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TRISUBSTITUTED HYDROXYCINNAMIC ACID SPERMIDINES FROM *QUERCUS DENTATA* POLLEN

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Key Word Index—*Quercus dentata*; Fagaceae; pollen; hydroxycinnamic acid amides; caffeic acid; *p*-coumaric acid; polyamines; electrospray mass spectrometry.

Abstract—Three novel and one known hydroxycinnamic acid amides were isolated from pollen of *Quercus dentata* Thunb. They were identified as N^1,N^5,N^{10}-tricaffeoylspermidine, N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine, N^1, N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine and N^1, N^5, N^{10}-tri-*p*-coumaroylspermidine, respectively. Information on the position of the hydroxycinnamoyl moieties was derived from electrospray mass spectral fragmentation patterns.

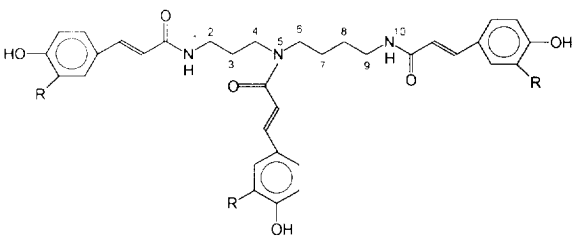
INTRODUCTION

Hydroxycinnamic acid amides (HCAAs) are known from the reproductive organs of many higher plants [1]. Previously, the accumulation of disubstituted hydroxycinnamoyl spermidines has been described from pollen of the Betulaceae, Fagaceae and Juglandaceae [2–4]. Apparently, the occurrence of these compounds constitutes a useful taxonomic marker in higher Hamamelidae. The trisubstituted N^1,N^5,N^{10}-tri-*p*-coumaroylspermidine, was found in flowers of *Crataegus* [5] and more recently reported to occur throughout the Rosaceae [6]. The occurrence of unknown hydroxycinnamic acid derivatives was previously noted from pollen of several *Quercus* species [4] and we report here the structural elucidation of three novel and one known trisubstituted hydroxycinnamic acid spermidines from pollen of *Quercus dentata*.

RESULTS AND DISCUSSION

A HPLC trace of 80% aqueous methanolic extract of pollen from *Q. dentata* is shown in Fig. 1. Further purification yielded the four major compounds (**1**–**4**). The four minor compounds were not identified. The first two peaks in the HPLC trace (A_1 and A_2) correspond to two positional isomers of di-*p*-coumaroylspermidine. These compounds were recently isolated from pollen of *Alnus glutinosa* [4] and identified by co-chromatography. Some chromatographic and UV-spectral properties of **1**–**4** are summarized in Table 1.

Strong alkaline hydrolysis of **1** yielded caffeic acid, **2** and **3**, *p*-coumaric acid and caffeic acid, and **4**, only *p*-coumaric acid. Strong acid hydrolysis of all compounds yielded only spermidine. Alkaline hydrolysis of all four compounds yielded a number of partially hydrolysed products, one of which could be identified by co-chromatography with a known compound from pollen of



R = H = p-coumaroyl, R = OH = caffeoyl

1 = N^1,N^5,N^{10}-tricaffeoylspermidine,
2 = N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine,
3 = N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine,
4 = N^1,N^5,N^{10}-tri-*p*-coumaroylspermidine.

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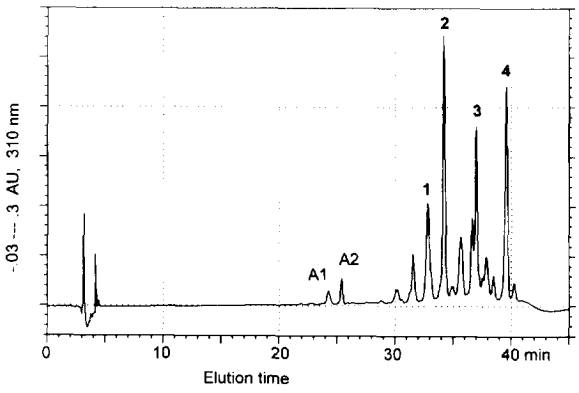

Fig. 1. HPLC-trace of a methanolic pollen extract of *Quercus dentata*. Compound **1** = N^1,N^5,N^{10}-tricaffeoylspermidine, **2** = N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine, **3** = N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine, **4** = N^1,N^5,N^{10}-tri-*p*-coumaroylspermidine. Compounds corresponding to peaks A1 and A2 were identified as di-*p*-coumaroylspermidines [4].

Pterocarya fraxinifolia [4]. Compound **4** yielded three intermediates with retention times and UV-spectral characteristics corresponding to disubstituted *p*-coumaroylspermidines. One of these intermediates co-eluted with N^1,N^5-di-*p*-coumaroylspermidine, the major compound in pollen of *P. fraxinifolia*. This intermediate was not detected after hydrolysis of **3**. N^1,N^5-di-*p*-coumaroyl-N^{10}-caffeoylspermidine was, therefore, excluded as one of the possible three positional isomers of **3**.

All 1D and 2D ^{1}H NMR spectra were complex because the compounds usually undergo configurational isomerization (*E*/*Z*) at the phenylpropanoid moiety and rotational isomerization (hindered rotation about the N^5-amidic bond). This may result in up to 16 different isomers per compound. The resulting problems in assigning the ^{1}H NMR signals have been discussed in a previous publication [4]; only the relevant data are discussed here. Characteristic signals in the aromatic region, δ 7.6–6.4, allowed ready identification of the nature and the relative number of hydroxycinnamic acid moieties present. Thus, the *p*-coumaroyl moiety had the major signals of the *E*-isomer at δ7.6–7.35 (H-7, H-2/H-6), 6.95–6.75 (H-3/H-5, H-8) and 6.5–6.4 (H-8), while the caffeoyl moiety had signals at δ7.6–7.4 (H-7), 7.1–6.7 (H-2, H-5, H-6, H-8) and 6.4–6.3 (H-8). There was no aromatic methoxyl signal in the spectra, which would indicate the presence of a feruloyl or sinapoyl derivative. Compound **1** showed only signals indicative of caffeoyl moieties, **4** only signals of *p*-coumaroyl units. The relative positions of the *p*-coumaroyl and caffeoyl moieties in **2** and **3** could not be deduced from the NMR spectra. Signals for the spermidine moiety in all compounds occurred in the regions δ3.7–3.3 (acyl-N-CH_2) and δ2.05–1.55 (internal CH_2-groups). There were no signals between δ3.1–2.7 indicative of non-acylated $-NH_2$ or $-NH-$ groups. The effect of acylation on the chemical shifts of ^{1}H NMR signals in the spermidine moiety has been thoroughly investigated by Meurer *et al.* [4]. Therefore, all compounds had acylated amide moieties.

All compounds show quasi-molecular ions in both their positive and negative ion electrospray mass spectra, $[M + H]^+$, $[M + Na]^+$ and $[M - H]^-$, respectively; $[M + H]^+$ ions were also detected by FAB mass spectrometry. These experiments confirmed the molecular formulae of the compounds (**1**, $C_{34}H_{37}N_3O_9$, M/W 631; **2**, $C_{34}H_{37}N_3O_8$, M/W 615; **3**, $C_{34}H_{37}N_3O_7$, M/W 599; **4**, $C_{34}H_{37}N_3O_6$, *m*/*z* 583).

In order to elucidate the positions of the different hydroxycinnamic acid moieties of **2** and **3**, MS/MS experiments were performed on their $[M + H]^+$ signals. After collision-induced decomposition, the spectrum shown in Fig. 2A was obtained from **2**. Major ions at *m*/*z* 470 and 454 could be assigned to fragments arising from cleavage of the amide bond between the monohydroxycinnamoyl residue or the dihydroxycinnamoyl residues and the spermidine moiety, respectively, accompanied by less intense satellites owing to loss of NH_3, or NH_4. The fragments at *m*/*z* 308 and 292 can be explained by the loss of a further cinnamoyl residue. Of special interest for the localization of the different cinnamoyl moieties, are the fragments at *m*/*z* 234 and 204. Since the former comprises the fragment containing the C_3-chain of the spermidine residue originating from the cleavage of

Table 1. Chromatographic and some spectroscopic characteristics of trisubstituted hydroxycinnamoyl spermidines from pollen of *Quercus dentata*

Compound	R_t* (min)	R_t† (min)	λ_{max}‡ (nm)	R_f§ (TLC)	Colour	
					UV_{365}	$+NH_3$
1	79	29.7	230, 295sh, 320	0.09	White	Green
2	85	32.0	230sh, 295, 312	0.19	Light blue	Green
3	91	34.4	226, 295, 310	0.34	Invisible	Dark blue
4	98	36.5	226, 295, 308	0.73	Absorbing	Dark blue

*Prep. HPLC conditions (see Experimental).

†Analytical HPLC conditions (see Experimental).

‡Recorded in MeCN–H_2O, 2% H_3PO_4 via photodiode array detection.

§TLC system: microcrystalline cellulose, $CHCl_3$–HOAc (2:1, H_2O satd), detection under UV-light (365 nm).

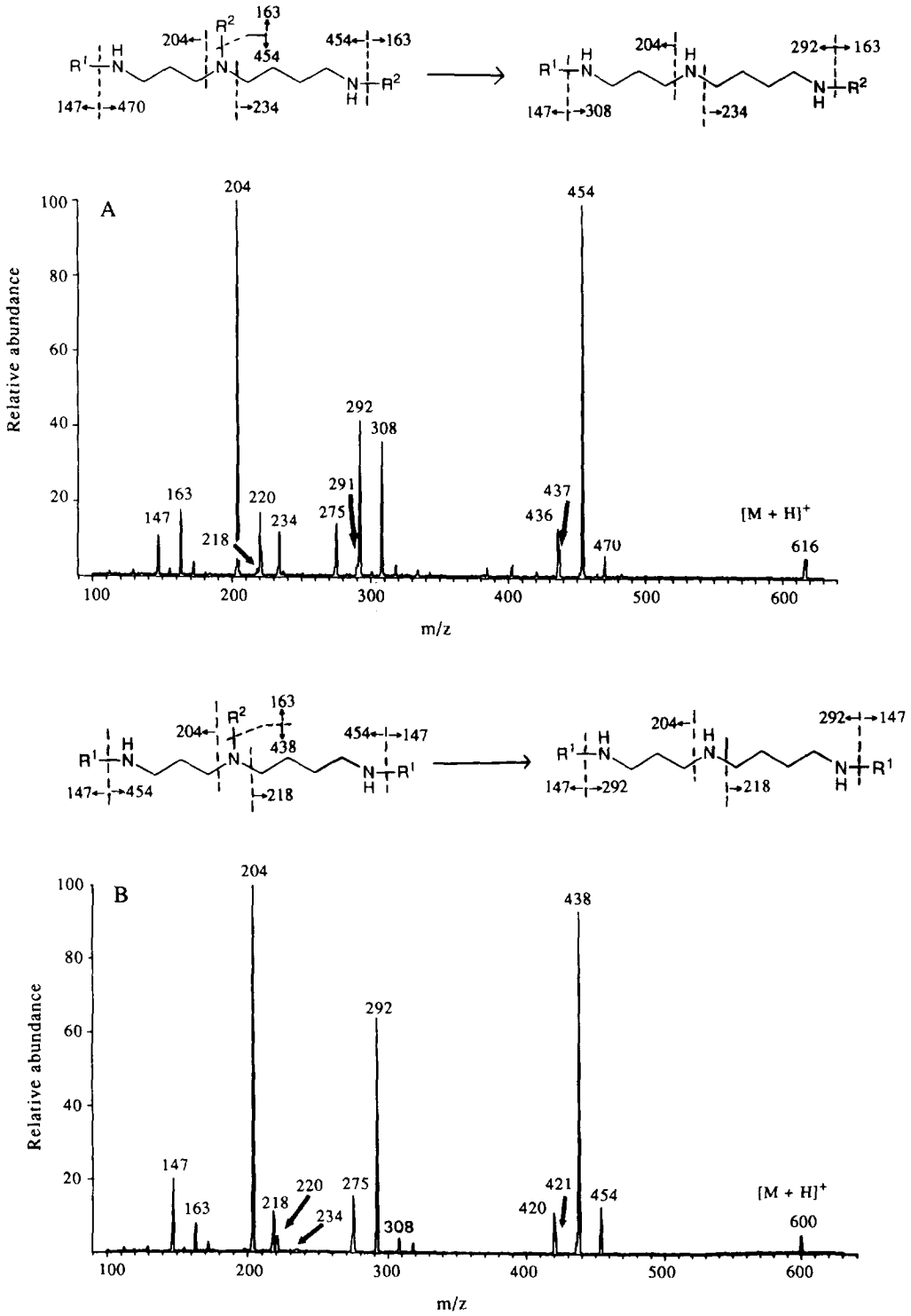

Fig. 2. ESI-MS/MS of $[M + H]^+$ ions of **2** (N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine) (A) and **3** (N^1, N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine) (B) with affixed fragmentation schemes. R^1 = 4-hydroxycinnamoyl = *p*-coumaroyl, R^2 = 3,4-dihydroxycinnamoyl = caffeoyl.

the bond between N^5 and C^6, it is obvious that N^{10} must be predominantly substituted by a dihydroxycinnamic acid. Consequently, the fragment at *m/z* 218 calculated to contain a monohydroxycinnamic acid is barely visible. The fragment at *m/z* 204 can be explained similarly as the C_3-chain containing a monohydroxycinnamoyl-substituted N^1. The presence of *ca* 10–20% of a dihydroxycinnamic acid-substituted N^1 is suggested by the

corresponding fragment at *m/z* 220. Alternatively, this fragment could arise by α-cleavage of the C_4-chain between C6 and C7. In either case, however, the dominant isomer present in fraction **2** is N^1-coumaroyl-N^5,N^{10}-dicaffeoylspermidine.

Compound **3** (Fig. 2B) yielded analogous fragments generated by the loss of one or two cinnamoyl residues at *m/z* 454 and 438 and 308 or 292, respectively. The characteristic fragment incorporating the C_4-chain of the spermidine residue was detected at *m/z* 218 with a weak signal at *m/z* 234, indicating the predominant substitution of N^{10} by a monohydroxycinnamoyl residue. The fragment ion comprising the C_3-chain yielded a strong signal at *m/z* 204 (less than 10% of the relative intensity was detected at *m/z* 220), suggesting at least 90% substitution of N^1 by the lighter cinnamoyl derivative. Therefore, the caffeoyl moiety must be linked to N^5 and the structure N^1,N^{10}-dicoumaroyl-N^5-caffeoylspermidine can be assigned to **3**. The substitution pattern of both spermidine derivatives is confirmed by the relative intensities of the fragment ions generated by loss of one or two cinnamoyl residues. Although in **3** the ratio between coumaroyl and caffeoyl residues is 2:1, the fragment ion arising from the loss of the single caffeoyl moiety is much more intense than the one originating after loss of one coumaroyl residue, clearly indicating preferential cleavage of the cinnamoyl residue linked to the unique secondary amine N^5. Obviously, after the loss of a second cinnamoyl residue, the chances of finding an intact caffeoyl residue linked to the N^5 are very low. This is exactly what is observed, since the respective ion at *m/z* 308 is very weak. For compound **2** one would expect a relatively strong signal at *m/z* 454 generated by loss of the caffeoyl residue linked to N^5 and *ca* equal intensities of the ions generated by the second fragmentation step at *m/z* 292 and 308 (loss of coumaroyl residue from N^1 or a caffeoyl residue from N^{10}), which is indeed observed (cf. Fig. 2A). From the above, it is clear that electrospray mass spectrometry is of general applicability to analysis of this class of compound and it affords, directly from the underivatized material, information on the substitution positions of aromatic moieties. In addition to this, it has the advantage over NMR spectroscopy of requiring less material (*ca* 500 pmol).

This is the second report of trisubstituted hydroxycinnamoylspermidine derivatives in nature. N^1,N^5,N^{10}-Tri-*p*-coumaroylspermidine (**4**) has been found in inflorescences of *Crataequs* [5] and, more recently, in flowers of many other Rosaceae [6]. The other three compounds, N^1,N^5,N^{10}-tricaffeoyl spermidine (**1**), N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine (**2**) and N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine (**3**) are reported for the first time. The occurrence of these compounds in floral parts of the Rosaceae and *Quercus dentata* (Fagaceae) has important chemotaxonomic implications, as recent investigations [7, 8] have revealed that the Rosaceae and other Rosidae are related much more closely to the families of the higher Hamamelidae (Fagaceae, Betulaceae, Juglandaceae, Myricaceae and Casuarinaceae) than previously assumed. Trisubstituted hydroxycinnamoyl spermidines have also been detected in other members of the Fagaceae, Betulaceae and Juglandaceae [9].

EXPERIMENTAL

Isolation. Pollen from *Quercus dentata* Thunb. was collected from a tree growing on the grounds of the New York Botanical Garden in spring 1992. A voucher is deposited in the Herbarium of the New York Botanical Garden (NY). Pollen (5 g) was extracted × 5 with 40 ml H_2O, and × 5 with 40 ml 80% aq. MeOH. The MeOH extracts were combined and subjected to semi-prep. HPLC (in 2 ml portions) on a C-18 Nucleosil column (2.1 × 25 cm, 10 μ, Phenomenex) using a linear gradient from 80% solvent A in B to 100% solvent B in 80 min, and continued isocratically at 100% B for 30 min at a flow rate of 10 ml min^{-1}. Solvent A was 4% HCO_2H in H_2O and solvent B was 4% HCO_2H, 46% H_2O and 50% MeOH. Elution was monitored by photodiode array detection from 220 to 400 nm. Frs corresponding to the peak apices were collected. Frs were evapd to dryness using repeated addition of H_2O to remove HCO_2H. The purity of the isolates was tested by TLC (microcrystalline cellulose, $CHCl_3$–HOAc, 4:2, H_2O satd) and by analytical HPLC. Analytical sepns were achieved on a C-18 column (Nucleosil, 4.6 mm × 250 mm, 5 μm, Phenomenex) using a linear gradient from 80% solvent A in solvent B to 20% A in B in 30 min, 20% A in B for 4 min, with 2% H_3PO_4 in H_2O as solvent A and 2% H_3PO_4, 48% H_2O and 50% MeCN as solvent B at a flow rate of 1 ml min^{-1}. Eluates were monitored at 220–400 nm.

Alkaline hydrolysis. Hydrolyses were carried out in a final concn of 4 N NaOH with the addition of $NaBH_4$ in a He-satd, tightly sealed glass tube for 2 to 4 hr at 100°. After acidification, hydrolysis assays were partitioned against EtOAc. The EtOAc phases were analysed for hydroxycinnamic acids and partially hydrolysed compounds by TLC (microcrystalline cellulose, toluene–HOAc, 2:1, H_2O satd) and the analytical HPLC method described above. Hydroxycinnamic acids were identified by co-chromatography with authentic standards.

Acid hydrolysis. Hydrolyses were performed for 4–6 hr in a final concn of 2 M HCl at 100°. After cooling, hydrolysis assays were partitioned × 2 with EtOAc and the aq. phase used for polyamine determinations. Hydrolysates and authentic polyamine standards were benzoylated using the method of ref. [10]. Benzoylated polyamines were determined by HPLC using the analytical method described above.

Mass spectrometry. FAB-MS were recorded with glycerol as matrix. A triple quadrupole mass spectrometer equipped with an electrospray ion-source was used for electrospray MS. Spermidine derivatives were dissolved in MeOH containing 2% HCO_2H and injected at a flow rate of 2 μl min^{-1} into the electrospray chamber. A voltage of 4.5 kV was applied to the electrospray needle. For CID experiments, parent ions were selectively transmitted by the first mass analyser and directed into the

collision cell (Ar was used as collision gas) with kinetic energy set at −35 eV.

NMR spectroscopy. 1D and 2D ^{1}H NMR spectra were recorded at ambient temperature locked to the major resonance of the solvent, CD_3OD.

REFERENCES

1. Martin-Tanguy, J., Cabanne, F., Perdrizet, E. and Martin, C. (1978) *Phytochemistry* **17**, 1927.
2. Meurer, B., Wray, V., Grotjahn, L., Wiermann, R. and Strack, D. (1986) *Phytochemistry* **25**, 433.
3. Meurer, B., Wiermann, R. and Strack, D. (1988a) *Phytochemistry* **27**, 823.
4. Meurer, B., Wray, V., Wiermann, R. and Strack, D. (1988b) *Phytochemistry* **27**, 839.
5. Jaggy, H. (1978) *Planta Med.* **33**, 285.
6. Strack, D., Eilert, U., Wray, V., Wolff, J. and Jaggy, H. (1990) *Phytochemistry* **29**, 2893.
7. Manos, P., Nixon, K. and Doyle, J. J. (1993) *Syst. Botany* **18**, 551.
8. Hufford, L. (1992) *Ann. Missouri Bot. Gard.* **79**, 218.
9. Meurer-Grimes, B. (1995) *Brittonia* (in press). Brittonia.
10. Flores, H. E. and Galston, A. W. (1982) *Plant Physiol.* **69**, 701.