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Article

Higher plant triterpenoids bound in resistant macromolecules in extant and Pliocene fossil *Liquidambar* fruits

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Abstract

Ester-bound compounds (compounds released by saponification) from the resistant macromolecules (RMMs) were analyzed in the extant and Pliocene fossil *Liquidambar* fruit from the Tokai Group distributed in Gifu Prefecture, central Japan. We compared the distributions of the ester-bound triterpenoids from the fossil with those of free and ester-bound triterpenoids of the extant fruits. The series of triterpenoid acids such as oleanolic and ursolic acids were identified in free compounds but hardly detected in ester-bound compounds from the extant *Liquidambar* samples. Most of free triterpenoids identified in the extant *Liquidambar* were also present in the fossil *Liquidambar* sample as the ester-bound compounds. These results indicate the occurrence of post-depositional incorporation of the free triterpenoids into the macromolecules such as the RMMs via ester bonds. Thus, it is suggested that the ester-bound triterpenoids in RMMs serve as excellent archives of the indigenous assemblage of triterpenoids. The class distributions of the oleanoids and ursanoids in the ester-bound compounds from the fossil sample are considerably different from those in the free polar compounds of the extant samples. Selective preservation of C-28 carboxyl group compared to C-3 oxygenated functions indicates that the triterpenoid acids were incorporated into RMM via ester-bound formed between C-28 carboxyl and hydroxyl groups of the RMM. These results indicate that the ester-bound triterpenoid acids obtained from the fossil sample are selectively preserved by incorporation into the RMM of the fruits.

1. Introduction

Terrestrial higher plant-derived biomarkers such as terpenoids are useful as chemotaxonomic indicators, and paleovegetation as well as palaeoclimatic proxies (van Aarssen et al., 2000; Lockheart et al., 2000; Otto and Simoneit, 2001; Hautevelle et al., 2006; Nakamura et al., 2010). Also, the compounds released from the refractory plant biomacromolecules (e.g. lignin phenols) in sediments have been used for evaluating paleo-vegetation. The major components such as polysaccharides in the macromolecular constituents of woody tissue are labile and preferentially degraded, so that the woody remains tend to enriched in ‘resistant macromolecule (RMM)’ such as lignin. The refractory parts of the RMMs can be preserved in ancient fossil wood (e.g. Paleogene; Yang et al., 2005). Several studies examined the chemotaxonomic utilities of the RMMs in ancient woody plant fossils (Ewbank et al., 1996; Abbott et al., 1998). On the other hand, the lipid biomarkers such as steroids have been known to be preserved by incorporation into the RMM including kerogen during early diagenesis (e.g. Sinninghe Damste et al., 1989; Filley et al., 1996; Koopmans et al., 1997). Such ‘bound’ type biomarkers have been mainly investigated in the sulfur-rich kerogens, especially preservation by sulfurization processes in kerogen, but there have been few reports for the other kerogens and the RMM in fossils. The indigenous biomarkers such as terpenoids preserved in the RMM of coal and plant fossils can be more clearly linked to source plant compared to free compounds, and thus, can be a powerful tool for reconstructing plant molecular paleontology and paleo-vegetation. We have reported the potentials for chemotaxonomic markers of the higher plant biomarkers (the wax-derived *n*-alkyl lipids and diterpenoids) bound in the RMMs of plant macrofossils collected from the Neogene Tokai Group in central Japan (Sawada et al., 2008; 2013).

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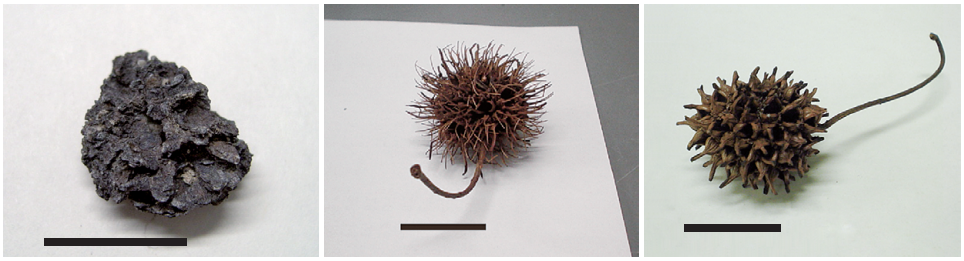

Fig. 1. Photographs of a *Liquidambar* fruit fossil (*L. formosana*) from the Toki Sand and Gravel Formation, Tokai Group in Ena City (Gifu Prefecture), central Japan, and extant fruits (*L. formosana* and *L. styraciflua*). Scale bars = 2.0 cm.

In the present study, we analyzed the ester-bound compounds from the RMMs in the Pliocene plant macrofossils, a fossil fruit of angiosperm *Liquidambar* (family Altingiaceae), from the Tokai Group distributed in Gifu Prefecture, central Japan. We compared the distributions of the ester-bound triterpenoids from the fossil with those of free and ester-bound triterpenoids of the extant fruits, and examine the preservation processes of the terpenoid biomarkers in the RMMs of plant fossil.

2. Materials and methods

2.1. Samples

A fruit fossil of *Liquidambar formosana* (Fig. 1) was collected from the Pliocene Middle Sand and Mud Member (Loc. NN06), Toki Sand and Gravel Formation (ca. 4 Ma; Todo Collaborative Research Group, 1994; Tsukagoshi and Todo Collaborative Research Group, 1995), the Tokai Group in Ena City, Gifu Prefecture, central Japan. The Toki Sand and Gravel Formation was deposited in a fluvial system and contained excellent-preserved plant macrofossils (Todo Collaborative Research Group, 1994). This fossil has been preserved within a bottle filled with the solution of ethyl alcohol in the Osaka Museum of Natural History, Japan. Therefore, most of the free (solvent-extractable) compounds have been removed from this fossil and probably added the contamination.

The fruits of extant *L. formosana* and *Liquidambar styraciflua* (Fig. 1) were collected in the Nagai Botanical Garden in Osaka City, Japan in November of 2002.

2.2. Lipid extraction and separation

The fruit fossil was treated by hydrolysis (saponification) with KOH/MeOH. Prior to the hydrolysis, the fossil was 'washed' by pure water, methanol (MeOH) and dichloromethane (DCM). The residue after this extraction was saponified within a sealed glass tube by 1M KOH in MeOH at 110ºC for 3 hours. The non-saponifiable (neutral) lipids were extracted by partitioning with hexane-ethyl acetate (8:1 v/v), and the carboxylic and hydroxy acids were extracted in the same way after acidification to pH 2 with HCl. The acid and neutral fractions were silylated by using bis(trimethylsilyl) trifluoroacetamide (BSTFA, Wako) at 60ºC for 1 hour.

The extant fruits were washed with distilled water and then cut into fine tips by pruning shears. Organic constituents were extracted from the fine tips of fruit samples by successive treatment with MeOH/DCM (3/1 v/v, x2), DCM (x2) for 20 min, and subsequently with MeOH by steeping overnight (for about 12 hours). The lipids were separated by adding pure water to the combined extracts, before the DCM layer was siphoned off and passed through an anhydrous Na_2SO_4 column for removing labile and polar compounds such as amino acid and sugar. The extract was dried in a rotary evaporator and then re-dissolved in hexane. The hexane extract was passed through a silica gel column (95% activated) as modified in Sawada et al. (2013), and the polar fraction was eluted with ethyl acetate - MeOH (1:1 v/v). The hydrolysis of the residue after the extraction was carried out as mentioned above. Also, the residues after extraction of extant fruits were treated by hydrolysis (saponification) with KOH/MeOH with the same methods for the fossil fruit sample as mentioned above.

2.3. Gas chromatography-mass spectrometry (GC-MS)

Identification of the lipid was carried out by GC-MS using a Hewlett Packard 6890 attached to a capillary GC (30 m x 0.25 mm i.d. DB-5HT column, J&W Scientific) directly coupled to a Hewlett Packard MSD quadrupole mass spectrometer. (electron voltage, 70 eV; emission current, 350 μA; mass range, m/z 50–650 in 2.91 scan/sec.) The GC temperature was programmed as follows: 60ºC for 5 min, 60–250ºC at 10ºC/min, 250–320ºC at 3ºC/min and 320ºC for 20 min in the polar fraction of free compounds, and 50ºC for 4 min, 50–300ºC at 4ºC/min and 300ºC for 20 min in the acid fraction. The lipids were quantified with a Hewlett Packard 6890 capillary GC equipped with a flame-ionization

Table 1. Peak assignments and concentrations of triterpenoids labelled in Fig. 3 in the acid fractions from extant and fossil *Liquidambar*.

Peak	Compound name	MW	Concentrations (μg/g)		
			fossil *L. formosana* Bound*1	extant *L. formosana* Free*2	extant *L. styraciflua* Free*2
1	olean-2, 12-dienoic acid	510	1.82	0.59	n.d.
2	ursan-2, 12-dienoic acid	510	1.29	2.28	n.d.
3	unknown triterpenoid	512	0.48	1.40	n.d.
4	3-epi-oleanolic acid	600	0.96	4.69	24.80
5	ursolic acid (isomer)	600	0.85	5.03	8.17
6	3-oxo-oleanolic acid (isomer)	526	1.30	0.80	n.d.
7	3-oxo-oleanolic acid	526	0.60	34.25	425.98
8	3-oxo-lup-20(29)-en-28-oic acid	526	n.d.	21.54	162.40
9	unknown triterpenoid	538	n.d.	4.39	n.d.
10	oleanolic acid	600	0.75	11.17	70.65
11	3-oxo-ursolic acid	526	0.54	17.60	51.10
12	unknown triterpenoid	614	n.d.	8.40	6.55
13	unknown triterpenoid	612	0.35	5.32	n.d.
14	ursolic acid	600	0.82	8.39	36.92
15	unknown triterpenoid	*3	n.d.	11.71	n.d.
16	23-oxo-ursolic acid	614	n.d.	11.67	28.40
17	unknown triterpenoid	658	n.d.	16.78	22.72
18	unknown triterpenoid	614	n.d.	11.06	n.d.
19	23 or 24-oxo-triterpenoic acid	614	n.d.	n.d.	74.22
20	unknown triterpenoid	658	n.d.	6.56	14.47

MW: Molecular weight, n.d.: Not detected
*1: Compounds bound in macromolecule (compound released from plant fossils by KOH/MeOH hydrolysis)
*2: Free (solvent-extractable) compounds
*3: Not determined

detector (FID), the capillary column and temperature program used being the same as those used for GC/MS. Identification of the compounds was made from mass chromatographic responses (mass fragmentation pattern and molecular ion *etc*.) and relative retention times in comparison with library data (NIST14) and the literature. Quantification of the compounds was made from the peak areas determined by the FID responses, and/or the responses of individual base peaks (e. g., *m/z* 203 for oleanoids) determined from the authentic standards of oleanolic acid (Wako co.) and betulin (Aldrich co.).

3. Results and Discussion

3.1. Free compounds

In the extant *L. formosana* and *L. styraciflua* samples, C_{16}-C_{24} even-carbon number alkanoic acids, C_{18} alkenoic acids, C_9 dicarboxylic acid, lignin phenols such as vanillic and syringic acids, and triterpenoid acids such as oleanolic and ursolic acids were identified as the major free polar compounds (Fig. 2a). The oleanolic and ursolic acids are angiospermous biomarkers, and several peaks in the mass fragmentogram (MF) of *m/z* 203 are attributed to their isomers and analogues (Table 1 and Fig. 3). Fig. 4 shows mass spectra of TMS derivatives of these compounds. The 3-oxo-oleanolic acid (oleanonic acid) and 3-epi-oleanolic acid can be identified according to the mass spectra of extracts from *Liquidambar* resins reported by Pastorova et al. (1998). The other triterpenoids were tentatively identified by molecular ions, mass fragment pattern and retention times.

Concentrations of the triterpenoid acids in the free polar fractions of the extant *L. formosana* and *L. styraciflua* are shown in Table 1 and Fig. 5. It was found that the concentrations of 3-oxo-oleanolic acids are the highest in both extant species (425.98 μg/g in *L. formosana* and 34.25 μg/g in *L. styraciflua*). The oleanolic acid and 3-oxo-ursolic acid (ursanonic acid) are also major components in these species.

3.2. Ester-bound compounds

Figure 2b shows the total ion chromatograms (TICs) of the compounds obtained by the KOH/MeOH hydrolysis (saponification) in the extant and fossil *L. formosana*, and extant *L. styraciflua* samples. In the extant samples, cutin acids, C_{16} and C_{18} alkanoic acids, C_4, C_9, and C_{16} dicarboxylic acids, and phenolic acids such as vanillic and syringic acids were identified as the major compounds released. On the other hand, C_{16}-C_{26} even-carbon number alkanoic acids and lignin phenols such as vanillic and syringic acids were mainly identified in the compounds released from the fossil *L. formosana*. These released compounds can be interpreted to have existed as the acyl moieties in the RMM of fruits, namely, is the ester-bound components. The cutin acids, which are derived from cuticle polyesters, detected in

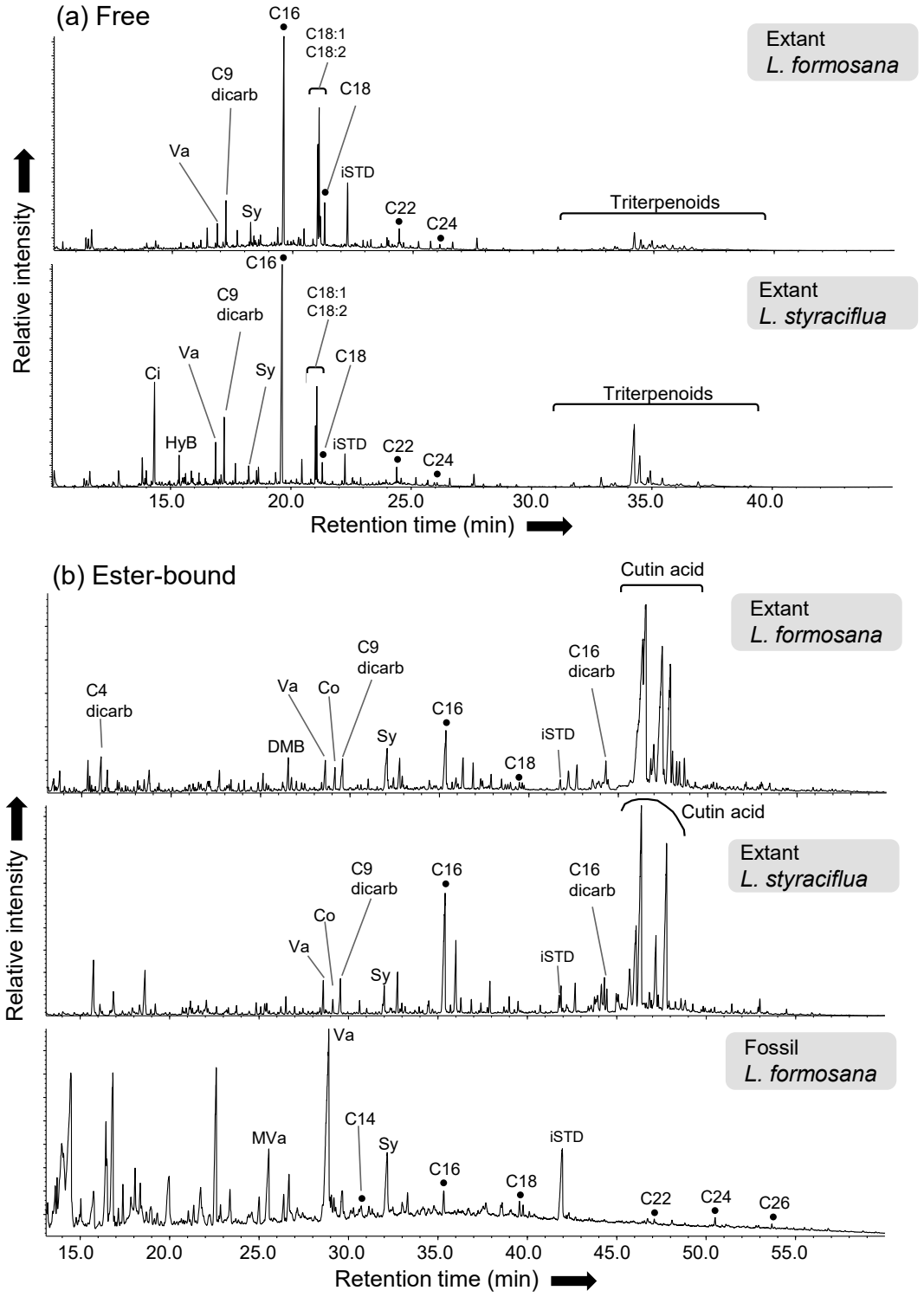

Fig. 2. Total ion chromatograms (TIC) of polar fractions of solvent-extracts (Free), and acid fractions after saponification (Ester-bound) of extant and fossil *Liquidambar*. iSTD: internal standard, Solid circle: *n*-alkanoic acid TMS ethers (Cx:y; x: carbon numbers, y: numbers of double bonds), dicarb: dicarboxylic acid diTMS ethers, Ci: cinnamic acid diTMS ethers, HyB: 4-hydroxybenzoic acid diTMS ethers, DMB: dimethoxybenzene, MVa: methyl vanillate TMS ethers, Va: vanillic acid diTMS ethers, Co: 4-coumaric acid diTMS ethers, Sy: syringic acid diTMS ethers.

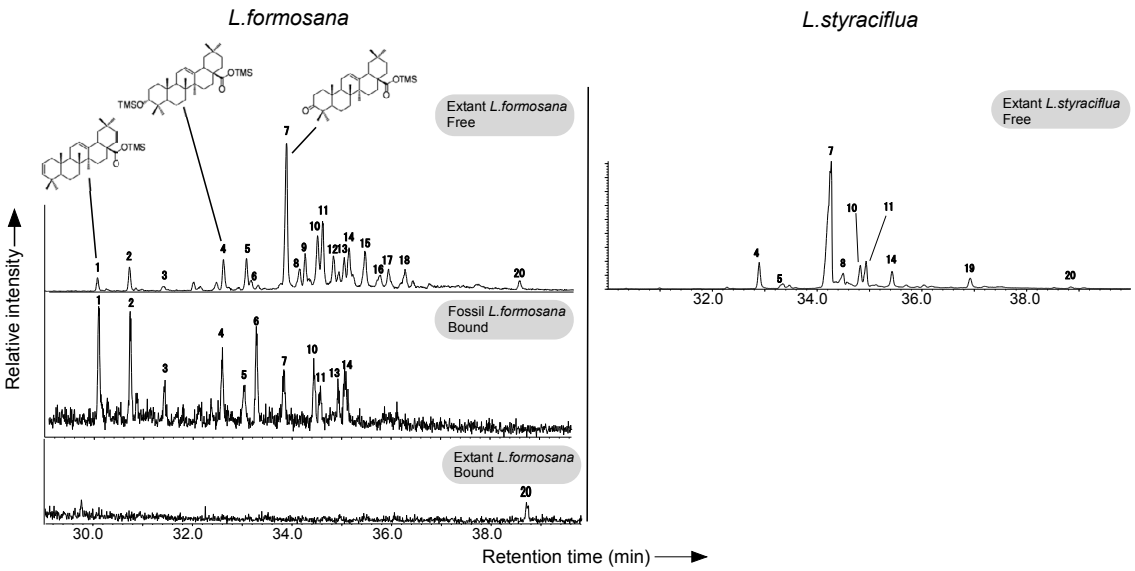

Fig. 3. Mass fragmentograms (*m/z* 203) of polar fractions of solvent-extracts (Free), and acid fractions after saponification (Bound) of the extant and fossil *Liquidambar*. Peak numbers are assigned as those of the triterpenoids in Table 1.

the extant samples are not preserved in the fossil sample. The phenolic acids detected are thought to be associated with lignin, and such components preserved in the fossil sample may be more refractory than the cuticle components in the RMMs of plant tissues including the fruit.

Interestingly, series of the free polar triterpenoids (oleanolic and ursolic acids) identified in the extant samples were also detected as the ester-bound compounds from the fossil *L. formosana* (Fig. 3). However, an unknown triterpenoid (peak 20 in Fig. 3) was the only released triterpenoid detected as ester-bound components from the extant *L. formosana*, and there are no released triterpenoids in the extant *L. styraciflua* (Fig. 3). Concentrations, which are defined as 'yields' by saponification, of the released triterpenoid acids are 0.35–1.82 μg/g in the fossil *L. formosana* (Table 1 and Fig. 5). Class distributions of the oleanoids and ursanoids in the compounds released from the fossil sample are quite different from those in the free polar compounds of the extant samples (Fig. 5). The concentrations of di-unsaturated triterpenoids such as olean-2, 12-dienoic and ursan-2, 12-dienoic acids are the highest in the compounds released from the fossil sample.

3.3. Possible incorporation of triterpenoids into the resistant macromolecules

Most of the triterpenoid acids were identified exclusively as free compounds in the extant *Liquidambar* samples and ester-bound compounds released from the fossil *Liquidambar* sample as mentioned above. These results indicate that there are an insignificant amount of triterpenoids bound to macromolecules via ester-bonds in living fruit tissues of *Liquidambar*, while the free triterpenoids are possibly incorporated into the macromolecules such as the RMMs during early diagenesis after deposition. The differences of class distributions for the triterpenoid acids between the free compounds of extant samples and ester bound compounds of fossil sample may be resulting from the diagenetic alteration. In particular, increases of the relative abundances of the olean-2, 12-dienoic and ursan-2, 12-dienoic acids imply that these triterpenoids were formed from the oleanolic and ursolic acids by diagenetic processes. Indeed, removal of the hydroxyl group (-OH) at the C-3 position and the formation of Δ^2 double bond by microbial activity occur during early diagenesis of triterpenoids as reported by ten Haven et al. (1992). Carboxyl group (-COOH) of the triterpenoid acid is also a target of microbial degradation (i.e. decarboxylation). Nevertheless, no 28-nor-triterpenoids, which are the homologues that the carboxyl group at C-28 are eliminated, were found in the compound released from the fossil sample. These results suggest that the carboxyl group at C-28 was forming ester bounds during diagenetic incorporation into the macromolecules: making it less accessible to microbial defunctionalization, which finally leads to the preferential occurrence of ester-bound triterpenoids with C-28 carboxyl group but with the variety of preservation of C-3 oxygenated functions. From these insights, we summarized the possible reactions for microbial degradation, dehydration condensation, and incorporation of triterpenoid acids

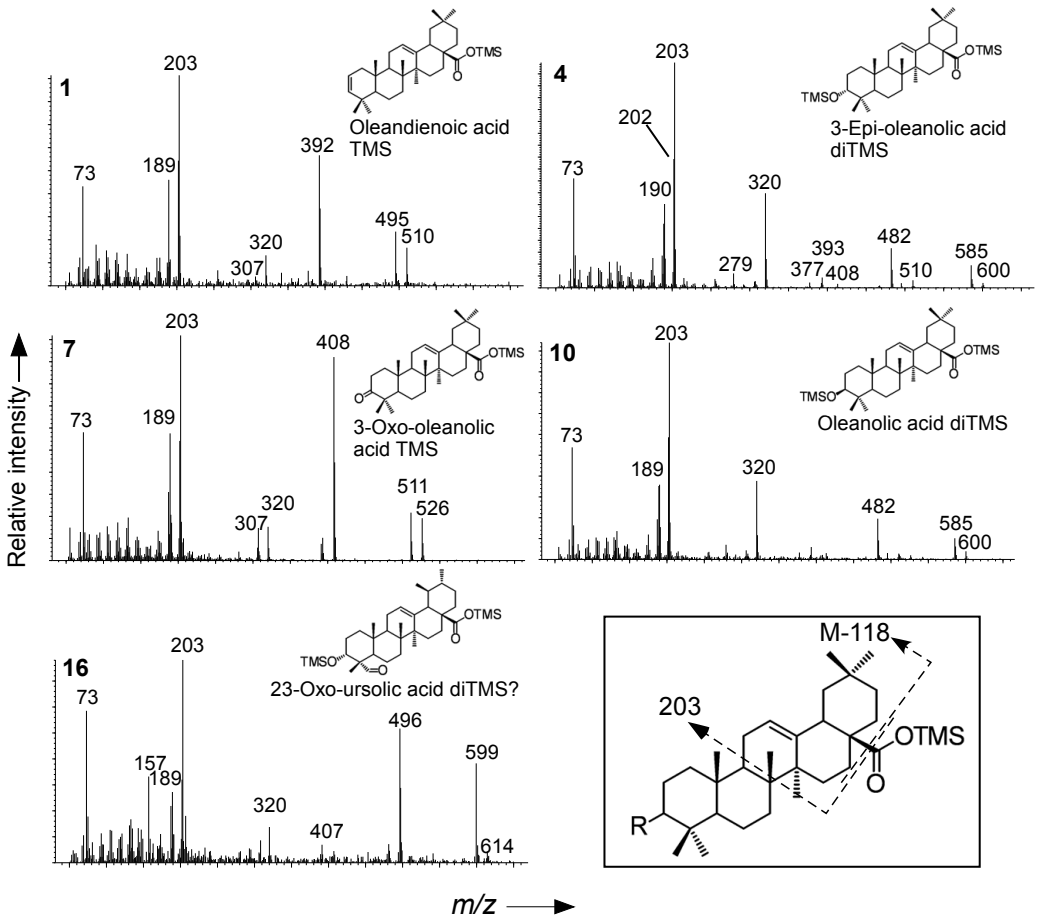

Fig. 4. Mass spectra of the triterpenoid acids in the polar and acid fractions from the extant and fossil *Liquidambar*. The numbers of mass spectra are assigned as those of the triterpenoids in Table 1.

into the macromolecules such as the RMM (Fig. 6). The triterpenoids are primarily synthesized as free compounds and have various physiological activities for secondary metabolisms in living plant tissues. On the other hand, some triterpenoids such as betulin have been known to be contained as ester-bound components in suberin in bark of genus *Betula* (Tegelaar, 1990; Tegelaar et al., 1995). This fact indicates that the triterpenoids are not entirely contained as free components in the living plant tissues, and thus, we also need to consider the bound compounds from the living sources. However, in the present study for the *Liquidambar* fruit, such ester-bound compound in living tissues is hardly considered because of the detection of only a small amount of an unknown triterpenoid as the compounds bound in the extant samples. In addition, the apparent differences of class distributions between the free compounds in the extant samples and bound compounds in the fossil sample are considered to evidence that the triterpenoid acids obtained from the fossil sample are selectively preserved by incorporation into the RMM of the fruits. Moreover, most of the ester-bound triterpenoids from the fossil were bioterpenoids with their intact structures, although some were partially altered by early diagenesis. This study shows that the RMM of plant fossils are a depository of less altered terpenoids, hence we suggest the analysis of bound triterpenoids, in addition to the kerogens and sediments, can serve as powerful indicators for chemotaxonomy, molecular paleontology and paleovegetation.

4. Conclusions

Ester-bound compounds (compounds released by saponification) from the RMMs were analyzed in the extant and Pliocene fossil *Liquidambar* fruit from the Tokai Group distributed in Gifu Prefecture, central Japan. We compared the distributions of the ester-bound triterpenoids from the fossil with those of free and ester-bound triterpenoids of the extant fruits. We conclude

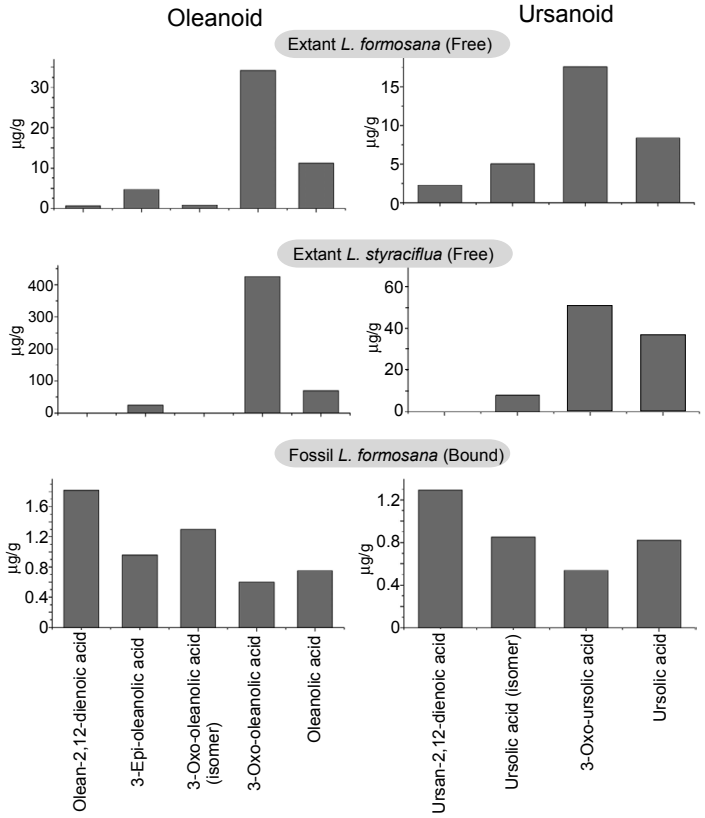

Fig. 5. Concentrations of the triterpenoid acids from the extant and fossil *Liquidambar*.

that;

1. The series of triterpenoid acids such as oleanolic and ursolic acids were identified in free compounds but hardly detected in ester-bound compounds from the extant *Liquidambar* samples. Most of free triterpenoids identified in the extant *Liquidambar* were also present in the fossil *Liquidambar* sample as the ester-bound compounds. These results indicate the occurrence of post-depositional incorporation of the free triterpenoids into the macromolecules such as the RMMs via ester bonds. Thus, it is suggested that the ester-bound triterpenoids in RMMs serve as excellent archives of the indigenous assemblage of triterpenoids.

2. The class distributions of the oleanoids and ursanoids in the ester-bound compounds from the fossil sample are considerably different from those in the free polar compounds of the extant samples. Selective preservation of C-28 carboxyl group compared to C-3 oxygenated functions indicates that the triterpenoid acids were incorporated into RMM via ester-bound formed between C-28 carboxyl and hydroxyl groups of the RMM. These results indicate that the ester-bound triterpenoid acids obtained from the fossil sample are selectively preserved by incorporation into the RMM of the fruits.

Hence, we suggest the triterpenoids bound in the RMM of plant fossils, in addition to the kerogens and sediments, are more powerful indicators for chemotaxonomy, molecular paleontology and paleovegetation.

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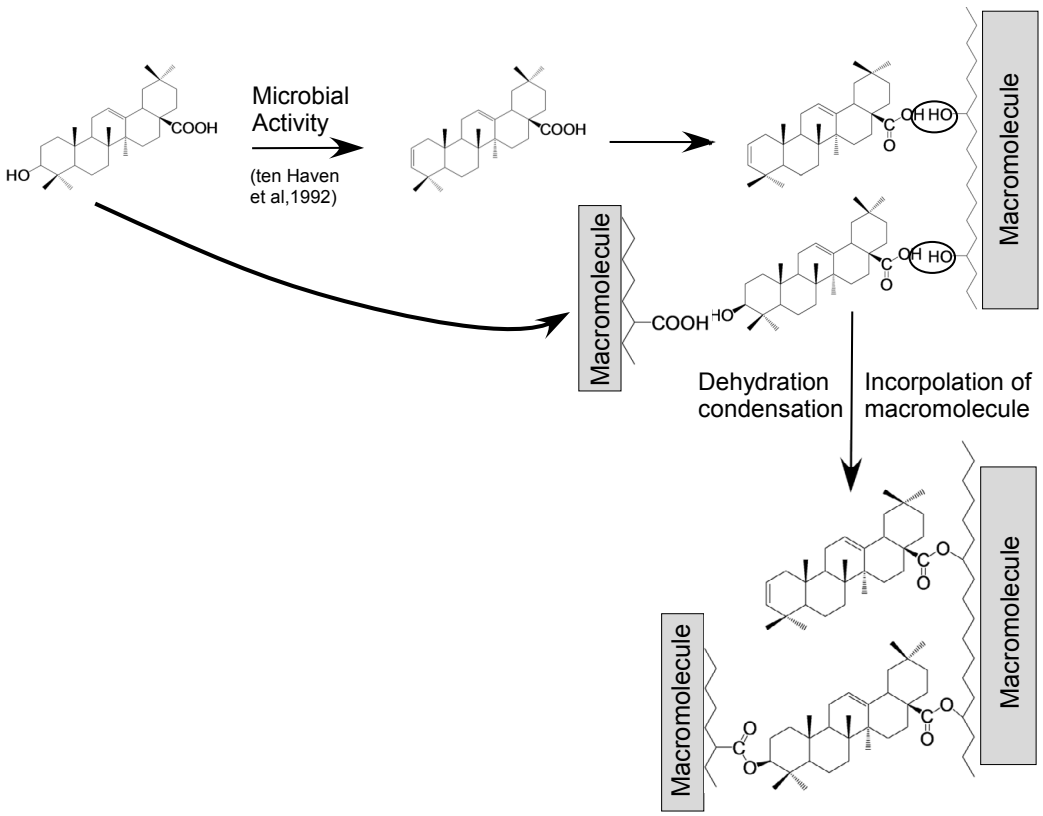

Fig. 6. Possible reactions for the diagenetic degradation (ten Haven et al., 1992), dehydration condensation, and incorporation of triterpenoid acids into resistant macromolecules.

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