Journal of Chromatography, 543 (1991) 113–121
Elsevier Science Publishers B.V., Amsterdam

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Synthesis of esters of acetyloxycaffeic acids and their occurrence in poplar bud exudates

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(First received October 30th, 1990; revised manuscript received January 16th, 1991)

ABSTRACT

The novel 3-methyl-3-butenyl, 2-methyl-2-butenyl, 3-methyl-2-butenyl, benzyl and 2-phenylethyl esters of 3-acetyloxycaffeic acid and 4-acetyloxycaffeic acid were synthesised on a micro scale and characterised by gas chromatography–mass spectrometry. These compounds are identified for the first time in poplar bud exudates.

INTRODUCTION

Poplar bud exudate is a complex mixture which may contain flavonoids, substituted benzyl and phenylpropenoic acids and their esters, terpenoids and hydrocarbons. The composition of bud exudate is characteristic of a species, or even clone [1] and there can be considerable differences in bud exudate composition between species. Thus in *Populus balsamifera* L. (section *Tacamahaca*) the principal components of the bud exudate are dihydrochalcones and terpenoids [2], whereas in bud exudate of *P. fremontii* S. Wats. (section *Aigeiros*) these compounds are entirely lacking, the principal components being flavanonols and chalcones [3].

In some Asiatic poplars, such as *P. ciliata* Wall. and *P. simonii* Carr. (both currently classified in section *Tacamahaca*), the bud exudate is distinctive in that caffeic acid and other acids, together with their esters, form a major part of the bud exudate (75% in *P. ciliata* and 45% in *P. simonii*). Preliminary study of mass spectra of these other unidentified acids suggested that they might be trimethylsilyl derivatives of acetyloxycaffeic acids and their esters. We here describe the synthesis of several series of acetyloxycaffeates and indicate how they may easily be located in complex chromatograms of poplar bud exudate by single ion reconstructions of gas chromatography–mass spectrometric (GC–MS) data, thus confirming our view of their identity. These acetyloxycaffeic acids and their esters have not been previously identified in poplar bud exudate.

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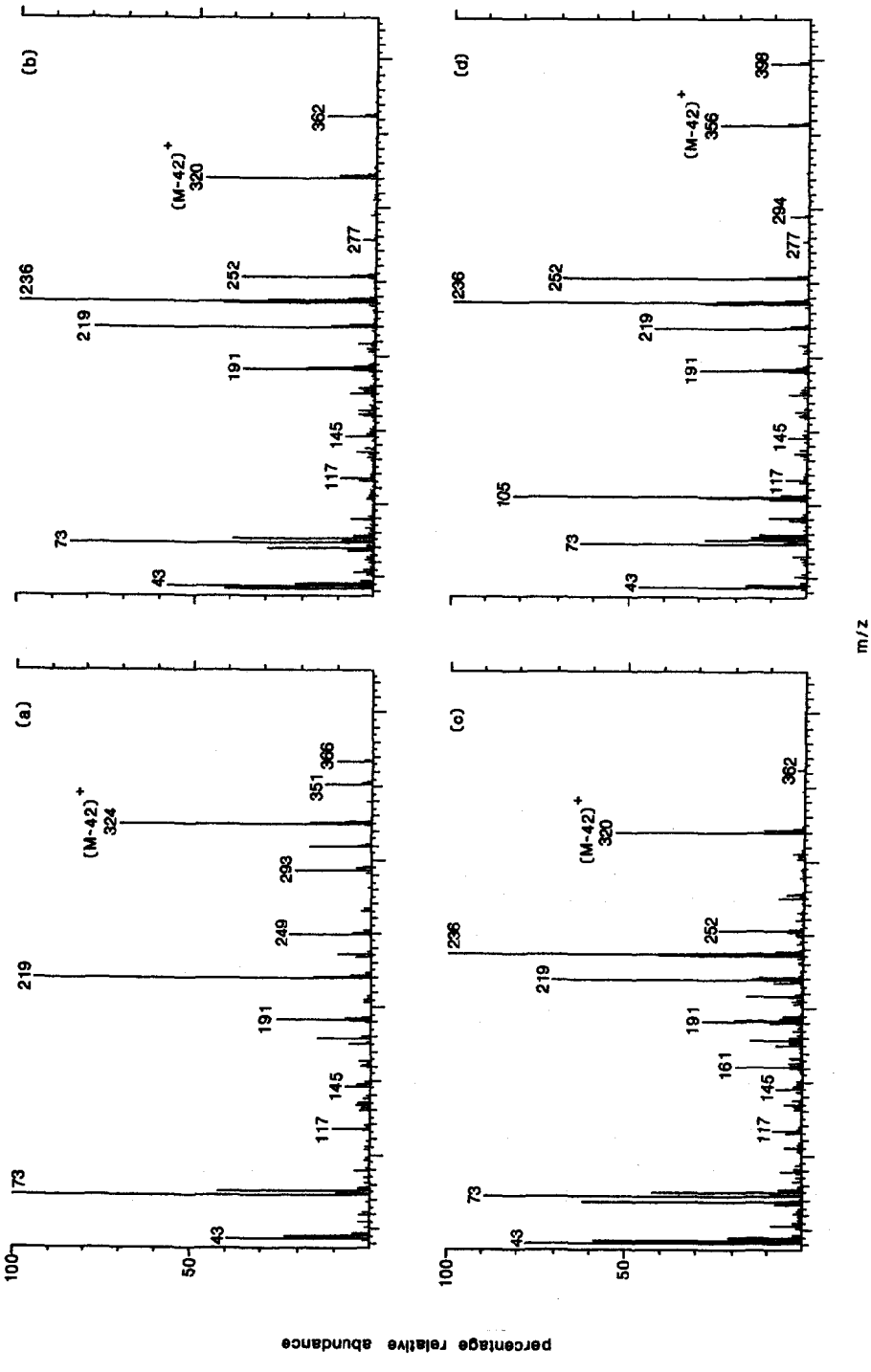


Fig. 1. Mass spectra recorded at 70 eV of (a) *trans*-3-acetyloxycaffeic acid bis-TMS, $[M]^+$ m/z = 366; (b) 3-methyl-3-butenyl *trans*-3-acetyloxycaffeate mono-TMS, $[M]^+$ m/z = 362; (c) 3-methyl-2-butenyl *trans*-3-acetyloxycaffeate mono-TMS, $[M]^+$ m/z = 362; (d) 2-phenylethyl *trans*-3-acetyloxycaffeate mono-TMS, $[M]^+$ m/z = 398. Spectra a–c are from *P. simonii*, spectrum d is from *P. ciliata*. The 4-acetyloxy-compounds have mass spectra very similar to those of the corresponding 3-acetyloxy-compounds.

EXPERIMENTAL

Reagents and materials

Acetic anhydride (Analar) was purchased from BDH (Dorset, U.K.), bis(trimethylsilyl)trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) from Sigma (Dorset, U.K.), N,N-dimethylformamide dineopentylacetal (DMF acetal) from Lancaster Synthesis (Lancashire, U.K.). Other chemicals were purchased from Aldrich (Dorset, U.K.) and Lancaster Synthesis, or provided by gift from Shell Research (Sittingbourne, U.K.).

Sample preparation

Bud exudate was obtained from buds of *P. ciliata*, clone ref D (originating from Dehra Dun, India) at the Forestry Commission Research Station (Alice Holt Lodge, Farnham, U.K.) and *P. simonii* (originating from Luozhenying, Shanxi, China) at the Poplar Bureau of Shanxi Province (Datong, Shanxi, China).

Exudate was collected by dipping five buds in 3 ml ethyl acetate for 10 s at room temperature. The ethyl acetate was evaporated in a screw-top conical glass tube under a stream of N_2 and the extract freeze dried for 10 min to remove residual water. After addition of 50 μl pyridine and 100 μl BSTFA containing 1% TMCS the tube was sealed and heated for 30 min at 100°C to produce trimethylsilyl (TMS) derivatives for GC.

GC–MS

The derivatised samples were separated and analysed in a Finnigan 1020 automated GC–MS system (incorporating a Data General Nova 3 computer). The GC–MS system was fitted with a 25 m × 0.32 mm I.D. Thames Chromatography (Maidenhead, U.K.) silica column coated with 0.5 μm of immobilized polydimethylsiloxane, and had a splitless injector with a flush 30 s after sample injection to remove residual gases. The end of the GC column was introduced directly into the mass spectrometer analyser chamber. The GC system was operated under the following conditions: He pressure, 13 p.s.i.; injector temperature, 310°C; GC temperature, 75–310°C at 3°C/min. 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 the injection of the sample (0.5–1 μl) into the GC.

Identification of compounds in bud exudate

This was by comparison with GC retention times in methylene units (MU; as defined by Dalgliesh *et al.* [4]) and mass spectra of trimethylsilyl (TMS) derivatives of reference compounds. The synthesis as reference compounds of acetyloxyferulic acid, acetyloxyisoferulic acid, the three acetyloxycaffeic acids and a number of esters of these acetyloxycaffeic acids is described in the Appendix.

RESULTS AND DISCUSSION

Preliminary studies of mass spectra suggested that a series of unidentified peaks in GC–MS analyses of bud exudate of *P. ciliata* and *P. simonii* were likely to be TMS derivatives of acetyloxycaffeic acids and their esters. The presence in these mass spectra of prominent m/z 43 and $[M - 42]^+$ ions (Fig. 1) representing CH_3CO and the

loss of CH_2CO from the mass ion, respectively, indicated the presence of an acetyl group.

The mass ions of the unidentified compounds added up to that expected for the TMS derivatives of 3- or 4-acetylated caffeic acids or their esters with benzyl alcohol, the methylbutenols or phenylethanol, all of which alcohols have previously been found in poplar bud exudates in the form of esters of caffeic acid.

Synthesis of acetyloxycaffeic acids and their esters enabled the methylene unit (MU) retention times and mass spectral patterns of several series of these compounds to be recorded (see Appendix). The two acetyloxy-derivatives produced from each parent compound, representing the 3-acetyloxy- and 4-acetyloxy-derivatives, had closely similar mass spectra but differed in MU retention times (Table I).

Caffeic acid, 3(3,4-dihydroxyphenyl)-2-propenoic acid, has two adjacent hydroxyl groups on the phenyl ring and the selective acetylation of only one of these groups presents difficulties. Ferulic acid, 3(3-methoxy-4-hydroxyphenyl)-2-propenoic acid,

TABLE I

CAFFEIC ACID, CAFFEATE ESTERS AND THEIR ACETYLOXY-DERIVATIVES IDENTIFIED IN POPLAR BUD EXUDATES BY GC–MS

Peak numbers correspond to those given in chromatograms shown in Figs. 3 and 4. GC retention times in methylene units (MU; defined by Dalgliesh *et al.* [4]) are given to two decimal places to indicate the elution sequence of peaks which chromatograph closely. Factors such as concentration of the compound concerned, 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 to only a single decimal place only.

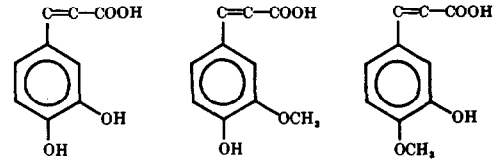
Peak[a] no.	Compound[b]	MU	Number of TMS groups	Distinctive ion	Composition
1	*trans*-4-Acetyloxycaffeic acid[c] (*trans*-3(3-hydroxy-4-acetyloxyphenyl)-2-propenoic acid)	21.42	2	366	$[M]^+$
2	*trans*-Caffeic acid	21.44	3	396	$[M]^+$
3	*trans*-3-Acetyloxycaffeic acid[c]	22.10	2	366	$[M]^+$
4	3-Methyl-3-butenyl *trans*-caffeate	23.47	2	392	$[M]^+$
5	3-Methyl-3-butenyl *trans*-4-acetyloxycaffeate[c]	23.52	1	320	$[M - 42]^+$
6	2-Methyl-2-butenyl *trans*-caffeate	23.83	2	392	$[M]^+$
7	2-Methyl-2-butenyl *trans*-4-acetyloxycaffeate[c]	23.88	1	320	$[M - 42]^+$
8	3-Methyl-2-butenyl *trans*-caffeate (prenylcaffeate)	23.96	2	392	$[M]^+$
9	3-Methyl-2-butenyl *trans*-4-acetyloxycaffeate[c]	23.98	1	320	$[M - 42]^+$
10	3-Methyl-3-butenyl *trans*-3-acetyloxycaffeate[c]	24.17	1	320	$[M - 42]^+$
11	2-Methyl-2-butenyl *trans*-3-acetyloxycaffeate[c]	24.49	1	320	$[M - 42]^+$
12	3-Methyl-2-butenyl *trans*-3-acetyloxycaffeate[c]	24.58	1	320	$[M - 42]^+$
13	Benzyl *trans*-caffeate	26.79	2	414	$[M]^+$
14	Benzyl *trans*-4-acetyloxycaffeate[c]	26.80	1	342	$[M - 42]^+$
16	2-Phenylethyl *trans*-caffeate	27.80	2	428	$[M]^+$
17	Benzyl *trans*-3-acetyloxycaffeate[c]	27.83	1	342	$[M - 42]^+$
18	2-Phenylethyl *trans*-4-acetyloxycaffeate[c]	27.85	1	356	$[M - 42]^+$
19	2-Phenylethyl *trans*-3-acetyloxycaffeate[c]	28.44	1	356	$[M - 42]^+$

[a] Location of peaks nos. 1–12 are shown in bud exudate of *P. simonii* (Fig. 3) and of peaks 13–19 in bud exudate of *P. ciliata* (Fig. 4).

[b] The names given do not include the TMS substituents.

[c] We are not aware of previous identifications of these compounds.

and isoferulic acid, 3(3-hydroxy-4-methoxyphenyl)-2-propenoic acid, differ from caffeic acid only in that one of the hydroxyls on the phenyl ring is methylated, leaving a single hydroxyl group available for acetylation (Fig. 2). With these as starting compounds it is possible to produce an acetylated derivative with the acetyl group in a defined position. Separate synthesis of acetyloxyferulic acid [3(3-methoxy-4-acetyloxyphenyl)-2-propenoic acid] and acetyloxyisoferulic acid [3(3-acetyloxy-4-methoxyphenyl)-2-propenoic acid] as described in the Appendix showed that, as trimethylsilyl derivatives, the 4-acetyloxy-compound (acetyloxyferulic acid), MU 20.73, chromatographed close to ferulic acid, MU 20.78, whereas the 3-acetyloxy-compound (acetyloxyisoferulic acid), MU 21.07, chromatographed much later than isoferulic acid, MU 20.65.



caffeic acid ferulic acid isoferulic acid

Fig. 2. Structures of caffeic acid, 3(3,4-dihydroxyphenyl)-2-propenoic acid; ferulic acid, 3(3-methoxy-4-hydroxyphenyl)-2-propenoic acid and isoferulic acid, 3(3-hydroxy-4-methoxyphenyl)-2-propenoic acid.

We believe that the differences in chromatographic retention time of acetyloxyferulic acid and acetyloxyisoferulic acid are likely to apply to the acetyloxycaffeates. We therefore assign the first of a pair of TMS–acetyloxycaffeates, *i.e.* that which chromatographs closely with the unacetylated parent compound, as the 4-acetyloxy-derivative and the second, which chromatographs later than the parental compound, as the 3-acetyloxy-derivative (see Table I, Figs. 3–5).

The 3-acetyloxy- and 4-acetyloxy-derivatives of caffeic acid, benzyl caffeate, 3-methyl-3-butenyl caffeate, 2-methyl-2-butenyl caffeate, 3-methyl-2-butenyl caffeate and 2-phenylethyl caffeate are identified in poplar bud exudate (Table I, Figs. 3, 4 show results from *P. simonii* and *P. ciliata*). The mass spectra of 3-acetyloxycaffeic acid bis-TMS, 3-methyl-3-butenyl 3-acetyloxycaffeate mono-TMS, 3-methyl-2-butenyl 3-acetyloxycaffeate mono-TMS and 2-phenylethyl 3-acetyloxycaffeate mono-TMS are shown in Fig. 1. Previous results have shown that the mass spectra of the different methylbutenyl esters of caffeic acid are very similar, and that the methylbutenyl esters are best differentiated by their characteristic MU retention times [5]. A similar, but more complex, situation occurs with the methylbutenyl esters of acetyloxycaffeate, in which the various methylbutenyl esters of both the 3-acetyloxy- and 4-acetyloxy-caffeates have very similar spectra. Here also the MU retention times provide the best means of identification (Table I). Although the 3,4-diacetyloxy-derivatives of caffeic acid and its esters were produced in the synthetic mixtures (see Appendix, Fig. 5), they appear not to occur in poplar bud exudates.

The acetyloxycaffeic acids and their esters occur primarily in those Asiatic poplars, such as *P. ciliata* [6], in which caffeic acid and its esters form the bulk of the

bud exudate. In such poplars the methylbutenyl acetyloxycaffeates may be major components: in one specimen of *P. ciliata* they formed *in toto* 26% of the bud exudate TIC, with 3-methyl-3-butenyl 4-acetyloxycaffeate present as both the *cis* (1%) and *trans* (12%) isomers [6].

We have previously demonstrated the potential of single ion reconstructions (SIR) of GC–MS data in resolving and identifying the components of complex mixtures [7,8] and of microsynthesis (as in the Appendix) and identification of reference compounds by GC–MS of the reaction mixture [5,9,10]. The work reported here confirms the potential of these methods.

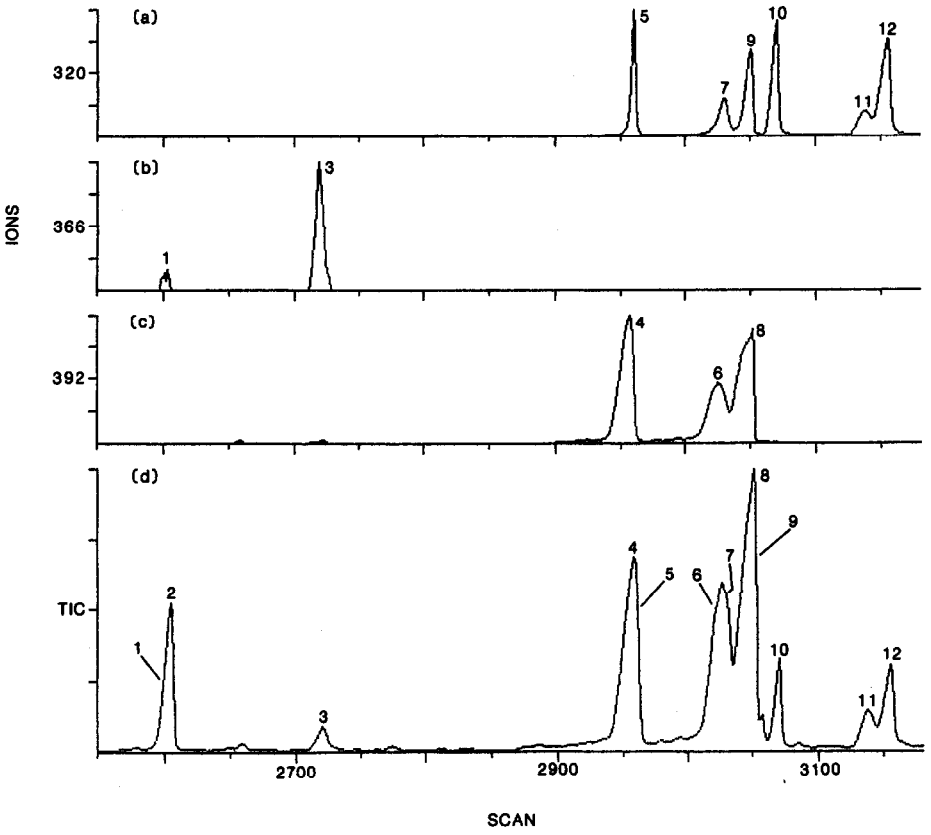


Fig. 3. Acetyloxycaffeic acids, methylbutenyl caffeates and methylbutenyl acetyloxycaffeates identified in bud exudate of *P. simonii*. (a) Single ion reconstruction (SIR) of m/z = 320 $[M - 42]^+$ locating 3-methyl-3-butenyl *trans*-4-acetyloxycaffeate mono-TMS (peak 5), 2-methyl-2-butenyl *trans*-4-acetyloxycaffeate mono-TMS (7), 3-methyl-2-butenyl *trans*-4-acetyloxycaffeate mono-TMS (9), 3-methyl-3-butenyl *trans*-3-acetyloxycaffeate mono-TMS (10), 2-methyl-2-butenyl *trans*-3-acetyloxycaffeate mono-TMS (11) and 3-methyl-2-butenyl *trans*-3-acetyloxycaffeate mono-TMS (12); (b) SIR of m/z = 366 $[M]^+$ locating *trans*-4-acetyloxycaffeic acid bis-TMS (1) and *trans*-3-acetyloxycaffeic acid bis-TMS (3); (c) SIR of m/z = 392 $[M]^+$ locating 3-methyl-3-butenyl *trans*-caffeate bis-TMS (4), 2-methyl-2-butenyl *trans*-caffeate bis-TMS (6) and 3-methyl-2-butenyl *trans*-caffeate bis-TMS (8); (d) total ion current between 21–25 MU. Peak 2 is *trans*-caffeic acid tris-TMS.

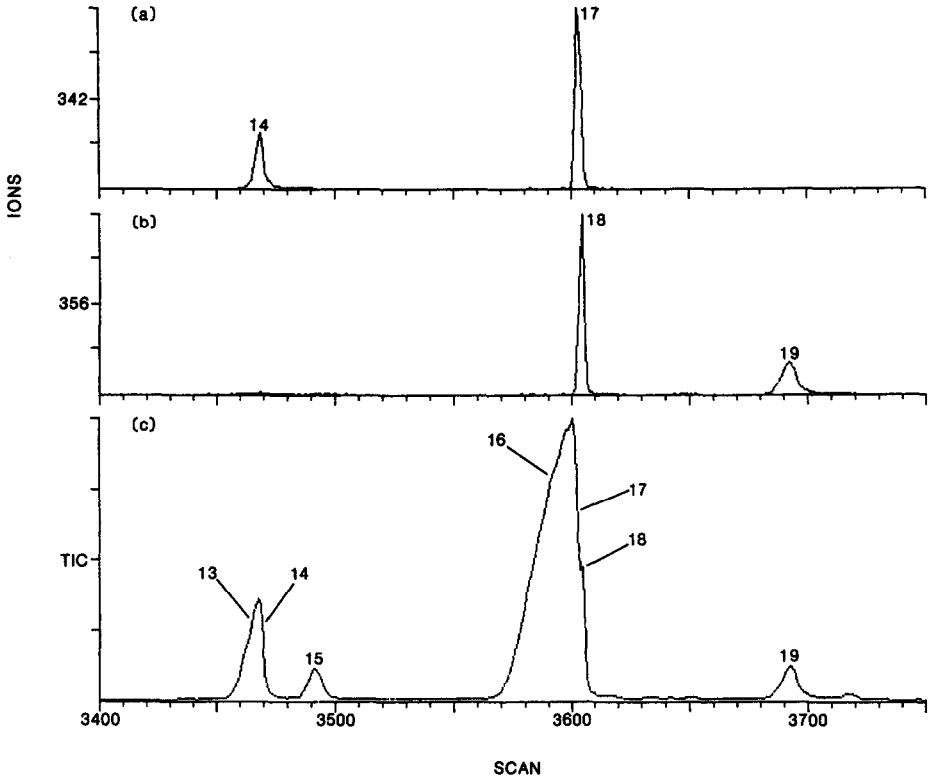


Fig. 4. Benzyl acetyloxycaffeates and phenylethyl acetyloxycaffeates identified in bud exudate of *P. ciliata*. (a) Single ion reconstruction (SIR) of m/z = 342 $[M - 42]^+$ locating benzyl *trans*-4-acetyloxycaffeate mono-TMS (peak 14) and benzyl *trans*-3-acetyloxycaffeate mono-TMS (17); (b) SIR of m/z = 356 $[M - 42]^+$ locating 2-phenylethyl *trans*-4-acetyloxycaffeate mono-TMS (18) and 2-phenylethyl *trans*-3-acetyloxycaffeate mono-TMS (19); (c) total ion current between 26–29 MU. Peak 13 is benzyl caffeate bis-TMS, peak 15 is heptacosane and peak 16 is 2-phenylethyl *trans*-caffeate bis-TMS.

ACKNOWLEDGEMENTS

We thank P. Howard, Forestry Commission, U.K. and Director Yang, Poplar Bureau of Shanxi Province, China for permission to collect plant material from their clonal collections of poplars and P. Jewess of Shell Research for providing a sample of 2-methyl-2-butenol.

APPENDIX

Synthesis of acetyloxyferulic and acetyloxyisoferulic acids

To 1 mg of ferulic acid, *trans*-3(3-methoxy-4-hydroxyphenyl)-2-propenoic acid, in a screw top glass derivatization tube, was added 75 μl pyridine and 25 μl acetic anhydride. The tube was heated at 60°C for 1 h, producing a mixture of unreacted ferulic acid and 4-acetyloxyferulic acid. The solvent was evaporated under a stream of

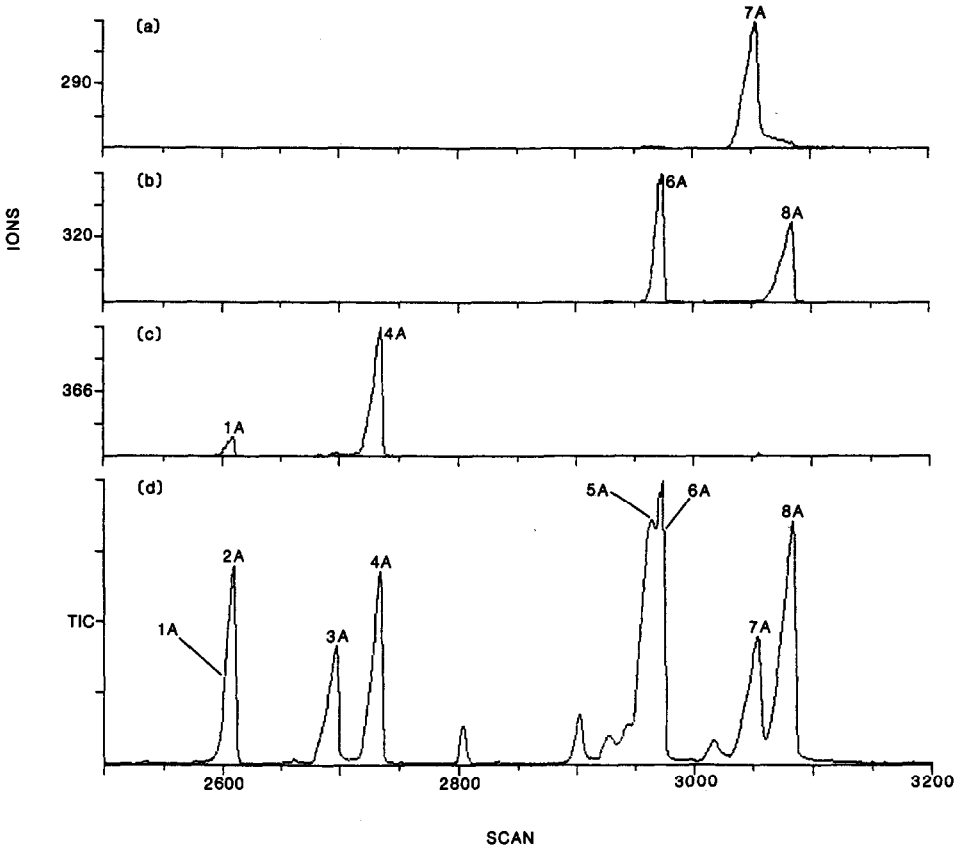


Fig. 5. Single ion reconstructions (SIR) and GC–MS total ion chromatogram (TIC) of caffeic acid derivatives produced by esterification of a mixture of acetyloxycaffeates with 3-methyl-3-butenol. (a) SIR m/z = 290 $[M - 42]^+$, locating 3-methyl-3-butenyl *trans*-3,4-diacetyloxycaffeate (7A), MU = 23.95; (b) SIR m/z = 320 $[M - 42]^+$, locating 3-methyl-3-butenyl *trans*-4-acetyloxycaffeate mono-TMS (6A), MU = 23.52, and 3-methyl-3-butenyl *trans*-3-acetyloxycaffeate mono-TMS (8A), MU = 24.17; (c) SIR m/z = 366 $[M]^+$, locating *trans*-4-acetyloxycaffeic acid bis-TMS (1A), MU = 21.42, and *trans*-3-acetyloxycaffeic acid bis-TMS (4A), MU = 22.10; (d) TIC between 21–25 MU. In addition to peaks identified above the following are present, *trans*-caffeic acid tris-TMS (2A), MU = 21.44; *trans*-3,4-diacetyloxycaffeic acid mono-TMS (3A), MU = 21.88; and 3-methyl-3-butenyl *trans*-caffeate bis-TMS (5A), MU = 23.47.

nitrogen and the residue briefly freeze dried. For gas chromatography 100 μl BSTFA (inc. 1% TMCS) and 50 μl pyridine was added to the mixture of acids and the tube was heated at 100°C for 1 h, to produce trimethylsilyl (TMS) derivatives with any unreacted hydroxyl or carboxyl groups. The same procedure, but using isoferulic acid, *trans*-3(3-hydroxy-4-methoxyphenyl)-2-propenoic acid, resulted in the synthesis of the TMS derivative of 3-acetyloxyisoferulic acid.

Synthesis of esters of acetylcaffeic acid

Caffeic acid, *trans*-3(3,4-dihydroxyphenyl)-2-propenoic acid (1 mg) was heated with pyridine and acetic anhydride as above. This resulted in a mixture of unreacted

caffeic, 3-acetyloxycaffeic, 4-acetyloxycaffeic and 3,4-diacetyloxycaffeic acids. The solvent was evaporated under a stream of nitrogen and the residue of acids briefly freeze dried as above. To this mixture of acids was added 10 μl of the appropriate alcohol (*e.g.*, 2-phenylethyl alcohol for 2-phenylethyl esters, 3-methyl-3-butenol for 3-methyl-3-butenyl esters, etc.), 150 μl of dichloromethane and 10 μl of DMF acetal. This latter compound mediates the esterification of acids with alcohols [11]. The tube was heated at 100°C for 1 h, the solvent evaporated under a stream of nitrogen and the residue briefly freeze dried. The resultant mixture of compounds was derivatized with BSTFA (inc. 1% TMCS) as above.

Whereas this mixture is complex, the various constituents can be located after GC separation by their characteristic ions (Fig. 5 shows the mixture resulting when 3-methyl-3-butenol is used as the alcohol). The appropriate methylene unit (MU) retention times were calculated after the addition of a series of straight chain hydrocarbon standards to the derivatized mixture.

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