On-Line Coupling of High-Performance Liquid Chromatography to Atmospheric Pressure Chemical Ionization Mass Spectrometry (HPLC/APCI-MS and MS/MS). The Pollen Analysis of *Hippeastrum* x hortorum (Amaryllidaceae)[1])

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In memory of Prof. Dr. *Wilhelm J. Richter*

A H_2O/MeOH extract of the pollen of *Hippeastrum* x hortorum (Amaryllidaceae) was analyzed. A mixture of different compounds (at the most 84) was found, namely the geometrically ((*E,E*), (*E,Z*), (*Z,E*), and (*Z,Z*) and structurally isomeric *N,N′*-dicoumaroyl (= *N,N′*-bis[3-(4-hydroxyphenyl)prop-2-enoyl]), *N,N′*-diferuloyl (= *N,N′*-bis[3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]), *N,N′*-disinapoyl (= *N,N′*-bis[3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoyl]), *N*-coumaroyl-*N′*-feruloyl, and *N*-feruloyl-*N′*-sinapoyl derivatives of spermidine (= 4-azaoctane-1,8-diamine = *N*-(3-aminopropyl)butane-1,4-diamine). Their structures were proven by using on-line-coupled high-performance liquid chromatography and atmospheric-pressure chemical-ionization mass spectrometry (HPLC-UV(DAD)/APCI-MS and MS/MS), UV-induced (*E*)⇌(*Z*) photoisomerization, and catalytic hydrogenation, as well by comparing their spectra and chromatographic behavior with those of synthetic standards. According to the physicochemical properties of these natural compounds, a proposed biological function is discussed.

Introduction. – Low-molecular-mass phenolic compounds occur in the primary and the secondary plant-cell wall. They are often linked to cell-wall polymers [1]. Common phenolic compounds are derivatives of 4-hydroxycinnamic acids (= 3-(4-hydroxyphenyl)prop-2-enoic acid; HC), such as coumaric acid[3]) (= 3-(4-hydroxyphenyl)prop-2-enoic acid), ferulic acid[3]) (= 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid), and sinapic acid[3]) (= 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid). Di- and polyamines can be linked to HC by an amide bond, forming the so-called hydroxycinnamamides (HCA) [2–4]. Mono-, di-, and trisubstituted hydroxycinnamamides (HCA) of spermidine (= 4-azaoctane-1,8-diamine = *N*-(3-aminopropyl)butane-1,4-diamine) have been isolated, and their structures have been determined from different organs of several species of many plant families [4][5]. More detailed studies were performed on pollen of plants from the families of Betulaceae, Fagaceae, Juglandaceae [3][6–9], and Acanthaceae [10]. The anthers may contain one type of HC derivatives, as described for *Aphelandra* where di- and tricoumaroyl-spermidines were isolated [10]. The pollen may also contain a complex mixture of compounds containing two or even three different HC rests, as reported for the pollen of *Quercus dentata* (Fagaceae) [8][9].

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[1]) Presented at the '31. Diskussionstagung der Arbeitsgemeinschaft Massenspektrometrie der Deutschen Physikalischen Gesellschaft', Cottbus, Germany, 5 June, 1998.

[2]) Part of the Ph. D. Thesis of *N.Y.*, University of Zürich.

[3]) Acronyme used in the phytochemical literature and defined for the (*E*)- or the (*Z*)-isomers.

During our studies on polyamine derivatives in higher plants, we detected relatively high amounts of their amidic conjugates in the extract from pollen of *Hippeastrum* sp. (Amaryllidaceae). Their diversity seems to be much higher than in *Aphelandra* species [10]. The genus *Hippeastrum* (Amaryllidaceae) is mainly distributed in Central and South America. There are about 60 known species. The *Hippeastrum* hybrids are widespread as indoor plants. The genus has been investigated for its alkaloidal and flavonoidal metabolites [11][12].

The isolation and structure analysis of hydroxycinnamic-acid derivatives, especially their amidic conjugates with polyamines, are quite complicated because of the occurrence of (*E*/*Z*) and/or structural isomerism. Thus, the number of theoretically possible geometrical and structural isomers of *N,N′*-dicoumaroylspermidines is 12, and in case of *N*-coumaroyl-*N′*-feruloylspermidines (with two different HC substituents), it is 24. The analysis and separation of such mixtures is difficult because these isomeric compounds show similar chromatographic and spectroscopic properties. The HCA are very sensitive to light exposure. In the case of the *N,N′*-dicoumaroylspermidines from *Aphelandra tetragona*, the UV-light-induced isomerization of the C=C bonds leads to the complicated mixture of (*E,E*)-, (*E,Z*)-, (*Z,E*)-, and (*Z,Z*)-isomers [10][13]. The conventional analytical methods like NMR spectroscopy need labor-intensive and time-consuming preparative isolation in order to obtain pure samples in milligram amounts. Additionally, NMR measurements give complicated and only partially useful spectra for this substance class. On the other hand, there are the difficulties of the limited quantities of pollen which can be collected. The rapid analysis and sensitive detection of natural products play an important role in phytochemical investigations. HPLC with UV/VIS diode array detection (DAD)[4]) is a powerful tool for quick analysis of complex mixtures and natural extracts. However, this method is inappropriate in the case of peak-overlap (substance co-elution) and/or when the substances do not contain a chromophore. These shortcomings of HPLC-UV(DAD) can be compensated for by using mass spectrometry. Soft ionization techniques, such as electrospray ionization (ESI) and atmospheric-pressure chemical-ionization (APCI) yield quasi-molecular ions with low fragmentation. This allows co-eluted substances with different mass-to-charge ratios (*m/z*) to be differentiated *via* their extracted-ion chromatogram. Selected quasi-molecular ions, after collision-induced dissociation (CID), yield daughter fragment ions, permitting deeper structural investigations. With the on-line coupling of HPLC to a mass spectrometer, it is possible to obtain the molecular mass as well as valuable structural information (HPLC/MS/MS) on the metabolites without isolation. Other strengths of mass spectrometry are its high sensitivity and selectivity. These are additional reasons for using mass spectrometry as a detector for separation techniques. With such an approach, the time-consuming isolation of natural products can be avoided.

In this work, we report a highly sensitive method for the separation and identification of differently substituted hydroxycinnamamides of spermidine obtained from the pollen of *Hippeastrum* x hortorum (Amaryllidaceae) by using HPLC-UV(DAD)/APCI-MS and HPLC/APCI-MS/MS.

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[4]) The scan of the UV(DAD) covers the region between 200 and 600 nm, *i.e.*, the ultraviolet (UV) and visible (VIS) parts of the light spectrum.

Results and Discussion. – *Isolation and Group Separation*. Our interest and investigations focus on hydroxycinnamamide derivatives of polyamines. In this work, we used an extraction and isolation procedure published some years ago [10]. The pollen was extracted with MeOH/H_2O. After solid-phase extraction on a weakly acidic ion-exchange resin (*Amberlite CG 50*), the residue was separated by thin-layer chromatography (TLC) to get three fractions. This simple preparation was necessary for further investigations, because the chromatographic behavior of the compounds in these fractions was quite different. *Fraction I* (R_f 0.17, 2.7 mg) was recovered and analyzed by HPLC-UV(DAD), HPLC-UV(DAD)/APCI-MS, and HPLC/APCI-MS/MS (*Fractions II* and *III* are currently under investigation).

HPLC Analysis of Fraction I. The best results from the HPLC-UV(DAD) analysis were obtained on a C_8 column with a mobile phase H_2O/MeCN containing 1% of AcOH (see *Exper. Part*). But even under these conditions, it was not possible to get well-separated UV signals (see *Fig. 1,g*). The chromatographic separation of the components in a classical sense failed. Hoping to determine the real number of compounds hidden under the peaks, we decided to analyze the mixture by a HPLC/MS on-line technique which is outlined in the *Scheme*.

HPLC-UV(DAD)/APCI-MS. A preliminary electrospray-ionization mass spectrometry (ESI-MS) experiment on *Fraction I* gave five quasi-molecular ions $[M+H]^+$ at *m/z* 438, 468, 498, 528, and 558. To get the molecular masses of the compounds corresponding to the UV chromatographic peaks (*Fig. 1,g*), the HPLC-UV(DAD) was on-line coupled with an APCI mass spectrometer. The experiments were performed in full-scan mode (FS). The reconstructed ion current (RIC) of the whole chromatogram is shown in *Fig. 1,f*. The two chromatograms detected by two different methods, HPLC-UV (280 nm) given in *Fig. 1,g*, and HPLC/APCI-MS (RIC) given in *Fig. 1,f*, are very similar to each other. For visualization of the five different quasi-molecular ions mentioned above, five extracted-ion chromatograms are presented in *Fig. 1,a–e* (for quasi-molecular ions of *m/z* 438, 468, 498, 528, and 558, resp.). They show quite a number of different chromatographic peaks, demonstrating that each quasi-molecular ion belongs to at least three (*m/z* 558), four (*m/z* 498), five (*m/z* 438 and *m/z* 528), and six (*m/z* 468) different types of molecules (isomers). Each signal in the chromatograms is denoted by a letter and a number, *e.g.*, **A1**–**A5** in *Fig. 1,a* ($[M+H]^+$ 438). To ensure that no important signals were lost by data analysis, the RIC was compared with the addition of the extracted-ion chromatograms *m/z* 438, 468, 498, 528, and 558 with normalized intensities (*Fig. 2*). Both are of very similar shape.

The analysis shows that *Fraction I* is a complicated mixture of at least 23 substances. The most intense signals are **C3** and **C4**, and the less intense ones are **E1**, **E2**, and **E3**. The peaks of the **E** group (**E1**–**E3**) are on their detection limits. These facts explain the obstacles hindering the interpretation of the HPLC-UV.

HPLC/APCI-MS/MS. The MS/MS techniques deliver valuable structural information, and they are very important for substance identification. The selectivity of triple-stage quadrupole mass spectrometers enables one to obtain the CID-MS not only of pure samples, but also to get independent CID-MS of two or more co-eluted substances in one HPLC run (in case their ionized molecules have different mass-to-charge ratios). Low-energy CID with Ar as the collision gas generally yields abundant fragment ions by non-radical reactions [14][15]. To obtain product-ion spectra, the first

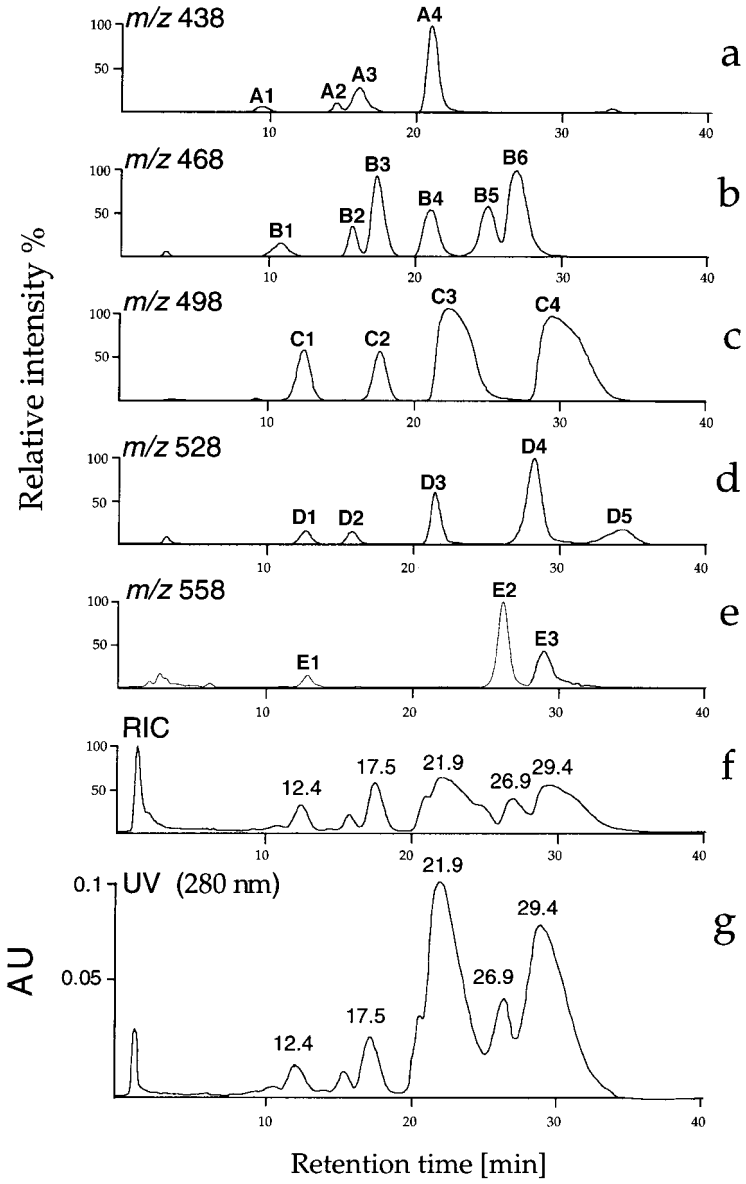
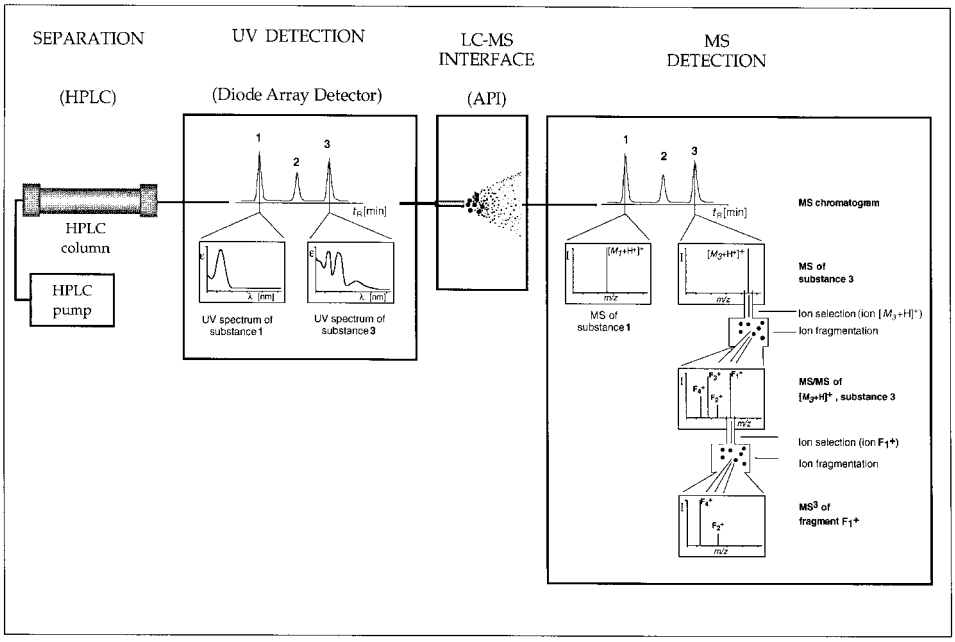


Fig. 1. *Summary of the HPLC-UV(DAD)/APCI-MS experiment on* Fraction I: g) *UV chromatogram detected at 280 nm*; f) *RIC chromatogram*; a)–e) *extracted-ion chromatograms of quasi-molecular ions* m/z *438, 468, 498, 528, and 558*. HPLC and MS conditions: see *Exper. Part*; AU = absorption units.

quadrupole is fixed to the *m/z* value of the selected quasi-molecular ion, and the third quadrupole is scanned over a particular *m/z* range. To get a higher sensitivity, the experiment can be performed in 'selected-reaction monitoring' (SRM) mode where specific CID reactions are detected.

Scheme. *General Overview of the Used HPLC-UV(DAD)/MS System*



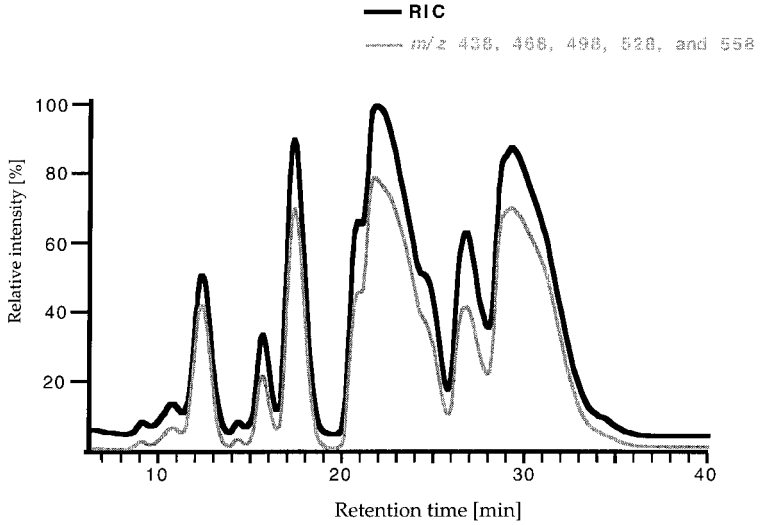


Fig. 2. *Comparison of the chromatogram obtained by addition of traces of* m/z *438, 468, 498, 528, and 558 and the RIC chromatogram*. The difference in the intensities of the curves is explained in terms of the negligence of the isotope peaks of the quasi-molecular ions.

HPLC/APCI-MS/MS experiments were performed on the five different $[M+H]^+$ ions present in *Fraction I* (*Fig. 3,a–d*). In all cases, the same HPLC and MS conditions were used. The interval between *m/z* 130 and 360 was taken as a fingerprint interval.

The analyzed substances can be identified by comparing the spectra with those of reference compounds of known fragmentation or by interpretation of the obtained spectra. In each of these three-dimensional graphics, the retention times in [min] of the RIC chromatogram are plotted on the *x*-axis, the MS/MS data (*m/z*) on the *z*-axis, and the relative intensity of mass spectra (above) and RIC (below) on the *y*-axis. An MS/MS is assigned to each chromatographic peak.

Signals of the **A** *Group* (**A1** – **A5**): $[M+H]^+$ *at* m/z *438*. The MS/MS of the group-**A** compounds were very similar, showing small relative-intensity differences (10 – 20%). The observed fragment-ion peaks are *m/z* 147, 204, 218, 221, 275, and 292 (see *Fig. 3,a*). After analysis of the obtained CID-MS and comparison with those of our established CID-MS library, *N,N′*-dicoumaroylspermidines **1**, **8**, and **15** were suggested as possible structures (see *Table 1*)[5]). The CID-MS of these compounds have been investigated in detail, and the mechanisms leading to the fragment ions have been published [16].

The three possible structural isomers **1**, **8**, and **15** gave the same product-ion spectra. They could not be differentiated by MS/MS techniques, since their fragmetation patterns are nearly the same. This has been explained by the occurrence of transamidation (the 'Zip' reaction), which takes place under mass-spectrometric conditions [16][17]. This reaction can be used to explain the fragment ions from the CID-MS in *Fig. 3,a*, and the results are presented in *Table 2*. The signal at *m/z* 147 arises after cleavage of the amide bond to give structure **a′**. The signal at *m/z* 204 can be assigned to the resonance-stabilized six-membered ring structure **b′**, and the signal at *m/z* 218 to the five-membered ring structure **c′**. The loss of one 3-(4-hydroxyphenyl)-prop-2-enoyl residue from $[M+H]^+$ ions could lead to the structures **f′** (*m/z* 292). The *m/z* 275 ions (**e′**) could be explained by loss of NH_3 from structures **f′** or by a neutral-loss of 163 u from $[M+H]^+$. This fragmentation pattern corresponds to two HC

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[5]) Systematic names of the compounds shown in *Table 1* (see also *Exper. Part*): **1**, *N*-(4-aminobutyl)-3,3-bis(4-hydroxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **2**, *N*-(4-aminobutyl)-3-(4-hydroxyphenyl)-3′-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **3**, *N*-(4-aminobutyl)-3-(4-hydroxy-3-methoxyphenyl)-3′-(4-hydroxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **4**, *N*-(4-aminobutyl)-3,3′-bis(4-hydroxy-3-methoxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **5**, *N*-(4-aminobutyl)-3′-(4-hydroxy-3,5-dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **6**, *N*-(4-aminobutyl)-3-(4-hydroxy-3,5-dimethoxyphenyl)-3′-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **7**, *N*-(4-aminobutyl)-3,3′-bis(4-hydroxy-3,5-dimethoxyphenyl)-*N,N′*-(propane-1,3-diyl)bis[prop-2-enamide]; **8**, *N*-(3-aminopropyl)-3,3′-bis(4-hydroxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **9**, *N*-(3-aminopropyl)-3-(4-hydroxy-3-methoxyphenyl)-3′-(4-hydroxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **10**, *N*-(3-aminopropyl)-3′-(4-hydroxy-3-methoxyphenyl)-3-(4-hydroxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **11**, *N*-(3-aminopropyl)-3,3′-bis(4-hydroxy-3-methoxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **12**, *N*-(3-aminopropyl)-3-(4-hydroxy-3,5-dimethoxyphenyl)-3′-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **13**, *N*-(3-aminopropyl)-3′-(4-hydroxy-3,5-dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **14**, *N*-(3-aminopropyl)-3,3′-bis(4-hydroxy-3,5-dimethoxyphenyl)-*N,N′*-(butane-1,4-diyl)bis[prop-2-enamide]; **15**, 3,3′-bis(4-hydroxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide]; **16**, 3-(4-hydroxy-3-methoxyphenyl)-3′-(4-hydroxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide]; **17**, 3′-(4-hydroxy-3-methoxyphenyl)-3-(4-hydroxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide]; **18**, 3,3′-bis(4-hydroxy-3-methoxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide]; **19**, 3-(4-hydroxy-3,5-dimethoxyphenyl)-3′-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide]; **20**, 3′-(4-hydroxy-3,5-dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide]; **21**, 3,3′-bis(4-hydroxy-3,5-dimethoxyphenyl)-*N,N′*-(4-azaoctane-1,8-diyl)bis[prop-2-enamide].

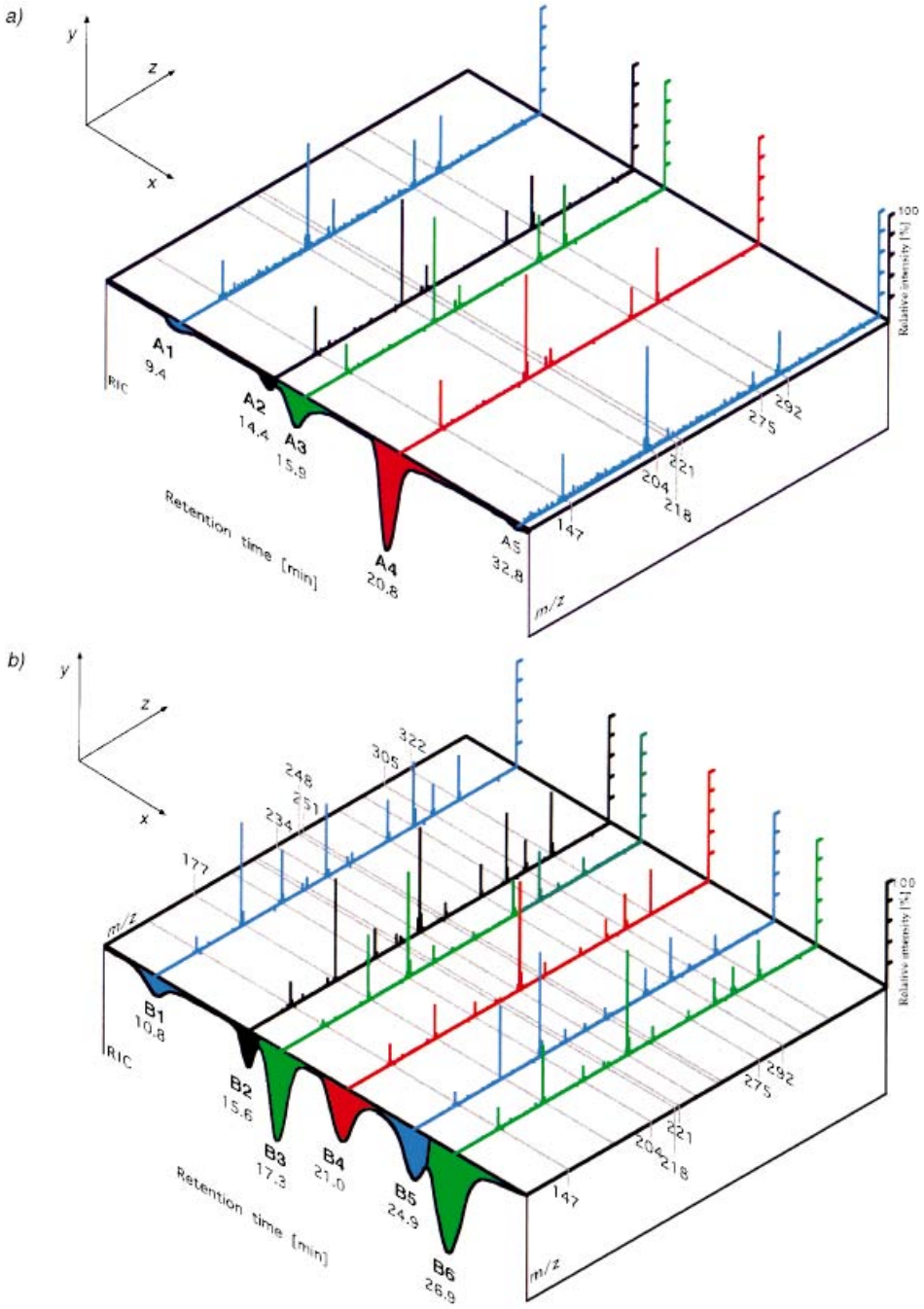


Fig. 3. *HPLC-APCI-MS/MS Experiment of* Fraction I *on parent ions* a) m/z *438*, b) m/z *468*, c) m/z *498*, *and* d) m/z *528*. *x*-Axis, t_R [min] of RIC chromatogram; *z*-axis, *m/z* of MS/MS signals, *y*-axis, relative intensity (above) and RIC (below). HPLC and MS conditions: see *Exper. Part.*

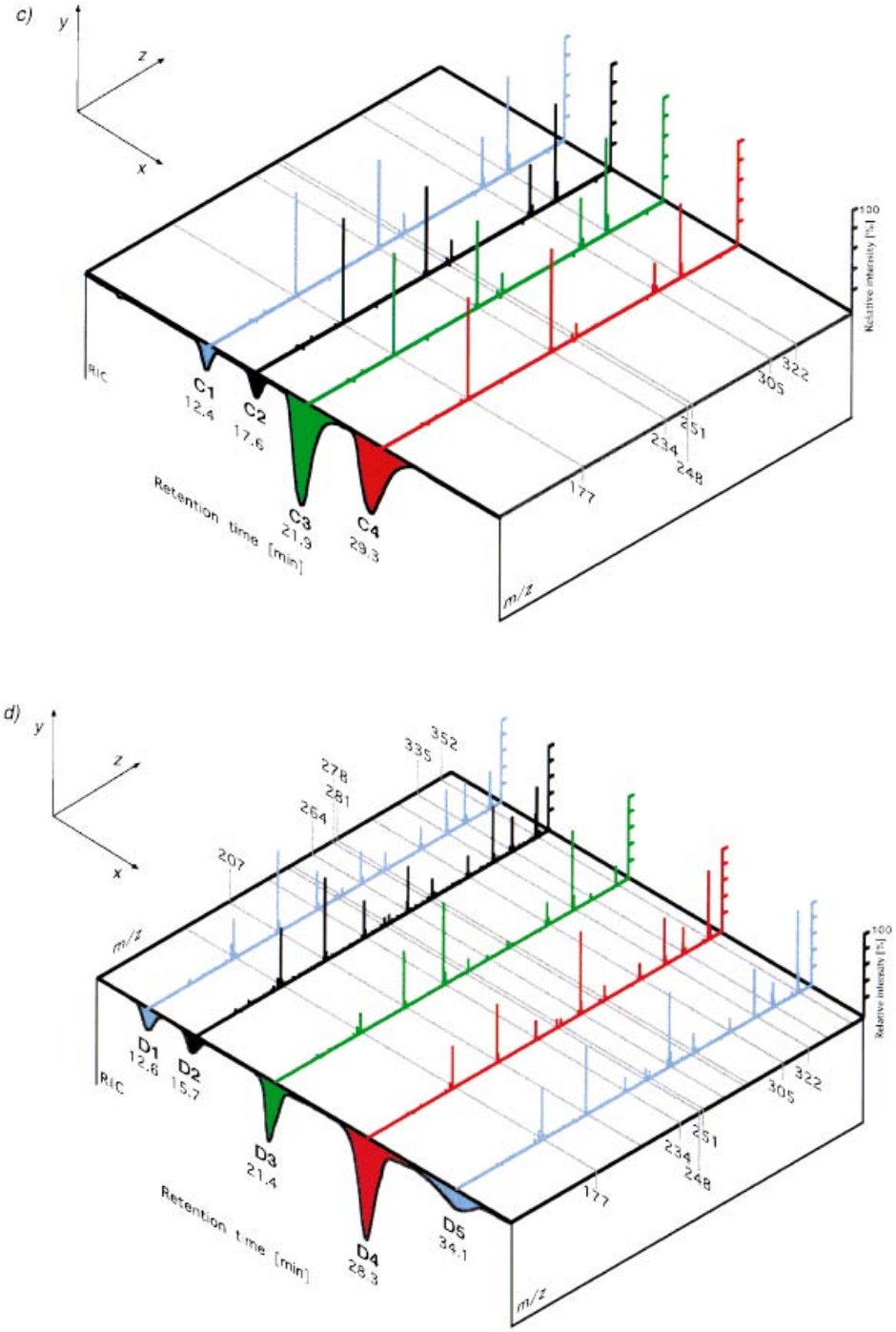
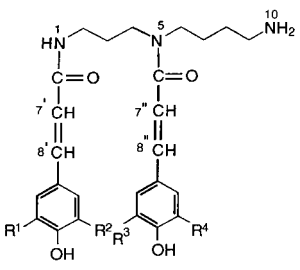
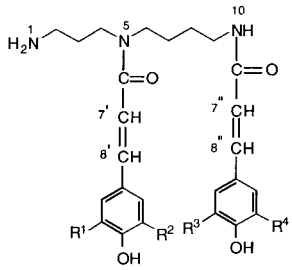

c)
y
z
x
RIC
C1
12.4
C2
17.6
C3
21.3
C4
29.3
Retention time [min]
177
234
248
251
305
322
m/z
100
Relative intensity [%]
d)
y
z
x
207
264
278
281
335
352
m/z
RIC
D1
12.8
D2
15.7
D3
21.4
D4
28.3
D5
34.1
Retention time [min]
177
234
248
251
305
322
m/z
100
Relative intensity [%]

Fig. 3 (cont.)

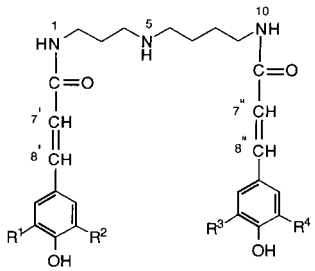
Table 1. *Presumed Disubstituted HCA of Spermidine, Quasi-molecular Ions* $[M + H]^+$, *and Possible Configurations*



1-7 a)



8-14 a)



15-21 a)

Group	Compound	R^1	R^2	C(7′)=C(8′)	R^3	R^4	(C(7″)=C(8″)	$[M + H]^+$
A	**1**, **8**, **15**	H	H	(*E*) or (*Z*)	H	H	(*E*) or (*Z*)	438
B	**2**, **9**, **16**	MeO	H	(*E*) or (*Z*)	H	H	(*E*) or (*Z*)	468
B	**3**, **10**, **17**	H	H	(*E*) or (*Z*)	MeO	H	(*E*) or (*Z*)	468
C	**4**, **11**, **18**	MeO	H	(*E*) or (*Z*)	MeO	H	(*E*) or (*Z*)	498
D	**5**, **12**, **19**	MeO	MeO	(*E*) or (*Z*)	MeO	H	(*E*) or (*Z*)	528
D	**6**, **13**, **20**	MeO	H	(*E*) or (*Z*)	MeO	MeO	(*E*) or (*Z*)	528
E	**7**, **14**, **21**	MeO	MeO	(*E*) or (*Z*)	MeO	MeO	(*E*) or (*Z*)	558

a) Arbitrary numbering; for systematic names, see *Footnote 5* and *Exper. Part.*

residues, connected *via* an amide bond to the spermidine backbone (substances **1**, **8**, and **15**) [16]. To confirm this supposition, the retention times of the observed HPLC peaks were compared with those of the synthetic standards. The synthesis of the (*E,E*)-isomers of *N,N′*-dicoumaroylspermidines **1**, **8**, and **15** and their spectroscopic data have been published [18]. Under the same chromatographic conditions, the synthetic standards of the (*E,E*)-isomers of **1** and **15** show retention times of 20.8 and 20.4 min, respectively (*Table 3*). The mixture of these two structural isomers is inseparable under our chromatographic conditions. The peak **A4** (*Fig. 1,a*) could be identified as the (*E,E*)-isomer(s) of **1** or **15** or both. The (*E,E*)-isomer of the synthetic compound **8** shows a retention time of 32.8 min, which corresponds to the signal **A5** observed in *Fig. 1,a* and *3,a*.

It is known that all HC conjugates are sensitive to UV light. An equilibrium mixture is formed by (*E*)⇌(*Z*) isomerization [13]. In the case of *N,N′*-dicoumaroylspermidines, four geometrically isomers, *i.e.*, (*E,E*), (*E,Z*), (*Z,E*), and (*Z,Z*), are possible (see *Table 1*). These photoisomerization properties were observed earlier [13]. The UV spectra of the (*E,E*)-, (*E,Z*)/(*Z,E*)-, and (*Z,Z*)-isomers are different and could be used for the determination of their configuration.

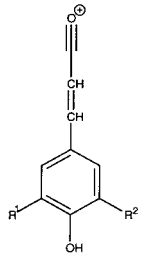
After UV photoisomerization (see *Exper. Part*) of the synthetic standards **1**, **8**, and **15**, which all have the (*E,E*)-configuration, mixtures of all possible geometrical isomers ((*E,E*), (*E,Z*), (*Z,E*), and (*Z,Z*)) were obtained. These mixtures were analyzed by HPLC-UV(DAD) under the same conditions as used for *Fraction I*. In all three cases, two additional peaks assigned to the (*Z,Z*)- and (*E,Z*)-/(*Z,E*)-isomers were obtained (see *Table 3*). Under these chromatographic conditions, the (*E,Z*)- and (*Z,E*)-isomers of **1**, as well as those of **8** and **15**, respectively, were not separable and gave only one

Table 2. *Summary of HPLC/APCI-MS/MS Experiments: HPLC Peaks with Retention Times, Quasi-molecular Ions [M + H]$^+$, and Daughter Ions [m/z] with Their Intensities [%] and Proposed Structures*

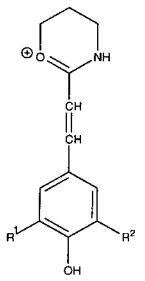
HPLC peaks[a]) (t_R [min])	Parent quasi-molecular ion $[M+H]^+$	Intensity [%] of MS/MS fragment[b])																		
		m/z 147 **a′**	*m/z* 177 **a″**	*m/z* 207 **a‴**	*m/z* 204 **b′**	*m/z* 234 **b″**	*m/z* 264 **b‴**	*m/z* 218 **c′**	*m/z* 248 **c″**	*m/z* 278 **c‴**	*m/z* 221 **d′**	*m/z* 251 **d″**	*m/z* 281 **d‴**	*m/z* 275 **e′**	*m/z* 305 **e″**	*m/z* 335 **e‴**	*m/z* 292 **f′**	*m/z* 322 **f″**	*m/z* 352 **f‴**
A1 (9.4)	438	38	–	–	100	–	–	8	–	–	30	–	–	45	–	–	52	–	–
A2 (14.4)	438	44	–	–	100	–	–	8	–	–	24	–	–	32	–	–	50	–	–
A3 (15.8)	438	28	–	–	100	–	–	8	–	–	22	–	–	44	–	–	58	–	–
A4 (20.8)	438	46	–	–	100	–	–	10	–	–	16	–	–	30	–	–	54	–	–
A5 (32.8)	438	43	–	–	100	–	–	10	–	–	5	–	–	5	–	–	47	–	–
B1 (10.8)	468	14	100	–	50	70	–	6	8	–	16	10	–	40	29	–	62	42	–
B2 (15.6)	468	24	98	–	28	100	–	11	1	–	7	14	–	28	30	–	66	62	–
B3 (17.3)	468	8	62	–	100	1	–	1	9	–	16	1	–	32	7	–	48	16	–
B4 (21.0)	468	17	32	–	10	100	–	6	1	–	4	10	–	6	32	–	19	42	–
B5 (24.9)	468	14	72	–	100	7	–	1	10	–	16	3	–	26	6	–	40	19	–
B6 (26.9)	468	6	54	–	6	100	–	8	2	–	4	12	–	10	23	–	22	34	–
C1 (12.4)	498	–	100	–	–	86	–	–	6	–	–	19	–	–	44	–	–	92	–
C2 (17.6)	498	–	100	–	–	78	–	–	9	–	–	16	–	–	41	–	–	80	–
C3 (21.9)	498	–	92	–	–	100	–	–	6	–	–	17	–	–	28	–	–	82	–
C4 (29.3)	498	–	98	–	–	100	–	–	7	–	–	14	–	–	29	–	–	72	–
D1 (12.6)	528	–	47	100	–	47	40	–	4	5	–	9	21	–	26	50	–	42	45
D2 (15.7)	528	–	70	100	–	44	55	–	10	1	–	11	22	–	25	41	–	68	61
D3 (21.4)	528	–	21	68	–	100	5	–	1	7	–	13	3	–	28	8	–	97	24
D4 (28.3)	528	–	51	70	–	24	100	–	8	4	–	7	18	–	19	33	–	57	83
D5 (34.1)	528	–	63	84	–	18	92	–	10	1	–	7	22	–	17	28	–	60	100
E′ (12.7)	558	–	–	88	–	–	42	–	–	4	–	–	–	–	–	17	–	–	100
E″ (20.3)	558	–	–	86	–	–	42	–	–	4	–	–	–	–	–	18	–	–	100

[a]) The group-**E** compounds give useful MS/MS only in the SRM mode, after UV irradiation of *Fraction I* (peaks **E′** and **E″**), see *Fig. 5*.

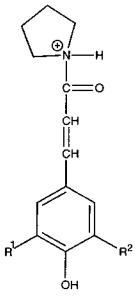
b)



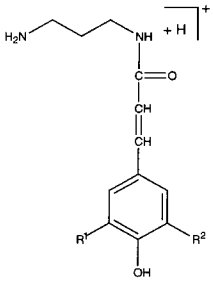
a' $R^1=R^2=H$ *m/z* 147
a'' $R^1=OCH_3$, $R^2=H$ *m/z* 177
a''' $R^1=R^2=OCH_3$ *m/z* 207



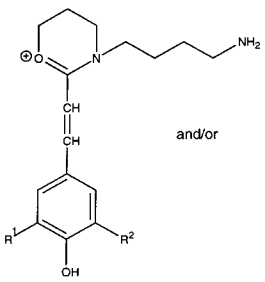
b' $R^1=R^2=H$ *m/z* 204
b'' $R^1=OCH_3$, $R^2=H$ *m/z* 234
b''' $R^1=R^2=OCH_3$ *m/z* 264



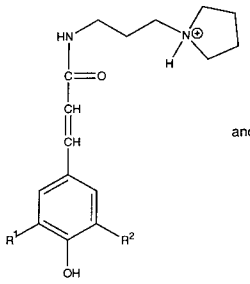
c' $R^1=R^2=H$ *m/z* 218
c'' $R^1=OCH_3$, $R^2=H$ *m/z* 248
c''' $R^1=R^2=OCH_3$ *m/z* 278



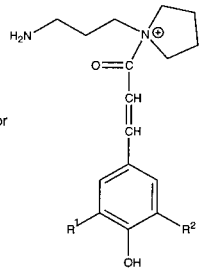
d' $R^1=R^2=H$ *m/z* 221
d'' $R^1=OCH_3$, $R^2=H$ *m/z* 251
d''' $R^1=R^2=OCH_3$ *m/z* 281



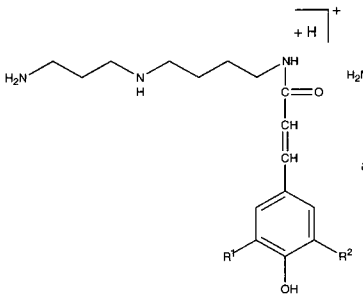
and/or



and/or



e' $R^1=R^2=H$ *m/z* 275
e'' $R^1=OCH_3$, $R^2=H$ *m/z* 305
e''' $R^1=R^2=OCH_3$ *m/z* 335



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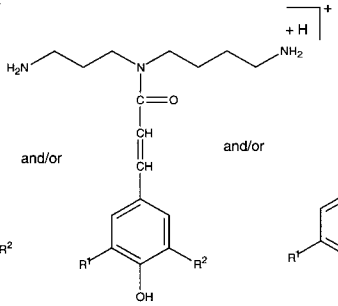
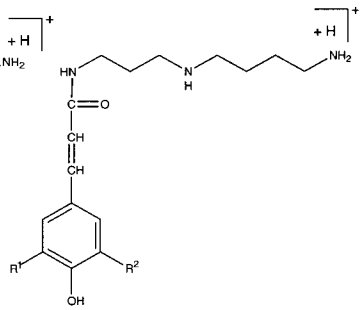


and/or



f' $R^1=R^2=H$ *m/z* 292
f'' $R^1=OCH_3$, $R^2=H$ *m/z* 322
f''' $R^1=R^2=OCH_3$ *m/z* 352

Table 3. *Retention Times of the Synthetic (*E,E*)-*N,N′*-Dicoumaroylspermidines* **1**, **8**, *and* **15**, *and of Their (*E,Z*)/(*Z,E*)- and (*Z,Z*)-Isomers.* HPLC conditions: see *Exper. Part.*

	Retention time [min]		
	(*E,E*)	(*E,Z*) and (*Z,E*)	(*Z,Z*)
1	20.8	15.8	9.3
8	32.8	22.3	15.7
15	20.4	14.4	9.7

chromatographic peak. The results summarized in *Table 3* permit the identification of peak **A1** as the (*Z,Z*)-isomer of **1** or **15** or their mixture, **A2** as the (*E,Z*)/(*Z,E*)-isomers of **15**, **A3** as the (*E,Z*)/(*Z,E*)-isomers of **1** and/or the (*Z,Z*)-isomer of **8**, **A4** as the (*E,E*)-isomer of **1** and/or **15**, and/or (*E,Z*)/(*Z,E*)-isomers of **8**, and **A5** as the (*E,E*)-isomer of **8**.

Signals of the **C** *Group* (**C1**–**C4**) *with* $[M+H]^+$ *at* m/z *498*. The CID-MS of the compounds represented by the four peaks **C1**–**C4** ($[M+H]^+$ 498) were very similar to each other. The peak-intensity differences are in the order of 5–20%, see *Fig. 3,c*, and *Table 2*. The observed peaks at *m/z* 177, 234, 248, 251, 305, and 322, can be compared with those of the CID-MS of the compounds of the **A** group. The fragmentation pattern is very similar to that of the already identified substances **1**, **8**, and **15** (peaks at *m/z* 147, 204, 218, 221, 275, and 292), but shifted by + 30 u. This shift is best interpreted as a *Biemann* shift. Similar compounds could give similar fragmentation reactions. Compounds with different substitution which do not influence the decomposition and which are not involved in the fragmentation processes could show in principle the same fragmentations. As a result, the fragmentation pattern or part of it is shifted by a defined number of units which depends on these substitutions. The mass difference of +30 u for the fragment ions and that of + 60 u for the quasi-molecular ions are explained by an additional methoxy substituent on both phenol moieties of the *N,N′*-dicoumaroylspermidines **1**, **8**, and **15**, to give the *N,N′*-diferuloylspermidines **4**, **11**, and **18** (*Table 1*). The observed fragment ions could be a result of the same CID processes, including acid-catalyzed isomerization, and give the same fragmentation pattern. Therefore, the fragment ions of the group-**C** compounds can be assigned as follows: the signal at *m/z* 177 (147 + 30), 234 (204 + 30), 248 (218 + 30), 251 (221 + 30), 305 (275 + 30), and 322 (292 + 30) correspond with structures **a″**, **b″**, **c″**, **d″**, **e″**, and **f″**, respectively (see *Table 2*). Other theoretically possible HCA derivatives of spermidine with the same molecular weight, but with a different substitution on the aromatic ring, for example, *N,N′*-coumaroylsinapoylspermidines, can be excluded. The fragment-ion signals corresponding to the coumaroyl (*m/z* 147) and sinapoyl residues (*m/z* 207) are missing. These conclusions were confirmed using the UV spectrum of **C4** which was obtained by DAD technique. It was identical to the UV spectrum of the (*E,E*)-isomer of 1,10-diferuloylspermidine **18** with λ_{max} 295 and 314 nm, which was isolated and characterized earlier [6]. The UV spectrum of **C1** (λ_{max} 271 and 292 nm) permits its identification as the (*Z,Z*)-isomer of *N,N′*-diferuloylspermidines **4**, **11**, or **18**, respectively. The UV spectrum of **C2** could not be used for determination of the configuration, because a substance co-elution took place.

Signals of the **B** *Group* (**B1**–**B6**) *with* $[M+H]^+$ *at* m/z *468*. The differences between the masses of the quasi-molecular ions of group **B** (*m/z* 468) and those of group **A** (*m/z* 438) or group **C** (*m/z* 498) are ±30 u. Furthermore, the CID-MS obtained from group-**B** compounds are more complicated than those from the groups **A** and **C** (*Fig. 3,b*, and *Table 2*). In these spectra, both of the already discussed fragment-ion patterns are present, namely fragment ions *m/z* 147, 204, 218, 221, 275, and 292 (group **A**) as well as *m/z* 177, 234, 248, 251, 305, and 322 (group **C**). Using the already-mentioned *Biemann*-shift method and arguments, it is possible to suggest that the substances present in the peaks **B1**–**B6** are the *N*-coumaroyl-*N*′-feruloylspermidines **2**, **3**, **9**, **10**, **16**, and **17** (*Table 1*). The two different substitutions enlarged the number of structural and geometrical isomers to 24. This fact can explain the larger number of chromatographic peaks in the **B** group, compared with those in the **A** and **C** groups. The differences in the relative intensities of the observed signals in the CID-MS (see *Fig. 3,b*, and *Table 2*) can also be explained with these alternative substitutions. The CID fragmentation of spermidine derivatives, *N*-substituted with two different 4-hydroxycinnamic acids (HC), is not well investigated. Further conclusions are hindered by difficulties like acid-catalyzed isomerization and the large number of possible fragmentation pathways (currently under investigation).

Signals of the **D** *Group* (**D1**–**D5**) *with* $[M+H]^+$ *at* m/z *528*. The CID-MS of the compounds of the **D** group (*Fig. 3,d*) are a combination of the fragment-ion pattern of group **C** (*m/z* 177, 234, 248, 251, 305, and 322) and a new one shifted by 30 u (*m/z* 207, 264, 278, 281, 335, and 352). For interpretation, the same method was used as for group **B**. These peak combinations could be assigned to *N*-feruloyl-*N*′-sinapoylspermidines **5**, **6**, **12**, **13**, **19**, and **20** (*Table 1*). The fragment ions of the group-**D** compounds can be assigned as follows: the fragment ions at *m/z* 207 (177 + 30), 264 (234 + 30), 278 (248 + 30), 281 (251 + 30), 335 (305 + 30), and 352 (322 + 30) correspond with proposed structures **a**‴, **b**‴, **c**‴, **d**‴, **e**‴, and **f**‴, respectively (see *Table 2*).

Signals of the **E** *Group* (**E1**–**E3**) *with* $[M+H]^+$ *at* m/z *558*. Due to the low peak intensity of the *m/z* 558 ions (about 500 times lower than those of the *m/z* 438–528 ions), no product-ion spectra were obtained. All attempts to inject a larger amount of sample were unsuccessful because they led to an overloading of the column and MS detector, followed by abnormal peak shape and drifting retention times. By using an SRM experiment, it was possible to compensate for this insufficient sensitivity (see below).

Further Proof of the Proposed Structures. To confirm the structures of the above-mentioned compounds, some chemical reactions were performed. Catalytic hydrogenation of disubstituted HCA of spermidine led to the corresponding tetrahydro derivatives. The preliminary experiments with synthetic standards **1**, **8**, and **15** were done in MeOH solution over 10% Pd/C under H_2 at atmospheric pressure in the presence of AcOH (see *Exper. Part*). The reaction was complete within 6 h in quantitative yield (ESI-MS). The reaction was repeated with 0.5 mg of *Fraction I*. An ESI-MS of the reaction product shows only the tetrahydro conjugates with quasi-molecular ion peaks at *m/z* 442, 472, 502, 532, and 562 instead of *m/z* 438, 468, 498, 528, and 558. This experiment is in agreement with the earlier conclusions about the structures of substances of the groups **A**–**D**.

It is well-known that HC derivatives isomerize under the action of UV light to form (*E*) ⇌ (*Z*) equilibrium mixtures [13]. A sample of *Fraction I* was irradiated at 366 nm,

and the product was analyzed by HPLC-UV(DAD)/APCI-MS under the same conditions as the original *Fraction I*. Both chromatograms were compared, and significant retention-time differences were observed for all groups of compounds (*Fig. 4*). Further irradiation at this wavelength did not lead to any additional changes, because the (*E*)⇌(*Z*)-photoisomerization equilibrium had been achieved. Some of the peaks on the extracted-ion chromatograms obtained after UV irradiation can be identified as the earlier observed peaks **A1**, **B1, C1**, **D1**, and **E1**. After irradiation, they are the highest-intensity peaks in the chromatograms. In contrast, the peaks of highest intensity from *Fraction I* are of low intensity or have disappeared after irradiation. The substances of group **A** can be identified: the peak **A4**, representing the (*E,E*)-isomers of **1** and **15**, disappeared after UV irradiation, peak **A1** ((*Z,Z*)-isomers of **1** and **15**) is now the most intense signal. These changes are typical for (*E,Z*)-isomerization of this class of compounds. The irradiation at 366 nm leads to predominantly (*Z,Z*)-isomers. In terms of these observations, peaks **B1**, **C1**, and **D1** could be assigned to (*Z,Z*)-*N*-coumaroyl-*N*′-feruloylspermidines, (*Z,Z*)-*N,N*′-diferuloylspermidines, and (*Z,Z*)-*N*-feruloyl-*N*′-sinapoylspermidines, respectively.

These two experiments (catalytic hydrogenation and UV photoisomerization) confirm the structures of the compounds of groups **A**, **B**, **C**, and **D**. Moreover, they make it possible to suggest structures for the compounds of group **E**.

Signals of the **E** *Group* (**E1**–**E3**) *with* $[M+H]^+$ *at* m/z *558*. Compounds of group **E** isomerize under UV-light irradiation (see *Fig. 4*). Their catalytic hydrogenation leads to tetrahydro conjugates. Their quasi-molecular ions (*m/z* 558) are shifted by + 30 u, compared to those of group **D**. In accordance with these properties, we presume that the substances of group **E** could be the *N,N*′-disinapoylspermidines **7**, **14**, and **21**. As

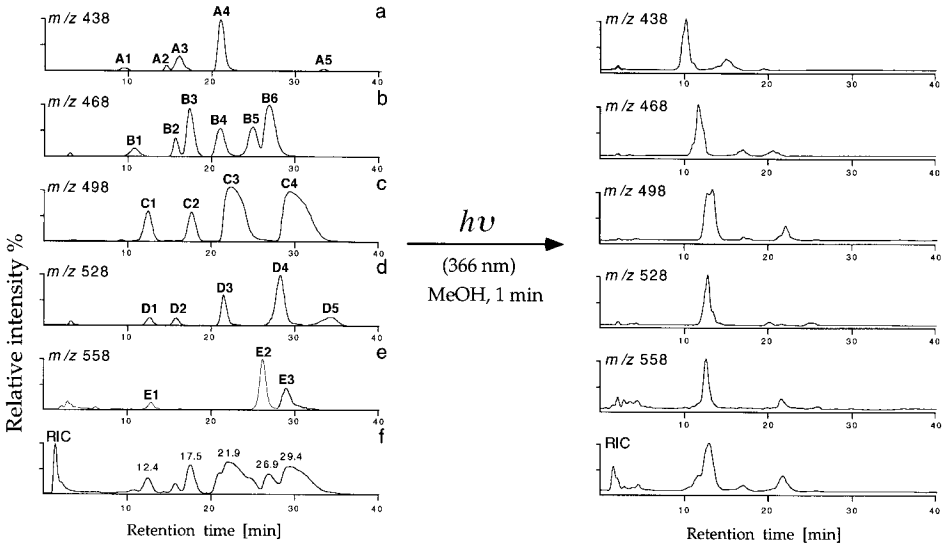


Fig. 4. *HPLC/APCI-MS Chromatograms* (FS mode) *of* Fraction I, *before* (left) *and after UV irradiation at 366 nm* (right): a)–e) *extracted-ion chromatograms of the quasi-molecular ions* m/z *438, 468, 498, 528, and 558, respectively*; f) *RIC*. HPLC and MS conditions: see *Exper. Part.*

shown in *Fig. 4,e* (right), the chromatographic peaks obtained after UV irradiation are narrower and of higher intensity than those before irradiation. This allowed MS/MS of group-**E** substances to be obtained by selected-reaction monitoring (SRM), using a triple-quadrupole mass spectrometer. This technique allowed the selection of one or several parent ions in the first quadrupole. The CID-MS of the chosen ions was achieved in the second one, and the third quadrupole scanned only a single or a set of *m/z* values and did not scan the whole mass range. In this way, higher sensitivity could be achieved than in product-ion spectra measured over a particular *m/z* range.

Because the analyzed compounds were thought to be the *N,N′*-disinapoylspermidines **7**, **14**, and **21**, the expected daughter-ion pattern (*m/z* 207, 264, 278, 335, and 352) was chosen for monitoring (reactions: *m/z* 558 → 207, 264, 278, 335, and 352, resp.). The HPLC/APCI-MS/MS (SRM) experiment confirmed the proposed fragmentation reactions (*Fig. 5* and *Table 2*). The monitoring of other *m/z* gave negative results (no signals). The structures of the daughter ions have already been discussed during the discussion of the CID-MS of group **D**. The daughter ions can be assigned as follows: *m/z* 207, 264, 278, 335, and 352 correspond with fragment structures **a‴**, **b‴**, **c‴**, **e‴**, and **f‴**, respectively. The observed daughter ions confirm that the suggested structures are *N,N′*-disinapoylspermidines (**7**, **14**, and **21**) corresponding to the signals **E1**, **E2**, and **E3**, respectively. The peak with the highest intensity after UV irradiation has the same retention time as peak **E1** of the original fraction. In accordance with earlier

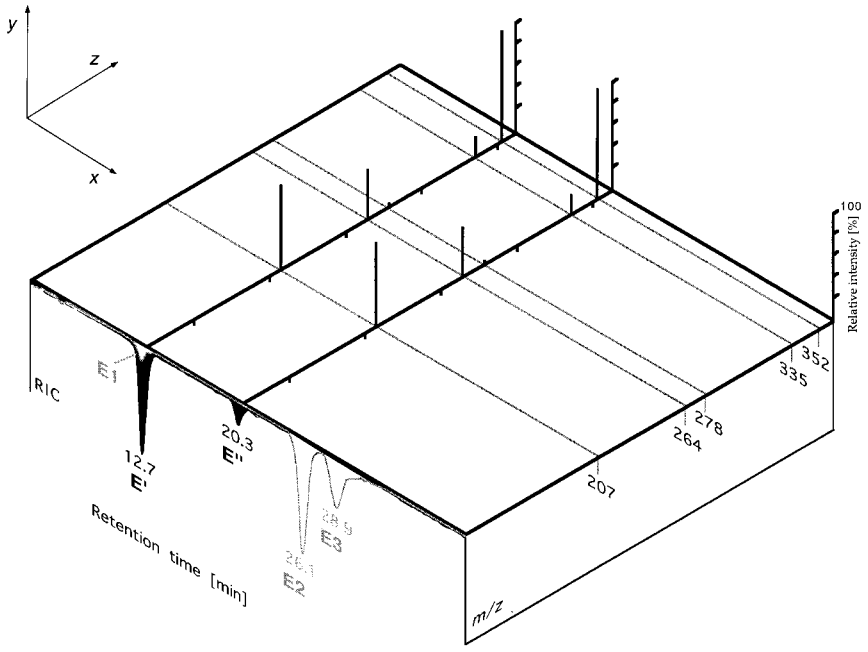


Fig. 5. *HPLC/APCI-MS/MS (SRM) Experiment of* Fraction I *after UV irradiation, observed reactions* m/z *558 → 207, 264, 278, 335, and 352*. *x*-Axis, t_R [min] of RIC chromatogram; *z*-axis, *m/z* of MS/MS signals; *y*-axis, relative intensity (above) and RIC (below). In gray, HPLC/APCI-MS extracted-ion chromatogram *m/z* 558 before irradiation (*Fig. 1*); HPLC and MS conditions: see *Exper. Part.*

observations, it can be assigned to the (*Z,Z*)-isomers of the *N,N′*-disinapoylspermidines (**7**, **14**, and **21**).

A Proposed Biological Function of HCA in Pollen. Studies of *in vitro* interactions between polyamines and nucleic acids have been reported [19–24]. The binding of the polyamines takes place mainly through the formation of H-bonds. Spermine interacts with DNA molecules much more strongly than spermidine, and NH_3^+ groups bind much more weakly than the NH_2^+ groups [22]. The DNA-binding properties of polyamine analogues can play a role in the stabilization of the DNA helix. In the plant cells, the HCA which are compounds of lower basicity compared with the polyamines, could also bind to DNA. This possibility is now under investigation, and the results will be published [25].

Due to their UV-absorbing properties, the function of the above-mentioned HCA could be to protect the pollen grains from damage by UV light. Pollen grains are highly exposed to light, and the protection of the integrity of DNA is of highest importance. Flavonoids are often proposed as the UV-protectant agents [26]. However, in plants with large quantities of HCA in the pollen, hydroxycinnamamides might well be involved in the UV protection of the genetic material. The solar UV reaching the surface of the earth is mainly in the UV-A region (320–390 nm), only partly in the UV-B region (280–320 nm), and it drops to zero beyond 290 nm. The absorption spectrum of DNA includes wavelengths from 240–310 nm. The absorption maximum of the HCA is in the area of 280–330 nm in their (*E*)-form and covers partially the UV region where the DNA of the pollen could be damaged.

Conclusions. – A highly sensitive method for the analysis of mixtures of 4-hydroxycinnamoylspermidine conjugates was developed. The detection limits by HPLC/APCI-MS (FS) are 4.5–5 pmol, determined by using the synthetic (*E,E*)-isomers of **1** and **8** as standards.

Using HPLC-UV(DAD)/APCI-MS (FS), HPLC/APCI-MS/MS, and HPLC/APCI-MS/MS in SRM mode (where very high sensitivity is required), a fraction of methanolic extract of pollen from *Hippeastrum* x hortorum (Amaryllidaceae) was analyzed. It contains at least 23 compounds. They were identified as the (*Z,Z*)-, (*E,Z*)-, (*Z,E*)-, and (*E,E*)-isomers of *N,N′*-dicoumaroyl-, *N*-coumaroyl-*N′*-feruloyl-, *N,N′*-diferuloyl-, *N*-feruloyl-*N′*-sinapoyl-, and *N,N′*-disinapoylspermidines (compounds **1**–**21**) by using UV, MS, and MS/MS data, synthetic standards, and some photochemical, chromatographic, and chemical behaviors.

According to their photochemical behaviors, hydroxycinnamamides (HCA) of spermidine take a part in the protection of DNA in the pollen against harmful UV irradiation.

For all experiments, only 1 mg of *Fraction I* was used.

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Experimental Part

General. MeOH, MeCN, and $CHCl_3$ (HPLC grade) from *Scharlau*, Barcelona, Spain, AcOH and 25% aq. NH_4OH soln. (*puriss p.a.* from *Merck*, Darmstadt, Germany), and 10% Pd/C from *Fluka*, Buchs, Switzerland. The H_2O was purified with a *MILLI-*Q_{RG} apparatus (*Millipore*, Milford, MA, USA). *Schlittler*-reagent: $K_2[PtI_6]$

in aq. HCl soln. for amines and amides. TLC: silica gel F_{254} plates (*Merck*, Darmstadt, Germany). Solid-phase extraction: *Amberlite CG 50* (*Fluka*, Buchs, Switzerland). HPLC: *Waters-626 LC* system, *Waters 996* photodiode array detector, and *Waters 600S* controller (*Waters* Corp., Milford, MA, USA) with *Millennium* chromatography manager *2010 v.2.15* (*Waters*) and *Rheodyne* rotary valve *7725i* with a 5 µl loop (*Rheodyne*, Cotati, CA, USA). MS: HPLC/APCI-MS and HPLC/APCI-MS/MS by means of a triple-stage quadrupole instrument (*Finnigan TSQ 700*, San José, CA, USA), equipped with a *Finnigan* atmospheric-pressure chemical-ionization (APCI) ion source.

UV Photoisomerization. Irradiations with low-pressure mercury lamp at 366 nm in MeOH soln., in concentrations of 0.5–1 mg/ml, for 1 min.

Plant Material. The plants originate from one *Hippeastrum* x hortorum plant bought on a local market (Zürich) in 1991). All plants were subcultivated from this plant by vegetative propagation in the greenhouse of our institute. During the flowering period (Feb. 1997), the anthers were cut and the pollen collected and stored at $-20°$.

Extraction. The pollen (1.0 g) was extracted twice with MeOH/H_2O 4:1 and once with MeOH/H_2O 1:1 by stirring for 3 h. After centrifugation, the combined supernatants were concentrated and loaded onto a weakly acidic cation-exchange column (*Amberlite CG 50*). The acidic and neutral components were successively eluted with H_2O, MeOH/H_2O 1:1, and MeOH/H_2O 4:1. According to [10], the basic substances were then eluted with 8M aq. AcOH/MeOH 1:1 (*v/v*). The eluent was evaporated, the residue (9 mg) dissolved in MeOH and separated by prep. TLC (silica gel F_{254}, $CHCl_3$/MeOH/aq. 25% NH_4OH soln. 78:19:3). Three UV-absorbing bands showed *Schlitter* positive reaction, at R_f 0.17 (*Fraction I*), 0.3 (*II*), and 0.4 (*III*). After evaporation, the *Fractions I* (2.7 mg), *II* (4.1 mg), and *III* (2.1 mg) were obtained. *Fraction I* was dissolved in MeOH (in concentrations 0.5–4 mg/ml) and analyzed (injected volumes 1–5 µl) with HPLC-UV(DAD), HPLC-UV(DAD)/MS, and HPLC/APCI-MS/MS. Fractions *II* and *III* are under investigation.

HPLC: *Waters* symmetry C_8 column (5 µm, 2.1 × 150 mm; *Waters* Corp., Milford, MA, USA); flow rate 0.22 ml min^{-1}; mobil phase: MeCN/H_2O 9:91 containing 1% of AcOH.

MS: The APCI operating conditions in positive mode were: vaporizer temp. 450°; corona voltage 5 kV; heated capillary temp. 220°; sheath gas N_2, with an inlet pressure of 50 psi; conversion dynode -15 kV. MS/MS Experiments: collision gas Ar with a relative pressure of 2.2–3.1 mTorr; collision induced dissociation offset (Coff) -28 eV. In the SRM mode, the observed reactions were *m/z* 558 → 207, 264, 278, 335, and 352, respectively. ESI Operating conditions: heated capillary temp. 200°; sheath gas N_2, with an inlet pressure of 40 psi; conversion dynode 15 kV; spray voltage 4.5 kV.

Catalytic Hydrogenation of Synthetic (E)-N-*(4-Aminobutyl)-3,3′-bis(4-hydroxyphenyl)*-N,N′-*(propan-1,3-diyl)bis[prop-2-enamide]* (=(*E*)-N-*(4-Aminobutyl)-3-(4-hydroxyphenyl)*-N-*{3-{[*(E)-*3-(4-hydroxyphenyl)-1-oxoprop-2-enyl]amino}propyl}prop-2-enamide*; (*E,E*)-**1**) *and* (E)-N-*(3-Aminopropyl)-3,3′-bis(4-hydroxyphenyl)*-N,N′-*(butane-1,4-diyl)bis[prop-2-enamide]* (=(E)-N-*(3-Aminopropyl)-3-(4-hydroxyphenyl)*-N-*{4-{[*(E)-*3-(4-hydroxyphenyl)-1-oxoprop-2-enyl]amino}butyl}prop-2-enamide*; (*E,E*)-**8**) *and isolated* Fraction I. A soln. of 0.5 mg of each sample (**1**, **8**, or *Fraction I*) in MeOH (1.5 ml) containing 10% Pd/C (0.2 mg) and AcOH (10 µl) was stirred for 6 h under H_2 at 1 atm and 25°. The mixture was filtered and analyzed by ESI-MS. Yield (tetrahydro conjugates): 99% (by ESI-MS).

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