tree species. When considering their low production in plants, the accumulation of these compounds in soils underlines their potential high preservation capacity that might favour their efficient transfer and archiving in sediments. Even if their occurrence in the sediments

remains to be verified, their detection in soils and other natural archives could attest to the evolution of forested surfaces with time, under natural or anthropogenic constraints. Filling a gap in specific biomarkers of arborescent vegetation, and in addition to diterpenoids, they potentially constitute a new powerful tool for palaeoenvironmental reconstruction. However, detailed studies of the distribution and variability of methoxy-serratenes in *Pinaceae* and on their behaviour during diagenesis are still necessary to fully exploit the possibilities offered by these new biomarkers.

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Table captions

Table 1

Main GC-MS characteristics of methoxy-serratenes detected in a soil under conifer forest in the catchment of Lake Aydat [compounds in bold identified with authentic standards (R. Tanaka, Osaka University, Japan].

N°[a]	Assignment	RT[b]	Δ[c]	R_1[d]	R_3[d]	R_2[d]	Formula	*m/z*[e]																
								a	a'	b	b'	c	c'	d	e	f	f'	g	h	i	j	k	k'	l	m	n
1	3β-methoxy-serrat-13-en-21-one	58,38	13	OMe	H	O	$C_{31}H_{50}O_2$	454	439	422	407	-		-	379	-	-	-	269	221	189	-	203	-	-	-
2	3α,21α-dimethoxy-serrat-14-ene	62,79	14	OMe	H	OMe	$C_{32}H_{54}O_2$	470	455	438	423	438	423	406	-	316	301	284	269	221	189	234	219	187	-	-
3	3β,21α-dimethoxy-serrat-14-ene	64,09	14	OMe	H	OMe	$C_{32}H_{54}O_2$	470	455	438	423	438	423	406	-	316	301	284	269	221	189	234	219	187	-	-
4	21β-methoxy-serrat-14-en-3-one	64,51	14	O	H	OMe	$C_{31}H_{50}O_2$	454	439	-	-	422	407	-	379	300	285	-	-	205	-	234	219	187	-	-
5	3-methoxy-serrat-14-en-21-one	65,71	14	OMe	H	O	$C_{31}H_{50}O_2$	454	439	422	407	-	-	-	379	-	-	284	269	221	189		203	-	-	-
6	21α-methoxy-serrat-14-en-3-one	66,03	14	O	H	OMe	$C_{31}H_{50}O_2$	454	439	-	-	422	407	-	379	300	285	-	-	205	-	234	219	187	-	-
7	3-methoxy-serrat-14-en-21α-yl acetate	69,96	14	OMe	H	OAc	$C_{33}H_{54}O_3$	498	483	438	423	466	451	406	-	316	301	284	269	221	189	262	247	187	-	-
8	3α-methoxy-serrat-13-en-21β-ol	57,95	13	OMe	H	OTMS	$C_{31}H_{52}O_2$	528	513	496	481	438	423	406	-	-	-	-	-	279	189	-	-	187	-	-
9	**3α-methoxy-serrat-14-en-21β-ol (PJ1)**	61,79	14	OMe	H	OTMS	$C_{31}H_{52}O_2$	528	513	-	-	438	423	406	-	316	301	284	269	221	189	292	277	187	-	-
10	**3β-methoxy-serrat-14-en-21β-ol (PJ2)**	62,64	14	OMe	H	OTMS	$C_{31}H_{52}O_2$	528	513	-	-	438	423	406	-	316	301	284	269	221	189	292	277	187	-	-
11	3-methoxy-serrat-14-en-21α-ol	66,65	14	OMe	H	OTMS	$C_{31}H_{52}O_2$	528	513	496	481	496	481	406	-	316	301	284	269	221	189	292	277	187	-	-
12	15-keto-21α-methoxy-serrat-13-en-3-one	72,73	13	O	O	OMe	$C_{31}H_{48}O_3$	468	453	-	-	436	421	-	393	-	-	-	-	-	-	250	-	-	315	287

[a] Refers to compound numbers in Figs. 1 and 3; [b] retention times (min); [c] location of double bond; [d] functional groups depicted in Fig. 2; [e] fragmentation patterns leading to fragments a to n are illustrated in Fig. 4. Compounds indicated in bold were identified with authentic standards (R. Tanaka, Osaka University, Japan).

Table 2

Inventory of methoxy-serratenes occurrences in plants (numbers in bold correspond to compounds detected in the soil developed under conifer forest in the catchment of Lake Aydat.

				1	2	3	4	5		6	7	8	9	10	11		12																															
Order	Family	Genus	Species	3β-methoxyserrat-13-en-21-one	3α,21α-dimethoxyserrat-14-ene	3β,21α-dimethoxyserrat-14-ene	21β-methoxyserrat-14-en-3-one	3α-methoxyserrat-14-en-21-one	3β-methoxyserrat-14-en-21-one	21α-methoxyserrat-14-en-3-one	3-methoxyserrat-14-en-21α-yl acetate	3α-methoxyserrat-13-en-21β-ol	3α-methoxyserrat-14-en-21β-ol (PJ1)	3β-methoxyserrat-14-en-21β-ol (PJ2)	3β-methoxyserrat-14-en-21α-ol	3α-methoxyserrat-14-en-21α-ol	21α-methoxyserrat-13-en-3,15-dione	3α,21β-dimethoxyserrat-14-ene	3β,21β-dimethoxyserrat-14-ene	3α-methoxyserrat-14-en-21β-yl formate	21α-methoxyserrat-13-en-3-one	21β-methoxyserrat-14-en-3β-ol	21α-methoxyserrat-14-en-3β-ol	3β,21α-Dimethoxyserrat-14-en-29-ol	3β-methoxyserrat-14-en-21α-yl acetate	21α-hydroxy-3β-methoxyserrat-14-en-29-yl acetate	3α-methoxy-21β-hydroxyserrat-14-en16-one	3α-methoxyserrat-14-ene-21α,30-diol	3β-methoxyserrat-14-ene-21α,29-diol	3β-methoxyserrat-14-en-21β,29-diol	29-hydroxy-3β-methoxyserrat-14-en-21-one	3β-methoxyserrat-14-ene-21α,30-diol	3β-methoxyserrat-14-ene-21α,30-diol, diacetate	29-nor-3α-methoxyserrat-14-en-21-one	29-nor-3β-methoxyserrat-14-en-21-one	21α-acetoxy-3β-methoxyserrat-14-en-29-al	3β-methoxy-21-oxoserrat-14-en-29-al	21α-hydroxy-3β-methoxyserrat-14-en-29-al	21α-hydroxy-3β-methoxyserrat-14-en-30-al	Jezananal A and B	Piceanonol A	Piceanonol B	13α,14α-epoxy-3β-methoxyserratan-21β-ol	13α,14α-epoxy-21α-methoxyserratan-3-one	14β,15β-epoxy-3β-methoxyserratan-21-one	14β,15β-epoxy-3α-methoxyserratan-21β-ol	14β,15β-epoxy-3β-methoxyserratan-21β-ol	References
Pinales	Pinaceae	Picea	abies							X			X	X																																		[1]
			glehni										X	X						X																					X							[2]
			jezoensis (ajaensis)	X			X	X	X				X	X			X				X						X	X				X		X	X			X	X	X	X	X	X	X	X	X	X	[3]; [4]; [5]; [6]; [7]; [8]; [9]; [10]; [11]; [12]; [13]; [38]
			obovata	X				X	X				X	X																																		[14]
			sitchensis					X	X			X		X		X		X																														[15]; [16]
		Pinus	armandii						X																X						X				X	X	X											[17]; [18]
			banksiana																			X																										[19]; [20]; [21]; [22]
			contorta																			X																										[22]
			lambertiana			X			X						X																																	[19]; [21]
			luchuensis			X			X						X											X																						[23]; [24]
			montelica			X			X					X	X				X			X							X	X		X			X													[22]; [25]; [26]; [27]
			strobus						X																																							[28]
			sylvestris		X	X		X	X																																							[29]
			taiw anensis												X																																	[30]
		Abies	nephrolepis						X	X					X						X																											[31]
		Cathaya	argyrophylla					X																																								[32]; [37]
	Taxodiaceae	Taiw ana	flousiana																							X																						[33]
Hypnales	Neckeraceae	Homalia	trichomanoides						X			X		X																																		[34]
Jungermanniales	Jungermanniaceae	Nardia	scalaris					X																																								[35]
Lecanorales	Parmeliaceae	Pseudevernia	furfuracea						X						X								X	X								X	X															[36]
Asterales	Asteraceae	Homogynes	alpina					X																																								[35]

[1]: Norin et al., 1972a; [2]: Tanaka et al., 2000; [3]: Chemenko et al., 1990; [4]: Tanaka et al.,1994; [5]: Tanaka et al, 1995; [6]: Tanaka et al., 1996; [7]: Tanaka et al, 1997; [8]: Tanaka et al, 1998; [9]: Tanaka et al, 1999a; [10]: Tanaka et al, 1999b; [11]: Tanaka et al, 2001; [12]: Tanaka et al, 2002; [13]: Doi et al., 2010; [14]: Chernenko et al., 1992b; [15]: Rogers and Rozon, 1970; [16]: Kutney et al., 1969b;[17]: Fang et al., 1991; [18]: Fang and Cheng, 1992; [19]: Rowe, 1964; [20]: Rowe, 1965; [21]: Rowe and Bower, 1965; [22]: Rowe et al., 1972; [23]: Cheng et al., 1975; [24]: Wada et al., 2001; [25]: Conner et al., 1980; [26]: Conner et al., 1981; [27]: Conner et al., 1984;[28]: Zinkel and Evans, 1972; [29]: Norin and Winell, 1972b; [30]: Cheng and Chao, 1979; [31]: Ou-Yang et al., 2011; [32]: He et al., 1981; [33]: Xiang et al., 2004; [34] : Wang and Lou, 2005; [35]: Benes et al., 1981; [36] Joulain and Tabacchi, 2009; [37] Ma et al., 1981; [38]: Chernenko et al., 1992a.

Figure captions

Fig. 1: Chromatograms of serratane-type triterpenes in the ketone (a) and alcohol (b and c) fractions. Numbers correspond to structures in Table 1, to mass spectra in Fig. 3 and to some compounds for which possible sources are listed in Table 2.

Fig. 2: Structure of serratane- and rearranged serratane-type compounds with carbon numbering, functional groups and double bond location. The correspondence between functional groups and structure names is indicated in Table 1 for compounds detected in the soil sample.

Fig. 3: Mass spectra and structures of methoxy-serratenes detected in the ketone fraction of lipids extracted from soil developed in the Aydat catchment. Mass spectra of **9** and **10** were recovered from the corresponding standards (PJ1 and PJ2). The structures of compounds tentatively identified are indicated with "?".

Fig. 4: Fragmentation patterns of methoxy-serratenes, modified from Tanaka et al. (1994). The name of fragments corresponds to fragmentation patterns reported in Table 1 and found in the spectra in Fig. 3.

Figure 1

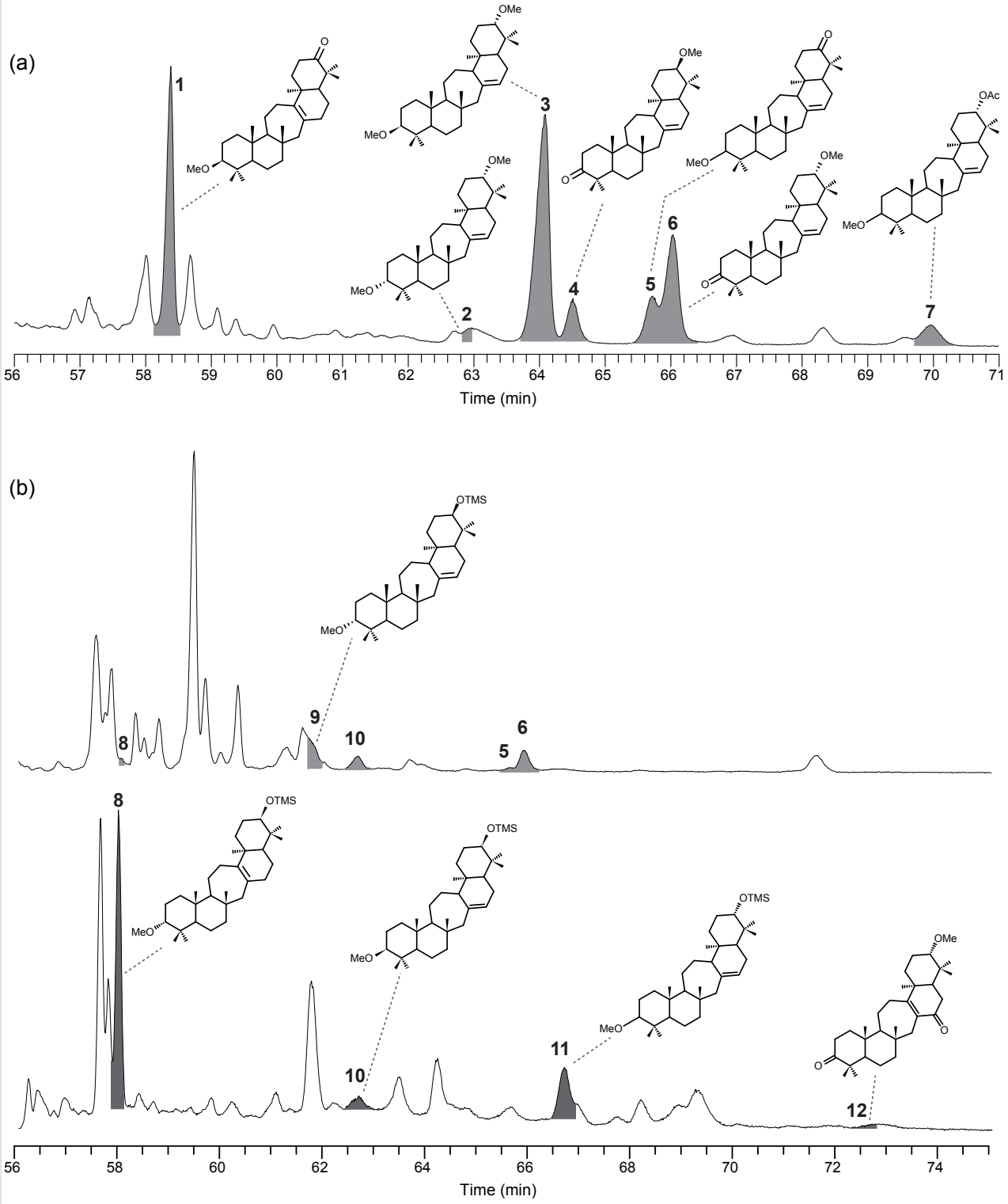
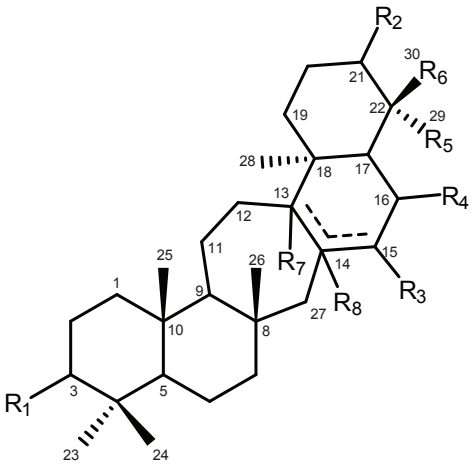


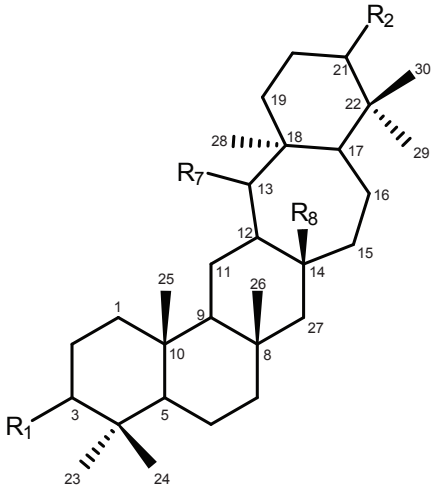
Figure 2

Serratenes

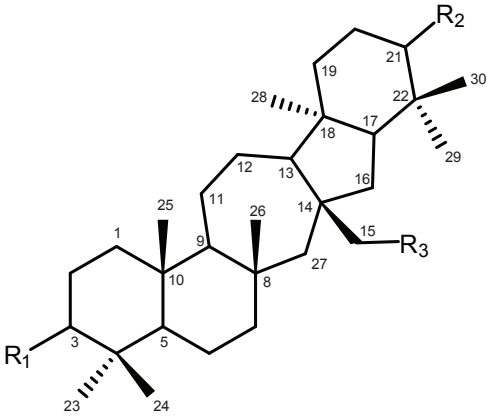


Rearranged serratenes :

Piceanane [14(13->12)abeo-12α(H)-serratane] :



Jezanane [16(15->14)abeo-13R,14S-serratane] :



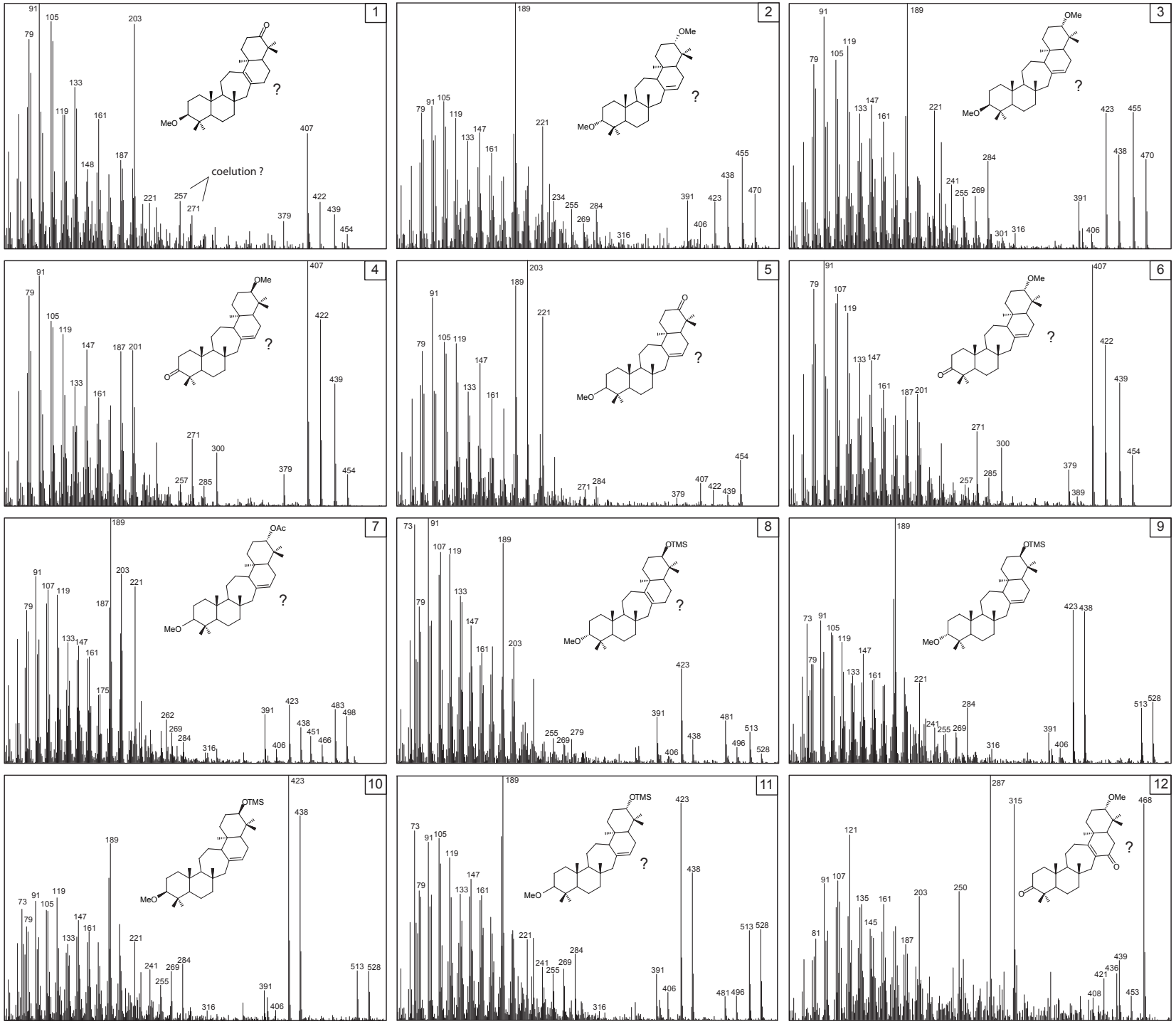
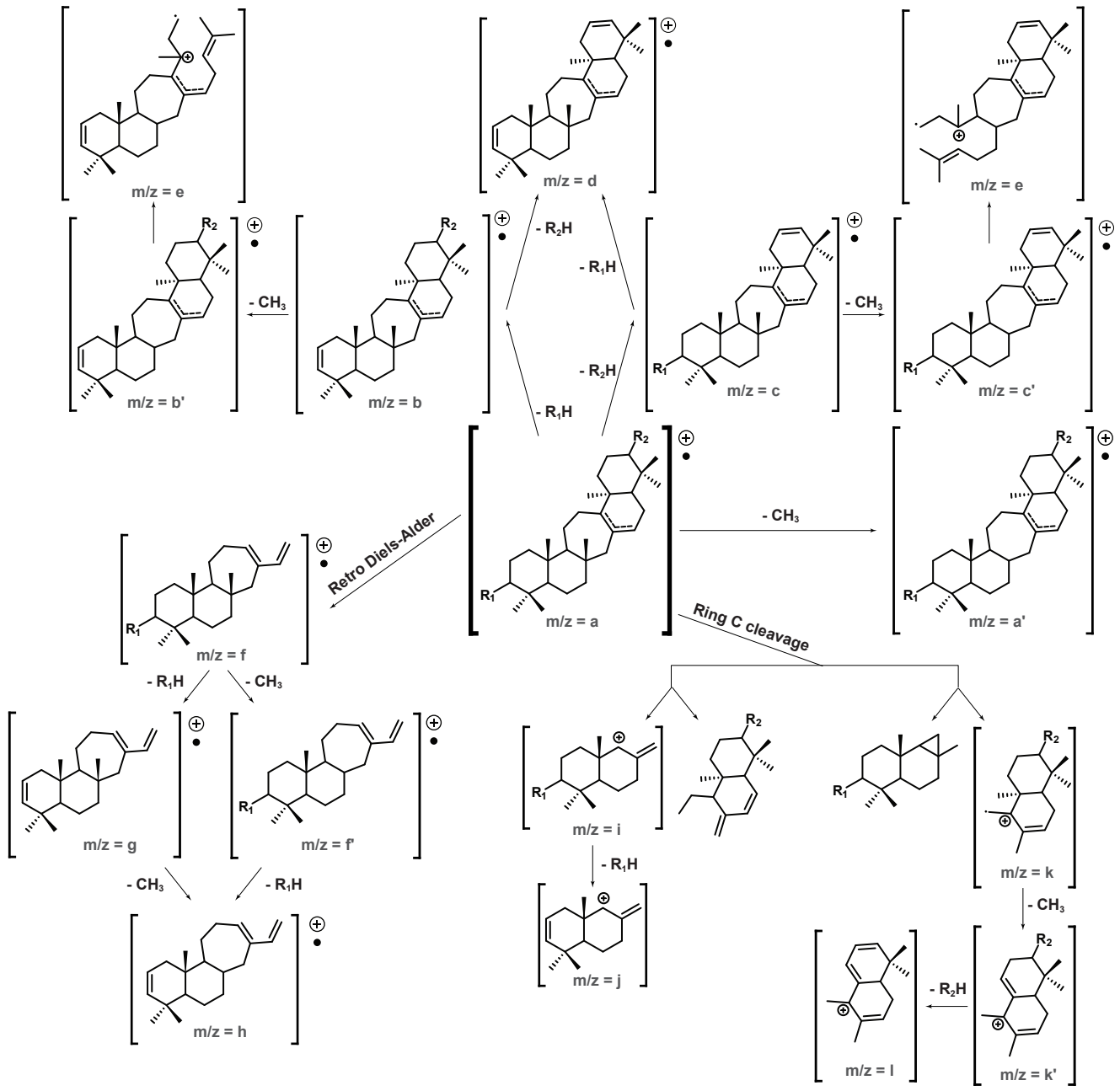


Figure 3

Figure 4



Additional fragments

21α-methoxy-serrat-13-en-3,15-dione