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DI-COUMAROYLSPERMIDINES AND TRI-COUMAROYLSPERMIDINES IN ANTHERS OF DIFFERENT SPECIES OF THE GENUS *APHELANDRA*

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Key Word Index—*Aphelandra*; Acanthaceae; anthers; di-coumaroylspermidines; tri-coumaroylspermidine.

Abstract—N^1,N^5-Di-*p*-coumaroylspermidine, N^5,N^{10}- di-*p*-coumaroylspermidine, and N^1,N^5,N^{10} tri-*p*-coumaroylspermidine were isolated from anthers of *Aphelandra tetragona* and *A. chamissoniana*. The correct substitution pattern of the di-coumaroylspermidines could be identified by their specific photoisomerization to four isomers which can be separated by HPLC.

INTRODUCTION

Hydroxycinnamic acid (HCA) spermidines have been reported in the reproductive organs from a large number of plant families [1–3]. Mono-, di-, and tri-substituted spermidine amides have been found as derivatives of hydroxycinnamic acids (*p*-coumaric, ferulic, caffeic or sinapic acid). The dihydroxycinnamoylspermidines can be substituted at the position N^1 and N^5, N^5 and N^{10} or N^1 and N^{10} (**1**, **2**, **3**).

During our work on the polyamine alkaloid producing plant *Aphelandra tetragona* (Acanthaceae) we analysed different plant parts for their hydroxycinnamic acid amide content which might be precursors of the polyamine alkaloid aphelandrine. We were able to isolate and identify di-coumaroylspermidines and tri-coumaroylspermidine (**4**) from anthers. The substitution pattern of the di-coumaroylspermidines could be clearly identified due to light induced isomerization. The *EE*, *EZ*/*ZE* and the *ZZ* isomers were separated by HPLC and compared with the synthesized reference substances.

RESULTS AND DISCUSSIONS

Di-p-coumaroylspermidines

Anthers of different *Aphelandra* species were collected during one flowering period and extracted (see the Experimental section). The extracts of *A. tetragona* and *A. chamissoniana* revealed on TLC one UV and Schlittler positive band ($R_f = 0.27$) which co-chromatographed with the synthetic reference samples N^1,N^5-di-[(*E*)-*p*-coumaroyl]-spermidine (**1**), N^5,N^{10}-di-[(*E*)-*p*-coumaroyl]-spermidine (**2**), and N^1,N^{10}-di-[(*E*)-*p*-coumaroyl]-spermidine (**3**). After elution with methanol from the TLC plate this sample was analysed by reversed phase HPLC using a modified two step linear gradient system according to Meurer *et al.* [2]. Six peaks were detected in this system with the R_t 13.7, 12.5, 11.5, 10.4, 9.2, and 8.1. They could be identified as the configuration isomers *EE*, the mixture of *EZ*/*ZE*, and *ZZ* of N^1,N^5-di-*p*-coumaroylspermidine and N^5,N^{10}-di-*p*-coumaroylspermidine by comparing with the synthetic reference samples. After photoisomerization, HPLC elution of the synthetic reference sample N^1,N^5-di-[(*E*)-*p*-coumaroyl]-spermidine changed from one peak (Fig. 1A) to three distinct peaks (Fig. 1B) with characteristic UV-spectra (Fig. 1C). Peak $R_t = 12.3$ is the synthetic product and known to be the *EE*-isomer according to the NMR spectra, peak $R_t = 8.1$ could be identified as *ZZ*-isomer and peak $R_t = 10.3$ should be the mixture of *EZ*/*ZE* isomers. An identical pattern of peaks in the HPLC diagram was obtained with the synthetic N^5,N^{10}-di-*p*-coumaroylspermidine and with N^1,N^{10}-di-*p*-coumaroylspermidine. The retention time of every group differs clearly and can be used for the identification of the naturally-occurring compounds (Table 1). In addition there is a pronounced difference in the shape of the UV-spectrum of the (*ZZ*)-N^1,N^{10}-di-*p*-coumaroylspermidine compared with the spectra of the *ZZ* isomers of the two other di-coumarolyspermidines. According to these results we were able to identify N^1,N^5- and N^5,N^{10}-di-*p*-coumaroylspermidine from anthers of *A. tetragona* and *A. chamissoniana*. In extracts of anthers of *A. fuscopunctata* and *A. squarrosa* the occurrence of the coumaroylspermidines could be shown on TLC. Further determination was not possible in these cases due to the very small amount of substances available.

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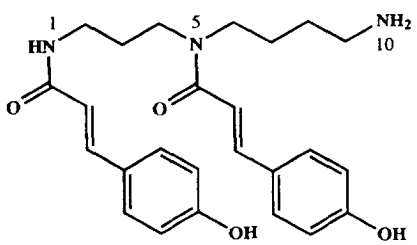

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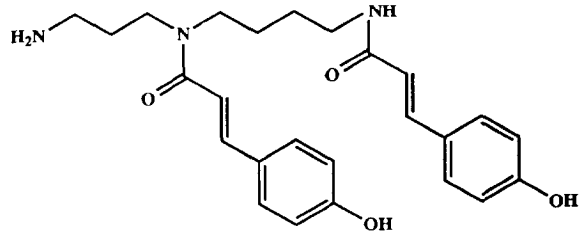

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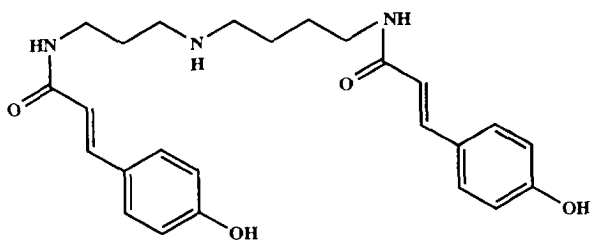

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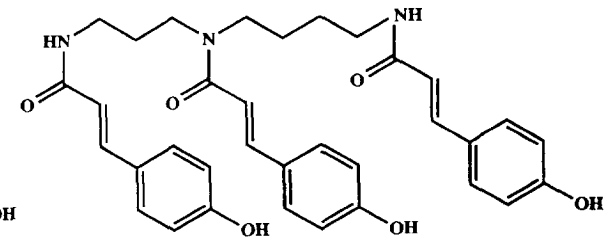

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Di-*p*-coumaroylspermidines have been isolated from pollen of Arales, Fagales, Juglandales and Myricales [1, 2]. The identification of these compounds was done by ^{1}H NMR and mass spectral data [4]. The authors report that the chromatographic separation of the differentially substituted di-coumaroylspermidines could not be achieved.

As the collection of pollens from *Aphelandra* plants is extremely difficult and the availability of flowers is limited we were forced to develop a method for the identification of very small amounts of products. With the help of the synthesized reference substances we were able to show the occurrence of di-coumaroylspermidines in the anthers of *Aphelandra* flowers. Their correct substitution pattern could be identified after photoisomerization and separation of the isomers by HPLC. Additional proof was the molecular peak of 438 $[M + 1]^+$ obtained by ESI-MS.

Tri-p-*coumaroylspermidine*

Different UV absorbing bands separated on TLC of the extracts of anthers of *A. tetragona* and *A. chamissoniana* were analysed by HPLC. An additional group of peaks with the typical UV spectra of coumaroyl derivatives was detected in the band with R_f 0.73. After purification from TLC the molecular peak of 583 $[M + 1]^+$ and 606 $[M + Na]^+$ obtained by ESI-MS indicated a tri-coumaroylspermidine. By co-chromatographing with the synthetic reference sample the identification of tri-coumaroylspermidine in the anthers of the *Aphelandra* species could be achieved (Table 2).

During our research on plants producing macrocyclic polyamine alkaloids [5, 6] we have not been able to isolate cinnamoyl- or hydroxy-cinnamoylpolyamines, possible precursors in the biosynthesis of these alkaloids, from any plant part up to now. The identification of the di-coumaroylspermidines and tri-coumaroylspermidine in the anthers of two *Aphelandra* species is the first indication of such substances occurring in these plants. To the best of our knowledge this is the first report about coumaroylspermidines in anthers of Acanthaceae.

EXPERIMENTAL

Plant material. Aphelandra tetragona (Vahl) Nees, *A. chamissoniana* Nees, *A. fuscopunctata* Markgr. and *A. squarrosa* Nees were cultivated in the greenhouse of our institute. During the flowering period the anthers were cut and stored at − 20°. The main work was carried out with anthers of *A. tetragona* and *A. chamissoniana.*

Extraction. The anthers were extracted ×3 with MeOH–H_2O (1:1) by stirring for 2–3 hr. After centrifugation the supernatants were combined, concd *in vacuo* and loaded onto a weakly acidic cation exchange column (Amberlite CG 50). Uncharged molecules were removed with water and the bound amides were eluted with 8 M HOAc–MeOH (1:1, v/v) according to ref. [7]. The eluant was evaporated *in vacuo*, dissolved in MeOH–H_2O (1:1) and chromatographed on TLC (Kieselgel F_{254}). The best solvent system for separating HCA amides from other substances is $CHCl_3$–MeOH–HOAc (14:4:1). The UV

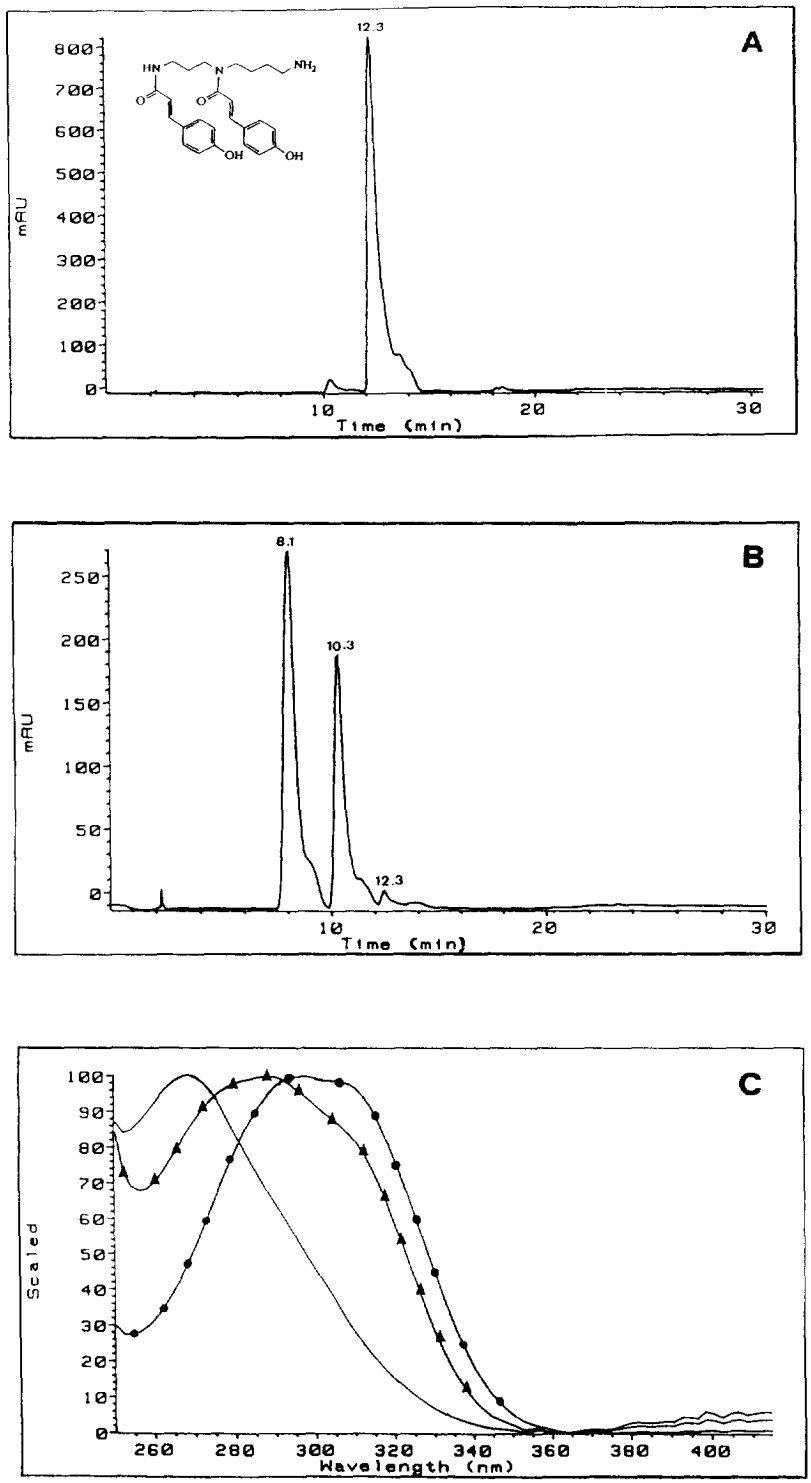

Fig. 1. Diagrams from HPLC analyses of N^1,N^5-di-*p*-coumaroylspermidine. (A) N^1,N^5-Di-[(*E*)-*p*-coumaroyl]-spermidine (**1**) obtained by chemical synthesis (R_t: 12.3). (B) N^1,N^5-Di-[(*E*)-*p*-coumaroyl]-spermidine (**1**) gave three peaks after irradiation with UV light (R_t: 8.1 = *ZZ*; R_t: 10.3 = *EZ*/*ZE*; and R_t: 12.3 = *EE* configurated isomer). (C) UV spectra from the *EE*, *EZ*/*ZE* and *ZZ* configured N^1,N^5-di-[(*E*)-*p*-coumaroyl]-spermidine. (●—●—●) *EE*, (▲—▲—▲) *EZ*/*ZE*, (——) *ZZ*.

Table 1. Retention times (min) of the di-[(*E*)-*p*-coumaroyl]-spermidines (**1**–**3**) and their isomers after irradiation at 366 nm, separated by reversed phase HPLC (for details see Experimental)

Substance	Retention time		
	EE	*EZ/ZE*	*ZZ*
N^1,N^5-Di-coumaroylspermidine (**1**)*	12.3	10.3	8.1
N^5,N^{10}-Di-coumaroylspermidine (**2**)*	13.7	11.5	9.2
N^1,N^{10}-Di-coumaroylspermidine (**3**)*	13.8	11.0	8.6
N^1,N^5-Di-coumaroylspermidine (from anthers of *Aphelandra*)	12.5	10.4	8.1
N^5,N^{10}-Di-coumaroylspermidine (from anthers of *Aphelandra*)	13.7	11.5	9.2

*Synthetic reference sample.

Table 2. Retention times (min) of N^1,N^5,N^{10}-tri-[(*E*)-*p*-coumaroyl]-spermidine (**4**) and its isomers after irradiation at 366 nm, separated by reversed phase HPLC (for details see Experimental)

Substance	Retention time			
	EEE	M1*	M2*	*ZZZ*
N^1,N^5,N^{10}-Tri-coumaroylspermidine (**4**)†	22.3	21.8	20.7	19.7
N^1,N^5,N^{10}-Tri-coumaroylspermidine (from anthers of *Aphelandra*)	22.5	21.7	20.9	19.9

*M1, Mixure 1 (supposed isomers: *ZZE*, *ZEZ*, *EZZ*); M2, Mixture 2 (supposed isomers: *EEZ*, *EZE*, *ZEE*).

†Synthetic reference sample.

absorbing bands were scraped from the plate and eluted with MeOH. After evaporation to dryness *in vacuo* they were taken up in a small amount of MeOH–H_2O (1:1) and analysed by HPLC equipped with a DAD detector.

Identification of the coumaroylspermidines. The samples were analysed by HPLC on Nucleosil C-8 column (5 μm, 200 × 4 mm i.d.) at a flow rate of 1 ml min^{-1} with a two step linear gradient: in 15 min from 30% 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) to 60% solvent B in A and subsequently in 5 min to 100% solvent B (modified after ref. [2]).

The UV and Schlittler active band at R_f 0.27, and a second UV absorbing band at R_f 0.73 from TLC showed the expected absorption spectra corresponding to the HCA amides. They were co-chromatographed with the synthesized reference compounds as well as analysed by ESI-MS.

Reference substances. The reference substances N^1, N^5-di-[(*E*)-*p*-coumaroyl]-spermidine, N^5,N^{10}-di-[(*E*)-*p*-coumaroyl]-spermidine, N^1,N^{10}-di-[(*E*)-*p*-coumaroyl]-spermidine and N^1,N^5,N^{10}-tri-[(*E*)-*p*-coumaroyl]-spermidine were synthesized in our laboratory [8].

N^1,N^5-*Di*-[(E)-p-*coumaroyl*]-*spermidine* (**1**). IR ν_{max} cm^{-1} (KBr): 1636*s*, 1600*s*, 1580*s*, 1510*s*. ESI-MS: 438 $[M + 1]^+$. ^{1}H NMR (300 MHz: in CD_3OD 5H are exchanged against 5D): 7.59–7.40 (*m*, 6H); 6.91–6.72 (*m*, 5H); 6.47 (*d*, J = 15.7, 1 olef. H); 3.63–3.51 (*m*, 4H); 3.40–3.29 (*m*, 2H); 2.98 (*m*, 2H); 2.00 (*m*, 2H); 1.72 (*m*, 4H). ^{13}C NMR (CD_3OD, mixture of conformers: some carbon atoms give more than one signal): 169.81 (*s*); 169.68 (*s*); 161.10 (*s*); 161.02; 145.67 (*d*); 143.47 (*d*); 131.46 (*d*); 131.13 (*d*); 130.86 (*d*); 127.43 (*s*); 127.18 (*s*); 116.84 (*d*); 116.50 (*d*); 116.31 (*d*); 113.66 (*d*); 47.74 (*t*); 47.28 (*t*); 40.39 (*t*); 38.28 (*t*); 29.90 (*t*); 28.06 (*t*); 25.76 (*t*).

N^5,N^{10}-*Di*-[(E)-p-*coumaroyl*]-*spermidine* (**2**). IR ν_{max} cm^{-1} (KBr): 1635*s*, 1600*s*, 1580*s*, 1510*s*. ESI-MS: 438 $[M + 1]^+$. ^{1}H NMR (300 MHz: in CD_3OD 5H are exchanged against 5D): 7.57 (*d*, J = 15.7, 1 olef. H); 7.51–7.36 (*m*, 5H); 6.89–6.76 (*m*, 5H); 6.40 (*d*, J = 15.7, 1 olef. H); 3.64–3.56 (*m*, 4H); 3.37 (*t*, J = 6.3, 2H); 2.92 (*t*, J = 6.7, H_2C (2)); 2.01–1.94 (*m*, H_2C (3)); 1.82–1.57 (*m*, H_2C (7) and H_2C (8)). ^{13}C NMR (CD_3OD, mixture of conformers: some carbon atoms give more than one signal): 173.20 (*s*); 170.43 (*s*); 161.62 (*s*); 161.20 (*s*); 147.72 (*d*); 146.28 (*d*); 131.64 (*d*); 126.93 (*s*); 126.36 (*s*); 116.92 (*d*), 113.66 (*d*); 45.26 (*t*); 41.73 (*t*); 38.08 (*t*); 27.78 (*t*); 27.37 (*t*); 26.62 (*t*); 25.84 (*t*).

N^1,N^{10}-*Di*-[(E)-p-*coumaroyl*]-*spermidine* (**3**). IR ν_{max} cm^{-1} (KBr): 1650*s*, 1600*s*, 1580*s*, 1510*s*. ESI-MS: 438

$[M + 1]^+$. ^1H NMR (300 MHz: in CD_3OD 5H are exchanged against 5D): 7.50 (*d*, $J = 15.7$, 1 olef. H); 7.47 (*d*, $J = 15.7$, 1 olef. H); 7.41 (*d*, $J = 8.5$, 4 arom. H); 6.79 (*d*, $J = 8.5$, 4 arom. H); 6.44 (*d*, $J = 15.7$m, 1 olef. H); 6.43 (*d*, $J = 15.7$, 1 olef. H); 3.44–3.34 (*m*, $H_2C(2)$ and $H_2C(9)$); 3.06–3.01 (*m*, $H_2C(4)$ and $H_2C(6)$); 1.97–1.92 (*m*, $H_2C(3)$); 1.78–1.68 (*m*, $H_2C(7)$ and $H_2C(8)$). ^{13}C NMR (CD_3OD, mixture of conformers: some carbon atoms give more than one signal): 170.21 (*s*); 161.27 (*s*); 160.85 (*s*); 143.66 (*d*); 143.64 (*d*); 131.28 (*d*); 131.01 (*d*); 126.94 (*s*); 126.69 (*s*); 116.92 (*d*); 116.84 (*d*); 116.12 (*d*); 114.89 (*d*); 46.34 (*t*); 40.80 (*t*); 37.86 (*t*); 37.78 (*t*); 27.12 (*t*); 26.78 (*t*); 24.46 (*t*).

N^1,N^5,N^{10}-*Tri*-[(E)-p-*coumaroyl*]-*spermidine* (**4**). IR ν_{max} cm^{-1} (KBr): 1652*s*, 1645*s*, 1600*s*, 1580*s*, 1510*s*. ESI-MS: 623 $[M + K]^+$, 606 $[M + Na]^+$, 584 $[M + 1]^+$. ^1H NMR (300 MHz: in CD_3OD 5H are exchanged against 5D): 7.57–7.34 (*m*, 9H); 6.89–6.71 (*m*, 7H); 6.45–6.35 (*m*, 2H); 3.57–3.24 (*m*, 8H); 1.95–1.56 (*m*, 6H). ^{13}C NMR (CD_3OD, mixture of conformers: some carbon atoms give more than one signal): 169.27 (*s*); 169.11 (*s*); 168.92 (*s*); 160.46 (*s*); 160.38 (*s*); 160.29 (*s*); 144.33 (*d*); 142.08 (*d*); 141.81 (*d*); 130.88 (*d*); 130.57 (*d*); 127.73 (*s*); 127.50 (*s*); 118.37 (*d*); 118.15 (*d*); 116.73 (*d*); 116.35 (*d*); 114.77 (*d*); 46.92 (*t*); 40.04 (*t*); 38.04 (*t*); 30.54 (*t*); 28.76 (*t*); 27.83 (*t*); 26.28 (*t*).

Photoisomerization of the di-coumaroylspermidines. 2 ml of a 3.0×10^{-3} M methanolic soln of N^1,N^5-, N^5, N^{10}- or N^1,N^{10}-di-[(*E*)-*p*-coumaroyl]-spermidine was introduced into a quartz vessel. The soln was deoxygenated by flushing with Ar for 20 min. The irradiation was carried out with a low-pressure mercury lamp at 366 nm for 50 min. The analysis of 15 μl of this soln by HPLC indicated a mixture of *EE*, *EZ*/*ZE* and *ZZ* isomers.

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