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Note

Novel isoferulate esters identified by gas chromatography–mass spectrometry in bud exudate of *Populus nigra*

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Previous investigations have shown that bud exudate of Populus spp. consists of a complex mixture of compounds including substituted benzoic acids and esters, substituted phenolic acids and esters and flavonoid aglycones[1–7]. Recent studies have identified 3-methyl-2-butenyl caffeate (prenyl caffeate) in bud exudate of *Populus nigra* L.[6] (black poplar) and a series of 3-methyl-2-butenyl- and 3-methyl-3-butenyl esters of caffeic, coumaric, ferulic and isoferulic acids in bud exudate of *P.* × *euramericana* (Dode) Guinier[2]. The prenyl esters are of particular interest because 3-methyl-2-butenyl caffeate has been found to be a major contact allergen in propolis[8], the "bee-glue" collected by bees from poplar buds[2–4,6,9], which finds wide use in homeopathic medical and cosmetic products[9,10].

We here report the identification from a fraction of *P. nigra* bud exudate (LB3)[6] of a series of esters of isoferulic acid with both aromatic and aliphatic alcohols. This series includes several novel esters of the prenyl type.

EXPERIMENTAL

Fractionation of poplar bud exudate

P. nigra bud exudate was extracted and fractionated as described previously[6].

Reagents and materials

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) was obtained from Sigma (Dorset, U.K.).

Esters of isoferulic acid were synthesised from the corresponding alcohol and *trans*-isoferulic acid as previously described[2]. The appropriate alcohols were purchased from Sigma, Aldrich (Dorset, U.K.), Lancaster Synthesis (Morecambe, U.K.), or provided as a gift by Shell Research (Sittingbourne, U.K.).

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Sample preparation

The samples (0.5–1 mg) were prepared for gas chromatography (GC) by heating for 30 min at 100°C with 50 μl pyridine and 100 μl BSTFA (including 1% TMCS) in a sealed glass tube to produce the trimethylsilyl (TMS) derivatives.

Gas chromatography–mass spectrometry (GC–MS)

The derivatized samples were separated and analysed in a Finnigan 1020 automated GC–MS system (incorporating a Data General Nova 3 computer); the GC system was fitted with a 30 m × 0.32 mm I.D. J & W Scientific silica column coated with 0.25 μm DB-1, and a splitless injector with a flush 30 s after sample injection to remove residual gases. The end of the column was introduced directly into the mass spectrometer analyser chamber. The system was operated under the following conditions: helium pressure 11 lbs/un.^{2}; injector temperature 300°C; GC temperature 75–300°C at 3°C min^{-1}. The mass spectrometer was set to scan 40–650 a.m.u. per nominal second with an ionizing voltage of 70 eV. The filament was switched on 250 s after injection of the sample into the gas chromatograph.

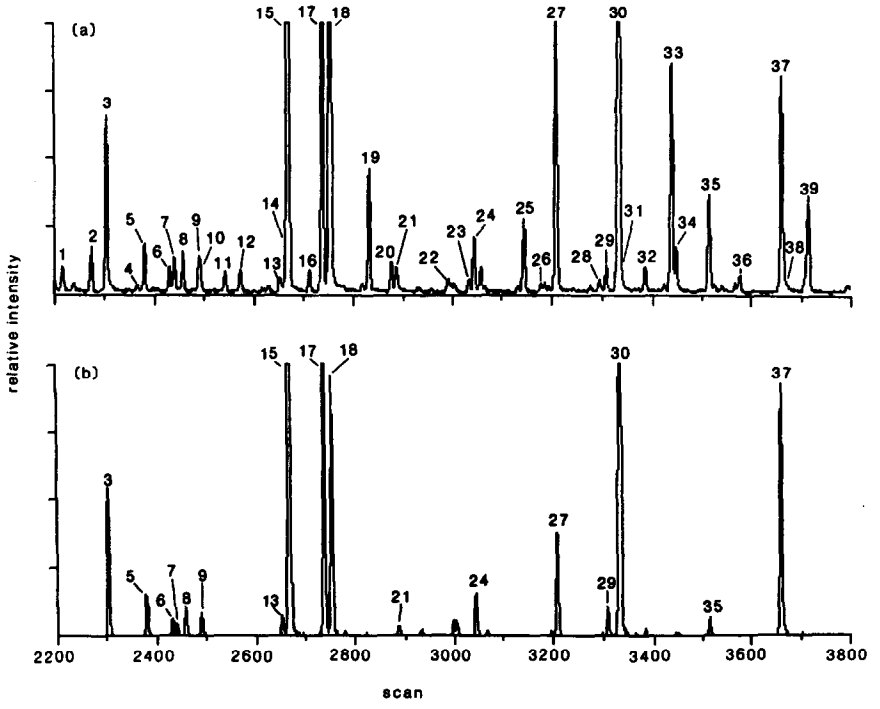
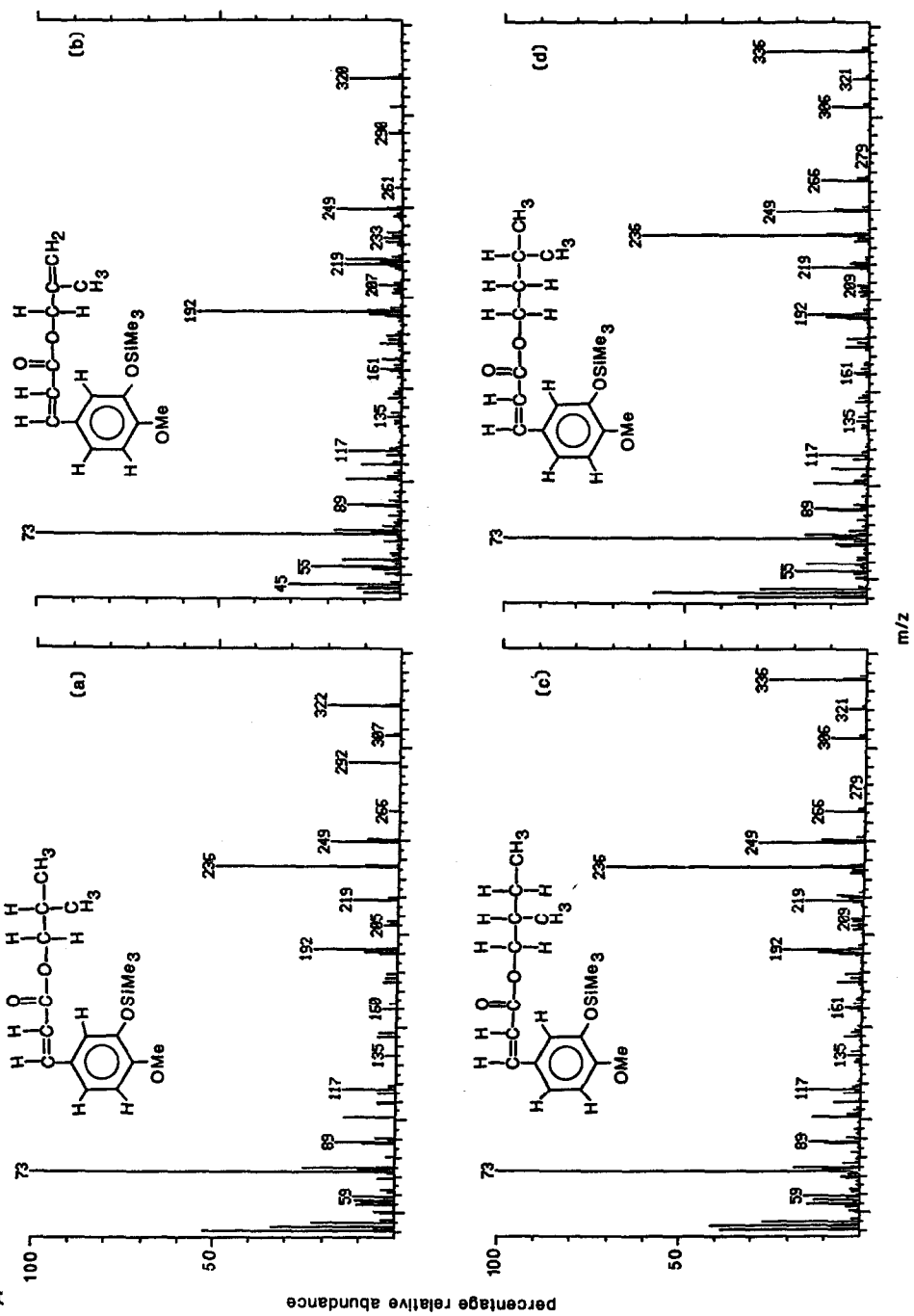


Fig. 1. (a) Reconstructed ion chromatogram of poplar bud exudate fraction LB3. The area shown [c MU 20–30; 175–260°C] contains all the isoferulic acid esters in the fraction. In addition to the isoferulic acid esters listed in Table I the following acids are identified: hexadecenoic (1); hexadecanoic (2); octadecenoic (12); phenylethyl *trans*-caffeate (34) and the following unbranched hydrocarbon alcohols are identified; C_{18}–C_{26} straight chain-1-ols (10, 14, 19, 23, 25, 28, 33, 36, 39), C_{18}–C_{24} straight chain-2-ols (4, 11, 16, 20, 23, 26, 31) and C_{24}–C_{26} iso branched chain-1-ols (32, 38). (b) Single-ion reconstruction of *m/z* 249 (for structure of *m/z* 249 see ref. 12) indicating positions of isoferulic acid and its esters.


A
percentage relative abundance
100
50
(a)
(b)
(c)
(d)
73
59
89
117
135
159
192
219
236
249
265
292
307
322
45
55
161
233
261
296
320
279
321
336
m/z

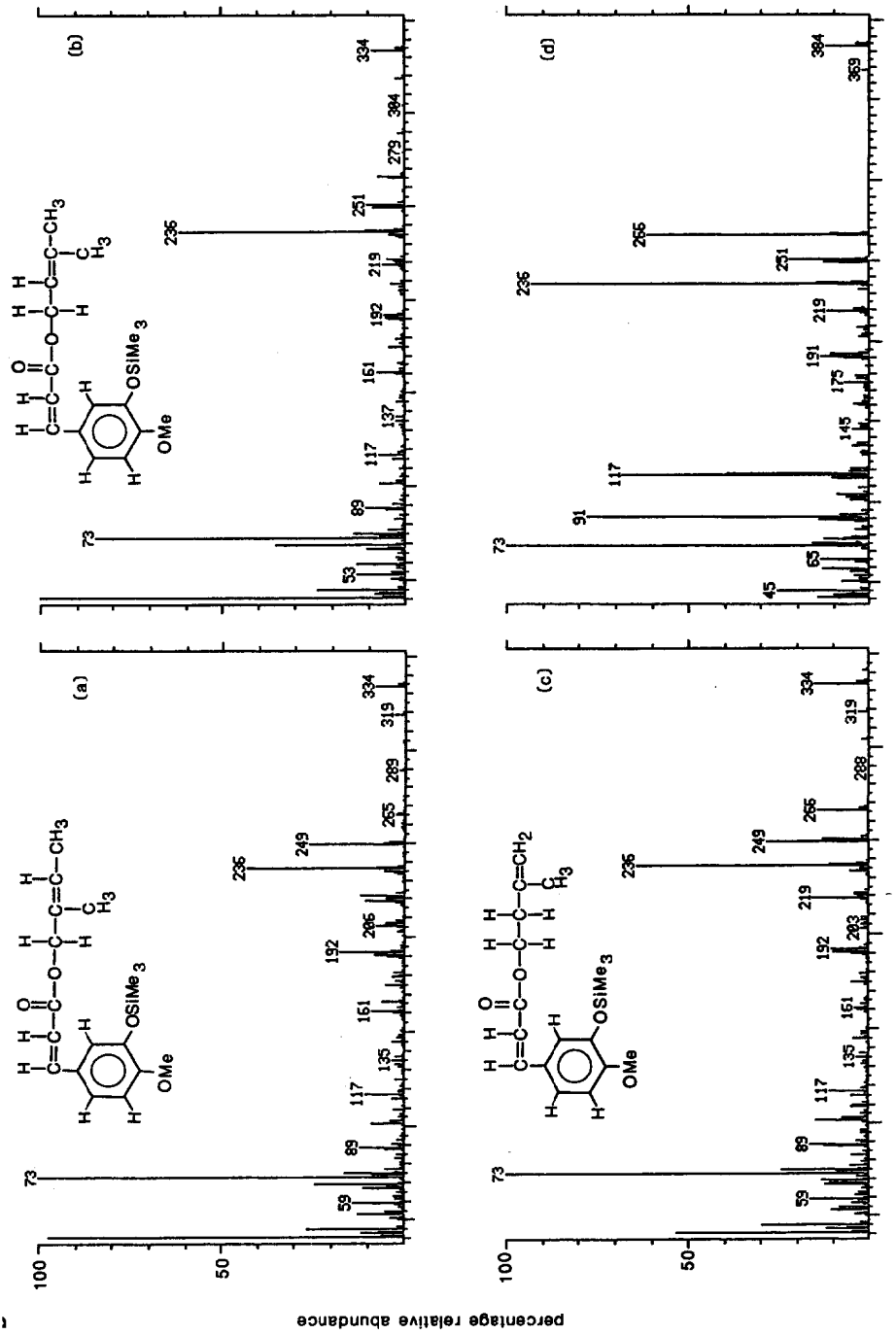


Fig. 2. (A) Mass spectra, recorded at 70 eV, of the 2-methylpropyl ester $[M]^+$ m/z = 322 (a), 2-methyl-2-propenyl ester $[M]^+$ m/z = 320 (b), 2-methylbutyl ester $[M]^+$ m/z = 336 (c), 3-methylbutyl ester $[M]^+$ m/z = 336 (d), of *trans*-isoferulic acid mono-TMS. Spectrum (a) is from poplar bud exudate; (b), (c) and (d) are reference standards, because (b) co-chromatographs with 1-octadecanol in bud exudate and (c) and (d) chromatograph together (see Results section). (B) Mass spectra, recorded at 70 eV, of the 2-methyl-2-butenyl ester $[M]^+$ m/z = 334 (a), 3-methyl-2-butenyl ester $[M]^+$ m/z = 334 (b), 3-methyl-3-butenyl ester $[M]^+$ m/z = 334 (c) and hydrocinnamyl ester $[M]^+$ m/z = 384 (d) of isoferulic acid mono-TMS. These spectra are from poplar bud exudate (Me = methyl).

Identification of compounds

Peaks were identified by computer search of user-generated reference libraries, incorporating GC retention times and mass spectra. Reference compounds were co-chromatographed with the experimental sample to confirm GC retention times and mass spectral patterns. Peaks were examined by single-ion chromatographic reconstructions to confirm their homogeneity; mixed peaks were resolved by a computer program aimed at resolving the mass spectral data of one compound from overlapping mass spectra of another.

RESULTS

Analysis by GC–MS and co-chromatography with appropriate reference compounds enabled separation and identification of *trans*-isoferulic acid (3-hydroxy-

TABLE I

ISOFERULIC ACID AND ISOFERULATE ESTER COMPONENTS OF FRACTION LB3 OF *P. NIGRA* BUD EXUDATE

GC retention times in methylene units (MU; defined by Dalgliesh *et al.*[11]) are given to two decimal places to indicate the elution sequence of peaks which chromatograph closely. Factors such as concentration of the compound concerned and concentration of adjacent compounds, together with the characteristics of a particular GC column are liable to affect the chromatography, and for general purposes the MU figures are probably reliable only to a single decimal place.

Peak No.	*Compound**	*Retention time (MU)*	*Percentage of total*** *isoferulate*
3	*trans*-Isoferulic acid	20.63	4.3
5***	3-Methyl-3-butenyl-*cis*-isoferulate	21.07	1.1
6***	2-Methyl-2-butenyl-*cis*-isoferulate	21.35	0.6
7***	3-Methyl-2-butenyl-*cis*-isoferulate	21.40	0.8
8	2-Methylpropyl-*trans*-isoferulate	21.49	0.9
9	2-Methyl-2-propenyl-*trans*-isoferulate	21.67	1.4
13§	2-Methylbutyl-*trans*-isoferulate 3-Methylbutyl-*trans*-isoferulate	22.50 22.55	0.5
15	3-Methyl-3-butenyl-*trans*-isoferulate	22.63	21.6
17	2-Methyl-2-butenyl-*trans*-isoferulate	23.04	10.9
18	3-Methyl-2-butenyl-*trans*-isoferulate	23.13	18.0
21***	Benzyl-*cis*-isoferulate	23.87	0.8
24***	Phenylethyl-*cis*-isoferulate	24.93	1.9
27	Benzyl-*trans*-isoferulate	26.00	8.0
29***	*trans*-Cinnamyl-*cis*-isoferulate	26.62	0.6
30	Phenylethyl-*trans*-isoferulate	26.79	19.7
35	Hydrocinnamyl-*trans*-isoferulate	28.13	2.9
37	*trans*-Cinnamyl-*trans*-isoferulate	29.13	6.0

* Isoferulic acid chromatographs as the bis-TMS derivative; all others listed chromatograph as mono-TMS derivatives.

** The total ion current generated depends on the characteristics of the compound concerned and is not a true quantitation (see ref. 2).

*** Identified on the basis of mass spectral and gas chromatographic characteristics. We have not confirmed our identification by co-chromatography with an authentic reference standard as we do not have *cis*-isoferulic acid available for syntheses of the *cis* derivatives.

§ The 2-methylbutyl- and 3-methylbutyl esters co-chromatograph and are not resolved (see Results).

4-methoxycinnamic acid) together with a series of *cis*- and *trans*-isoferulate esters and of aliphatic alcohols (Fig. 1a). A single-ion reconstruction of $m/z = 249$ enables the isoferulic esters to be identified (Fig. 1b). *Cis* and *trans* isomers of 2-methyl-2-butenyl-, 3-methyl-3-butenyl-, 3-methyl-2-butenyl-, benzyl-, phenylethyl- and *trans*-cinnamyl isoferulate were identified, as were the *trans* isomers of 2-methylpropyl-, 2-methyl-2-propenyl-, 2-methylbutyl-, 3-methylbutyl- and hydrocinnamyl isoferulate (Table I).

The 2-methylbutyl- and 3-methylbutyl esters have similar GC retention times (MU 22.50; 22.55) and mass spectra which are noticeably different only in some of the lower mass ions ($m/z = 40$–60, see Fig. 2A). Both esters chromatograph very close to the 3-methyl-3-butenyl ester (Fig. 1b), a major peak, and this causes some problems in attempting to exactly co-chromatograph reference standards to confirm positively the presence of both the 2-methylbutyl- and 3-methylbutyl esters. From our co-chromatography results and examination of the mass spectra we consider both to be present, although it is difficult to be certain of this.

Mass spectra of the esters of isoferulic acid with saturated and unsaturated aliphatic alcohols of the prenyl type and with hydrocinnamyl alcohol are shown in Fig. 2A and B.

DISCUSSION

Preliminary analysis of this fraction of poplar bud exudate (LB3)[6] indicated that it contained esters of ferulic and isoferulic acid. Our current, more detailed, analysis confirms the identification of isoferulic acid esters only. Although spectra of isoferulate (3-hydroxy-4-methoxycinnamate) and ferulate (4-hydroxy-3-methoxycinnamate) esters are virtually identical, esters of the former compound chromatograph about 0.1–>0.2 MU before those of the latter[2], and co-chromatography with appropriate reference compounds enables them to be clearly identified. Esters identified include the 2-methylpropyl-, 2-methyl-2-propenyl-, 2-methyl-2-butenyl-, 2-methylbutyl-, 3-methylbutyl- and hydrocinnamyl esters. Isofar as we are aware this is the first report of the natural occurrence of these esters.

There are traces of a further novel isoferulate ester at MU 24.45 and the $[M]^+$ ($m/z = 348$) suggest it to be a methylpentenyl ester.

We have identified from mass spectral patterns and GC retention times the *cis*-isoferulate esters of several compounds (Table I). We have consistently found that *cis* isomers are not formed from *trans* isomers of isoferulate esters during derivatization and chromatography. We conclude therefore that these occur naturally in poplar bud exudates, although we do not know whether the *cis* isomers are secreted as such, or whether they are subsequently formed from *trans* isomers due to environmental conditions.

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