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HYDROXYCINNAMIC ACID SPERMIDINE AMIDES FROM POLLEN OF *CORYLUS AVELLANA* L.

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Key Word Index—*Corylus avellana*; Betulaceae; hazelnut; pollen; hydroxycinnamic acid; polyamine; spermidine; amide.

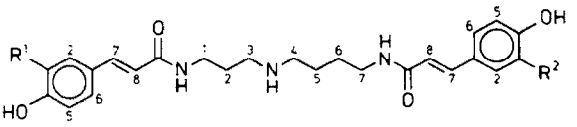
Abstract—Two hydroxycinnamic acid amides from the pollen of *Corylus avellana* L. have been identified as (*E*)-caffeoyl-(*E*)-feruloylspermidine and di-(*E*)-feruloylspermidine on the basis of ^{1}H NMR, ^{13}C NMR and mass spectral data.

INTRODUCTION

Hydroxycinnamic acid (HCA) amides, such as putrescine, spermidine or tyramine conjugates, have frequently been found to accumulate in the reproductive organs of higher plants [1]. Production of these acylated amines may be linked to the physiology of flowering [2, 3]. They may also play a fundamental role as antiviral agents [4] or antibiotics against bacteria [5].

HCA amides were repeatedly shown to be associated with anthers, most likely with the pollen grains, and they appear to determine pollen fertility [6, 7]. The pollen in general is characterized by accumulation of large amounts of secondary plant products, especially carotenoids and flavonoids [8]. For the pollen of *Corylus avellana* a flavonol content of up to 3–4% of pollen dry weight was found [9, 10] and there is evidence that flavonoids are localized in or at the outer pollen wall [11]. 'Simple' phenylpropane conjugates (hydroxycinnamic acid esters or amides) have not been intensively studied with regard to localization, biochemistry and function in the pollen wall.

In a previous paper we reported the identification of quercetin 3-*O*-glucosylgalactoside from pollen of *Corylus avellana* and we mentioned the presence of two major ferulic acid conjugates which awaited structural elucidation [10]. We report here an investigation of these conjugates which are shown to be caffeoylferuloylspermidine (**1**) and diferuloylspermidine (**2**).



1 $R^1 = OH; R^2 = OCH_3$ or $R^1 = OCH_3; R^2 = OH$

2 $R^1 = R^2 = OCH_3$

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RESULTS AND DISCUSSION

Successive extractions of freeze-dried pollen of *Corylus avellana* with water and 80% aq. methanol showed that flavonoids (quercetin 3-*O*-glucosylgalactoside as the main constituent) [10] could be easily totally removed from the grains by water [9], whereas the hydroxycinnamic acid (HCA) amides (**1** and **2**) only could be extracted with an organic solvent, such as 80% aq. methanol (Fig. 1). Total extraction of the HCA amides was difficult and a repeated extraction was necessary to give sufficient quantities of these amides. Combining six extractions, the amount of these conjugates was *ca* 2% of the dry wt, composed of 80% **1** and 20% **2**.

In the present study, **1** and **2** were purified by means of ion-exchange chromatography of CM-Sephadex (Na^+ or NH_4^+ form) and the column was developed with a sodium chloride (Fig. 2) or ammonium chloride gradient. The structures of the two amides, (*E*)-caffeoyl-(*E*)-feruloylspermidine (**1**) and di-(*E*)-feruloylspermidine (**2**) were assigned on the basis of the data presented below. The UV spectral data (λ_{max}^{MeOH}, nm) for **1** were 219, 231, 295, 320 and for **2** were 212, 216, 295, 314. Strong alkaline hydrolysis of **1** gave caffeic and ferulic acids, while **2** gave only ferulic acid.

The structure of **1** followed from the ^{1}H and ^{13}C NMR data, and the positive and negative ion fast atom bombardment (FAB) mass spectral data. The ^{1}H NMR spectrum of **1** in methanol-d_4 showed characteristic signals to low field for the two aromatic residues and to higher field for the spermidine residue in a ratio of 1 : 1 : 1. The unambiguous assignment of the signals of all three residues was deduced from the cross peaks of the two-dimensional ^{1}H homonuclear shift-correlated spectrum. Each aromatic system was identified separately and their corresponding vinyl groups were found from the nuclear Overhauser enhancements between the olefinic protons and the aromatic protons. The characteristic chemical shifts and the cross peak between the methoxyl group and H-2 of the feruloyl system allowed unambiguous assignment of the aromatic system signals. Although the amount of material

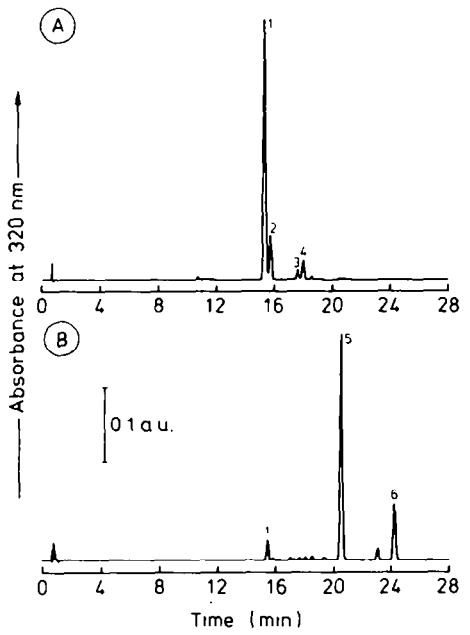

Fig. 1. HPLC resolution of extracts (A, three H_2O extractions combined; B, six 80% aq. MeOH extractions combined) from pollen of *Corylus avellana* (8.3 mg dry wt per ml solvent) on a Nova-Park® C_{18} column. Peak identification: 1, quercetin 3-*O*-glucosylgalactoside (R_t 15.5 min); 2, unidentified quercetin glycoside; 3 and 4, unidentified kaempferol glycosides; 5, caffeoylferuloylspermidine (**1**) (R_t 20.5 min); 6, diferuloylspermidine (**2**) (R_t 24.3 min). Development: linear gradient elution with 40 min from 2 to 80% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) at a flow rate of 1.5 ml/min. At the detection wavelength of 320 nm the quercetin glycosides showed *ca* 75% of the maximal absorptivity (at 355 nm)

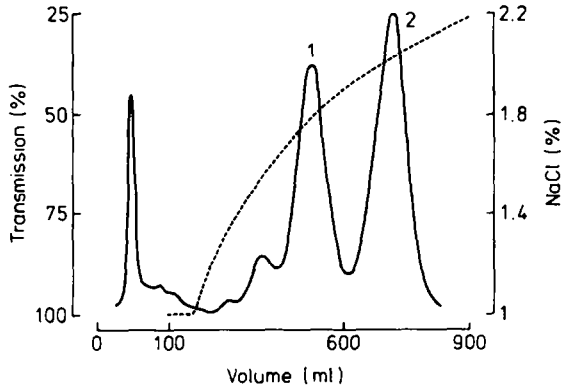

Fig. 2. Elution profile (solid line) of UV absorbing compounds of an extract (80% aq. MeOH) from pollen of *Corylus avellana* on a CM-Sephadex-C-25 column. 1, Diferuloylspermidine (**2**); 2, caffeoylferuloylspermidine (**1**). Dashed line shows the profile of the NaCl gradient.

available only allowed the proton-attached carbons to be observed in the ^1H broadband decoupled ^{13}C spectrum, their multiplicity was obtained from a DEPT spectrum. This confirmed the presence of both the aromatic residues and that there were seven methylene groups in the molecule with shifts compatible with a spermidine residue. The ^1H spectrum in DMSO-d_6 showed, in addition to the signals observed in methanol-d_4, two triplet signals to low field of equal intensity for the amidic protons which indicated the position of attachment of the amide functions on the spermidine residue.

Unfortunately, although **1** was a single compound, the absolute positions of the aromatic residues could not be deduced unambiguously from either the NMR data nor from the fragmentation pattern in the FAB mass spectrum.

Component **2** had a ^1H spectrum that closely resembled **1**. Instead of the caffeoyl system a second feruloyl residue was detected, as most of the aromatic protons were doubled and their intensities were in a 1:1 ratio. The nature of the compound was confirmed by FAB mass spectra which indicated an M_r of 497, fourteen mass units greater than **1**.

Initial difficulties were experienced in the spectroscopic determination of **1** and **2** as both compounds readily undergo isomerization of the double bonds leading to four distinct compounds in each case. In such preparations the signals of the aromatic residues were complex and up to four sets of signals could be detected for each proton. Such spectra and the observation of only one set of signals, after extraction and purification under more stringent conditions, clearly indicate that the natural products have the *E*-configuration of the double bonds and **1** exists in only one of the two possible forms.

Compounds **1** and **2** are closely related to several alkaloids found in the seeds of *Lunaria* species [12] and **1** corresponds to the open form of codonocarpine found in the bark of *Codonocarpus australis* A. Cunn. [13]. In this latter respect they also resemble maytenine, di-*trans*-cinnamoyl spermidine with unsubstituted aromatic residues, from *Maytenus Chuchuhuasha* [14]. The occurrence of compound **2** in anthers and pollen grains seems to be widespread [3, 6, 15].

EXPERIMENTAL

Plant material. Pollen of hazelnut (*Corylus avellana* L.) was collected from the area around Münster, West Germany, and was freeze-dried immediately after harvest.

Extraction and isolation. Pollen was first treated for 4 min with Ultra turrax homogenizer in H_2O (10 g/20 ml) and then allowed to stand for 1 hr with continuous stirring. The suspension was centrifuged at 3500 *g* for 15 min. This was repeated 4 times and the supernatants combined. Subsequent extraction of the hydroxycinnamic acid amides (**1** and **2**) was accomplished by treatment of the remaining pellet with 80% aq. MeOH as described above. The extract was evapd to dryness *in vacuo* and the residue suspended in a few ml of H_2O. Compounds **1** and **2** were purified by chromatography on CM-Sephadex-C-25 column [5] (2 × 28 cm; Pharmacia) equilibrated with 0.9% NH_4Cl or 1% NaCl. The column was developed with a convex gradient from 0.9 to 2.7% NH_4Cl and 1–3% NaCl, respectively, in H_2O (Fig. 1) at a flow rate of *ca* 3.5 ml/min. Elution of phenolic compounds was monitored by UV at 254 nm (Uvicord; LKB). Fractions containing **1** or **2** were evapd to dryness *in vacuo*. The residues were redissolved in 10 ml MeOH and excess salt was removed by centrifugation (3500 *g*, 15 min). The MeOH solns were evapd to dryness.

Extractions in analytical work (Fig. 1) were carried out with 200 mg pollen dry wt, repeatedly extracted with 2 ml of H_2O and 2 ml of 80% aq. MeOH, respectively.

TLC. Compounds **1** and **2** were chromatographed on microcrystalline cellulose (Avicel) in BAW (*n*-BuOH–HOAc–H_2O, 6:1:2) and CAW ($CHCl_3$–HOAc, 3:2, H_2O satd) and on polyamide in WMMA (H_2O–MeOH–MeCOEt–acetylacetone, 13:3:3:1) and gave the following R_fs: **1**, 0.51 in BAW, 0.62 in CAW, 0.69 in WMMA; **2**, 0.63 in BAW, 0.94 in CAW, 0.74 in WMMA. Compounds were detected under UV at 350 nm. Compound **1** changed from bright blue to bright greenish-blue fluorescence when treated with NH_3 vapour and **2** from blue to greenish-blue. After hydrolysis for 4 hr at 100° in 4 M NaOH (air was displaced by a current of N_2), acidification with HCl and extraction with Et_2O, **1** gave caffeic and ferulic acids, and **2** ferulic acid (R_fs in toluene–HOAc, 2:1, H_2O satd: caffeic acid 0.23, ferulic acid 0.70; under UV at 350 nm caffeic acid changed from blue to white-blue and ferulic acid from blue to green-blue when treated with NH_3 vapour).

HPLC. The liquid chromatograph used was from LKB. Injection was done via a 20 μl loop. Quantitative calculations were obtained with a Shimadzu Data Processor Chromatopac C-R3A. The chromatographic column was prepacked with Nova-Pak® C_{18}, 5 μm, 3.9 mm × 15 cm (Millipore/Waters). For elution system see Fig. 1. For quantifications a log ε value of 4.33 was taken [13].

^1H *NMR spectra* (400 MHz) and ^{13}C *NMR spectra* (75 MHz) were recorded on Bruker WM 400 and AM 300 NMR spectrometers, respectively, at ambient temp. and locked to the deuterium resonance of the solvent. Shifts are reported in ppm relative to TMS and couplings in Hz. The 2D ^1H homonuclear shift-correlated (COSY) spectrum was recorded with a 90°-t_1-90°-FID(t_2) pulse sequence. The spectral widths were 2994.0 Hz for F2 and ± 1497.0 Hz for F1 with 1K data points in t_2 and 512 data points in t_1. For each t_1 increment 112 pulses were accumulated with a relaxation delay of 0.5 sec between pulse sequence to give a total accumulation time of 12.9 hr. The data were multiplied by a Sine-Bell function and one level of zero filling was used in both t_1 and t_2. All 1D and 2D spectra were taken with the standard Bruker Aspect 3000 software.

FAB mass spectra were recorded in both the positive and negative modes of operation on a Kratos MS 50 spectrometer with a Kratos FAB source; glycerol was used as matrix.

(E)-*Caffeoyl*-(E)-*feruloylspermidine* (**1**). ^1H NMR (MeOH-d_4): *caffeoyl residue:* δ7.462 (*d*, H-7, $J_{7,8}$ = 15.7 Hz), 7.079 (*d*, H-2, $J_{2,6}$ = 1.9 Hz), 6.946 (*dd*, H-6, $J_{5,6}$ = 8.1 Hz), 6.810 (*d*, H-5), 6.460 (*d*, H-8); *feruloyl residue*: 7.490 (*d*, H-7, $J_{7,8}$ = 15.7 Hz), 7.179 (*d*, H-2, $J_{2,6}$ = 1.9 Hz), 7.069 (*dd*, H-6, $J_{5,6}$ = 8.1 Hz), 6.843 (*d*, H-5), 6.545 (*d*, H-8), 3.929 (*s*, 3-OMe); *spermidine residue*: 3.465 (*t*, H-1, $J_{1,2}$ = 6.8 Hz), 3.409 (*t*, H-7, $J_{6,7}$ = 6.8 Hz), 3.109(*t*, H-4, $J_{4,5}$ = 6.8 Hz), 3.093 (*t*, H-3, $J_{2,3}$ = 7 Hz), 2.009 (*m*, H-2), 1.837 (*m*, H-5, $J_{5,6}$ = 7 Hz), 1.743 (*m*, H-6). In DMSO-d_6 the amidic protons appeared as two triplets of equal intensities at 8.17 and 8.39. ^1H nuclear Overhauser enhancements in the caffeoyl residue were observed for the signals of H-2 and H-6 upon irradiation of H-8, and in the feruloyl residue at H-2 and H-6 upon irradiation of H-8 and at H-5, H-7 and H-8 upon irradiation of H-6. ^{13}C NMR (MeOH-d_4): *caffeoyl and feruloyl residues:* δ142.51, 141.81 (*d* × 2, C-7 × 2), 123.01, 122.08 (*d* × 2, C-8c, C-6f), 119.31, 118.53, 117.62, 116.20, 114.75, 111.30 (*d* × 6, C-2 × 2, C-5 × 2, C-6c, C-8f), 56.22 (*q*, OMe); *spermidine residue*: 48.34 (*t*, C-4), 46.15 (*t*, C-3), 39.25 (*t*, C-7), 36.74 (*t*, C-1), 27.48, 27.24, 24.37 (*t* × 3, C-2, C-5, C-6). FAB-MS: positive ion *m/z*: 484 $[M + H]^+$, negative ion *m/z*: 482 $[M - H]^-$.

Di-(E)-*feruloylspermidine* (**2**). ^1H NMR (MeOH-d_4): *feruloyl residues*: 7.509, 7.483 (*d* × 2, H-7 × 2; $J_{7,8}$) = 16, 15.6 Hz, 7. 208, 7.196 (*d* × 2, H-2 × 2, J_{2-6} = 2.1 × 2 Hz), 7.090 (*dd*, H-6 × 2, $J_{5,6}$ = 8.2 × 2 Hz), 6.870, 6.866 (*d* × 2; H-5 × 2), 6.568, 6.565 (*d* × 2, H-8 × 2), 3.929 (s, 3-OMe × 2); *spermidine residue*: 3.314 (*t*, H-7), 3.112 (*t*, H-4, H-3), 2.034 (*m*, H-2), 1.836 (*m*, H-5), 1.734 (*m*, H-6). The signal for H-1 was hidden under the solvent signal. FAB-MS: Positive ion *m/z*: 498 $[M + H]^+$, negative ion *m/z*: 496 $[M - H]^-$.

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