The Acylpolyamines from the Venom of the Spider *Agelenopsis aperta*[1])

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Dedicated to Prof. Dr. *H. J. Veith* on the occasion of his 60th birthday

The lyophilized venom of the spider *Agelenopsis aperta* (Aranea: Agelenidae) has been re-analyzed by on-line coupled high-performance liquid chromatography and atmospheric-pressure chemical-ionization mass spectrometry (HPLC-UV(DAD)-APCI-MS and MS/MS). Thus, 33 acylpolyamines with 11 different molecular masses, present in various concentrations, were detected, and their structures were disclosed. Different types of possible fragmentation as well as principles of structure elucidation are discussed. The method can be used for rapid separation and analysis of spider-venom constituents.

1. Introduction. – Spider venoms are complex mixtures of different classes of biologically active compounds and, therefore, of great interest to scientists who are investigating their composition, biological activity, and possible application of venom constituents. But on the other hand, they are available mostly in small amounts. For classical analytical methods, spider venoms are too complicated, only the application of modern and sensitive techniques can solve the analytical problems.

In the last ten years, spider venoms were widely investigated with the aim to elucidate their structures and to test their biological activity [1–5]. Quite interesting results were obtained during these studies: in the spider venom, depending on the species, one could find in different ratios various compounds, such as amino acids, purine bases, polyamines, acylpolyamines, peptides, polypeptides, and proteins [6–9]. Moreover, the biological action of some of these classes was examined, especially for the species from the families Agelenidae and Araneidae [10–13]. For the spider *Agelenopsis aperta* (Aranea: Agelenidae) (see *Fig. 1*), peptides (μ-agatoxins) and polypeptides (ω-agatoxins) [14] are responsible for long-acting and irreversible paralysis of insects. On the contrary, the acylpolyamines (α-agatoxins) effect is immediate but reversible [1][15][16]. These acylpolyamines exhibit neuromodulatory activity within the mammalian central nervous system, and, therefore, could be applied for investigating diseases such as stroke, *Alzheimer*'s disease, and epilepsy [17][18].

A large number of spider acylpolyamines were synthesized [2][5][19][20]; last year, several of them were even obtained by solid-phase synthesis [21]. The structures of almost all known acylpolyamines isolated from the family Agelenidae were elucidated by mass spectrometry or partly by NMR spectroscopy (for the major compounds) and verified by total synthesis [19].

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[1]) Presented by *S. C.* at the '34. Diskussionstagung der Deutschen Gesellschaft für Massenspektrometrie', on March 4–7, 2001, Technical University of Munich, Germany.

[2]) Part of the Ph.D. Thesis of *S. C.*

Fig. 1. Agelenopsis aperta *spider*. Body size 2.5 cm (without legs). With permission of Dr. *Ch. Kristensen, Spider Pharm, Inc.*, Yarnell, AZ, U.S.A.

Last year, high-performance liquid chromatography (HPLC), coupled on-line with atmospheric-pressure chemical-ionization mass spectroscopy (APCI-MS), has been successfully used for the isolation and structure elucidation of polyamines from the venom of the spider *Paracoelotes birulai* (Aranea: Amaurobiidae) [22]. It gave an idea that this selective and sensitive method could be used for analysis and structure elucidation of new acylpolyamines present in *Agelenopsis aperta* venom in low concentrations.

Since APCI-MS produces by 'collision-induced decomposition' (CID) at low activation energies (< 100 eV) quasi-molecular ions with low fragmentation, it was interesting to compare results obtained by on-line-coupled HPLC and APCI mass spectrometer (HPLC-APCI-MS and HPLC-APCI-MS/MS) with other analytical methods such as chemical-ionization (CI), matrix-assisted laser-desorption ionization (MALDI), fast-atom-bombardment (FAB), continuous-flow-frit-FAB, and electro-spray-ionization (ESI) mass spectrometry.

In this work, a number of novel polyamine-containing toxins from the venom of *Agelenopsis aperta* are reported.

2. Results and Discussion. – 2.1. *Sample Preparation and HPLC-UV(DAD)-APCI-MS.* Lyophilized *Agelenopsis aperta* spider venom was dissolved in a H_2O/MeCN solution of CF_3COOH and filtered, giving a clear solution, which was analyzed without additional handling.

The same optimized conditions as previously described were used [22]. The problem of the small amount of available venom was solved by on-line coupling of HPLC, UV/VIS diode array detection (DAD), and APCI-MS. Screening the crude venom solution

by HPLC-UV(DAD), amino acids, purine bases, acylpolyamines, peptides, polypeptides, as well as proteins were differentiated according to their t_R (*Fig. 2,a*). The reconstructed ion chromatogram (RIC, see *Fig. 2,b*) obtained from the full-scan mode (FS) HPLC-APCI-MS analysis is in good agreement with the UV chromatogram (254 nm).

The fraction selected for further investigations (20–40 min) was located between the purine bases and the peptide-containing fractions. *Fig. 3* shows more detailed views of the RIC (*Fig. 3,g*) and the UV chromatogram (*Fig. 3,h*) in this time domain. Its complexity forced us to split the fraction on several, according to their UV-absorption properties. The analysis of the UV spectra indicated the presence of at least three different types of α-agatoxins. The first type (compounds I) possessed three maxima

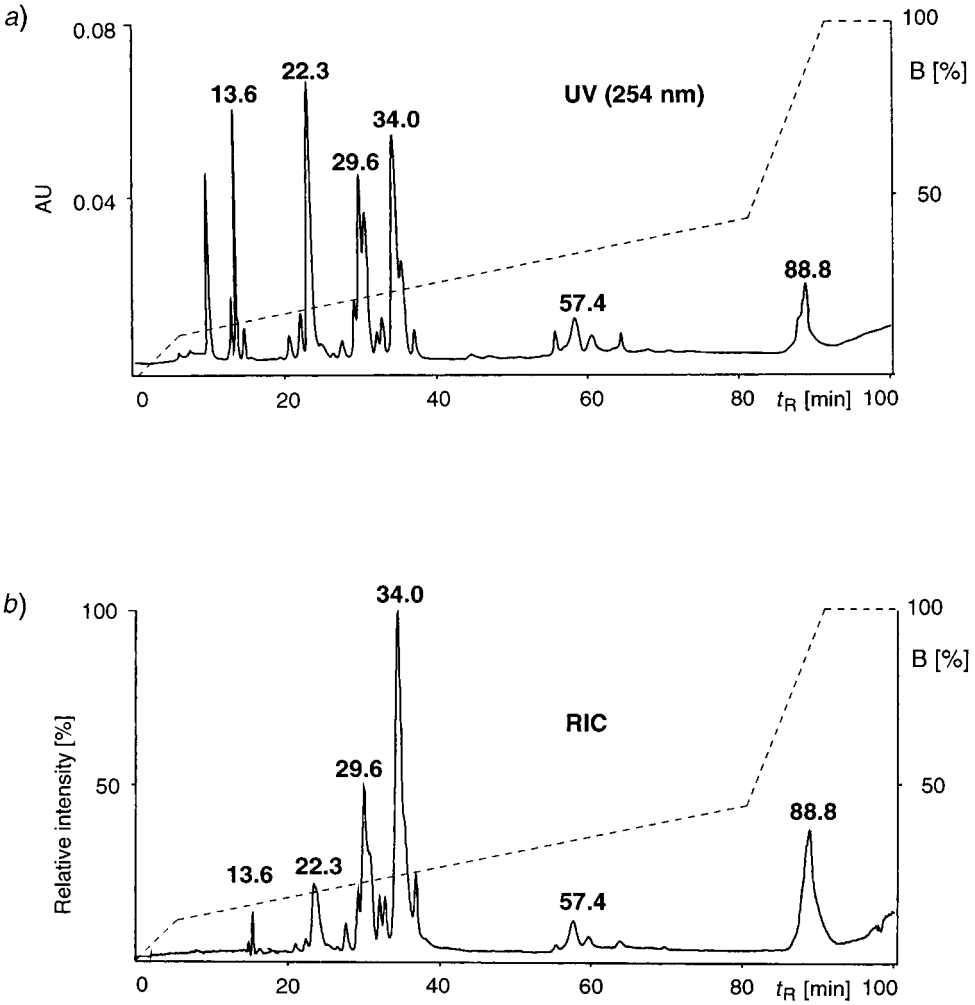

Fig. 2. Agelenopsis aperta *venom: results of HPLC-UV(DAD)/APCI-MS.* a) *UV detection at 254 nm*; b) *RIC.* B [%] = concentration of the solvent B; AU = arbitrary unit. The dashed line shows the gradient of the concentration of the solvent B. For HPLC and MS conditions, see *Exper. Part.*

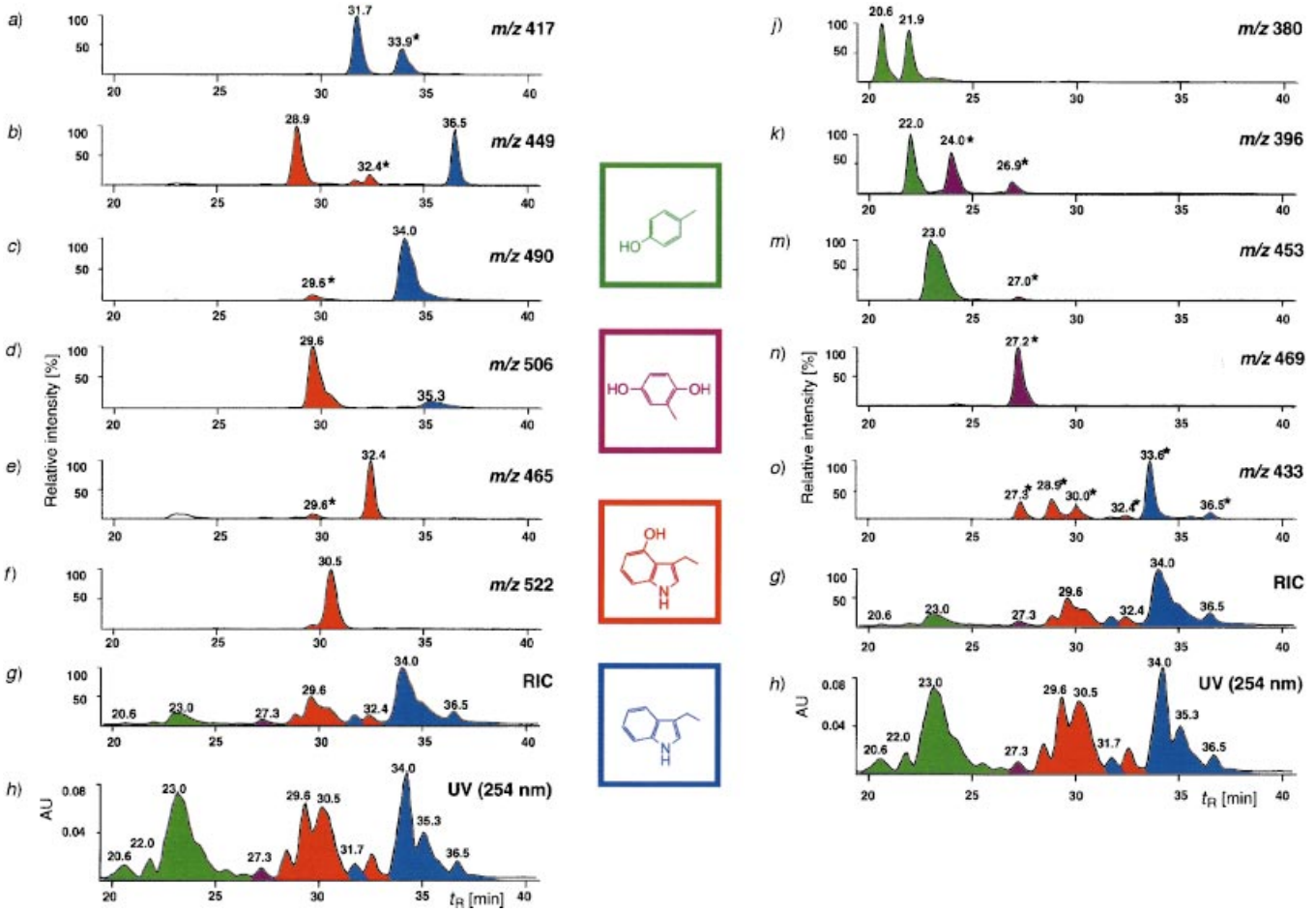

Fig. 3. Agelenopsis aperta *venom: acylpolyamine-containing fraction analyzed by HPLC-UV(DAD)/APCI-MS.* a)–g) *and* j)–o) *extracted-ion chromatogram* (EIC) *of quasi-molecular ions* m/z *417, 449, 490, 506, 465, 522, 380, 396, 453, 469, and 433.* g) *RIC*; h) *UV Detection at 254 nm.* AU = arbitrary unit. The starred signals belong to compounds co-eluted with the major constituents, where the chromophore type was established from MS/MS data. For HPLC and MS conditions, see *Exper. Part*.

(λ_{max} at 218, 279, and 287 nm), the second type (compounds II) four (λ_{max} at 218, 266, 281, and 291 nm), and the third type (compound III) only one UV maximum (λ_{max} at 251 nm).

The acylpolyamines from spider venoms are thermolabile compounds. To get their molecular masses, the HPLC-UV(DAD) was on-line coupled with an APCI mass spectrometer. This soft and sensitive ionization technique yields quasi-molecular ions with low fragmentation.

Compounds I (λ_{max} at 218, 279, and 287 nm): According to the RIC, the group of compounds I is the main one, and was chosen as the starting point for further investigations. In the UV chromatograms, signals at t_R 31.7 (*m*/*z* 417), 36.5 (*m*/*z* 449), 34.0 (*m*/*z* 490), and 35.3 min (*m*/*z* 506) were observed (*Fig. 3*, *a*–*d*, blue-colored signals). Comparing these UV spectra with those from our in-house library, we could assume the presence of the 1*H*-indole-3-acetamide chromophore (IndAc). Several peaks in every extracted-ion chromatogram (EIC) indicated the presence of additional isomers.

Compounds II (λ_{max} at 218, 266, 281, and 291 nm): The absorptions of compounds II, typical for the 4-hydroxy-1*H*-indole-3-acetamide (4-OH-IndAc) chromophore, were observed for signals at t_R 28.9 (*m*/*z* 449), 29.6 (*m*/*z* 506), 32.4 (*m*/*z* 465), and 30.5 min (*m*/*z* 522), and their EIC are shown in *Fig. 3*, *b* and *d*–*f* as the red-marked signals.

Compounds III (λ_{max} at 251 nm): On the HPLC-UV(DAD), three signals possessing this type of absorption (green-colored peaks) at t_R 20.6 (*m*/*z* 380), 21.9, and 22.0 (compounds yielding quasi-molecular ions *m*/*z* 380 and 396 are co-eluted having similar signal intensities), and at 23.0 min (*m*/*z* 453) were observed (*Fig. 3*, *j*–*m*). According to a previous report [5], the 4-hydroxybenzoate moiety (4-OH-Bz) could be postulated as a chromophore group for compounds III. Moreover, for the other, at least fourteen compounds (starred signals in *Fig. 3*), co-eluted with the above-listed UV-active constituents, the determination of their absorption maxima was not possible. This problem, as well as the later identification of the violet-colored signals (2,5-dihydroxybenzoic acid derivatives), could be solved by on-line coupled HPLC-APCI-MS/MS.

2.2. *HPLC-APCI-MS/MS.* Previously, the analysis of spider toxins has been achieved by CI-MS, FAB-MS, continuous-flow (Frit) FAB-LC/MS, MALDI-MS, and HPLC-ESI-MS [4][9][23–25]. The FAB-MS/MS of **AG 489**[3]) was the only spectrum of α-agatoxins available in the literature [6]. It agreed with ours, which was measured in the APCI-MS/MS mode (*Fig. 4*), the only differences being the signal intensities.

This sensitive and selective approach, which was already efficiently applied for the structure elucidation of acylpolyamines present in the *Paracoelotes birulai* [22] spider venom, can be employed for the characterization of new compounds present in low concentrations as well as to those, co-eluted with another one.

All HPLC-APCI-MS/MS experiments were independently performed for the eleven different quasi-molecular ions presented in *Fig. 3*. For every HPLC run, the first quadrupole was fixed to the corresponding *m*/*z* value and the third quadrupole was scanning over a particular *m*/*z* range.

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[3]) According to the procedure used in the pertinent literature, this and the further abbreviations are based on the species of the spider from which they are derived and the molecular mass of the respective compounds. Thus, **AG 489**, means the *Agelenopsis* toxin with the molecular mass 489. The letters following some names of toxins serve to differentiate compounds with the same molecular mass from the same species, *e.g.* **AG 489a**. For systematic names of all compounds described in this report, see *Footnotes 4* and *5*.

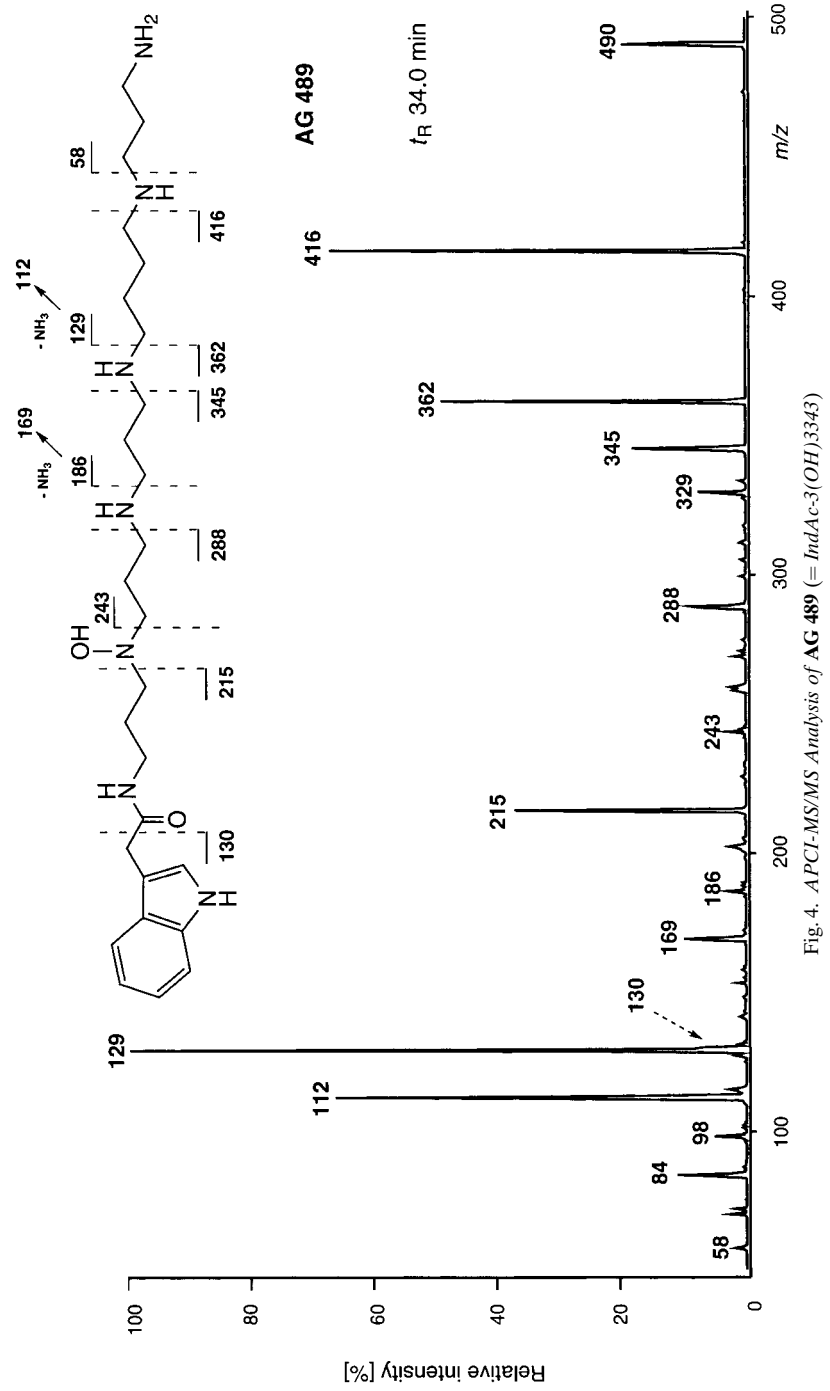

Fig. 4. *APCI-MS/MS Analysis of* **AG 489** (= *IndAc-3(OH)3343*)

In spite of different optimal collision energies (30% signal intensity of the quasi-molecular ion), all the data shown and discussed in this paper were obtained under the same conditions (the value of collision energy *Coff* was set up to -27 eV).

To simplify the discussion of the large number of possible isomers, a specific nomenclature informing about the chromophore types, the number of CH_2 groups between the N-atoms, as well as the position of substituents is generated. In the case of **AG 489** (*Fig. 4*), the short form *IndAc-3(OH)3343* is proposed. This means, that either three or four CH_2 groups separate the six N-atoms of the hexamine, which is modified at one terminus by the indoleacetyl moiety. Furthermore, the N(4)-atom (for atom numbering, see *Footnote 4*) is substituted with an OH group.

The principal CID-fragmentation patterns could be explained by the occurrence of a quasi-molecular ion protonated at different N-atoms. The mechanisms were previously extensively studied for some di- and triamine derivatives [26][27]. This investigation allowed the structure determination of polyamine backbones. The position of the OH group either at the chromophore or at the N-atoms was deduced as for acylpolyamines from the *Paracoelotes birulai* spider venom [22].

2.3. *Indole Derivatives of Hexamines: Compounds* **AG 489** *and* **AG 505b**. In the MS/MS analysis of **AG 489** (*Fig. 4*), a signal at *m*/*z* 130, characteristic for the indole moiety, was observed. The same fragment was typical for some other compounds of the venom with quasi-molecular ions at *m*/*z* 506, 417, 433, and 449 and possessing the absorption of the indole chromophore. For several indole-containing acylpolyamines present in minor concentrations, no UV spectra of pure compounds were available (starred signals in *Fig. 3*). These acylpolyamines had similar molecular masses, and according to the literature and the 'nitrogen rule', they could be split into hexamine and pentamine derivatives.

The signals at *m*/*z* 58, 112, 129, 169, 186, and 243 in MS/MS analysis of **AG 489** are characteristic for a hexamine moiety, where the atoms N(12) and N(17) are separated by four and all the other N-atoms by three CH_2 groups (polyamine *PA33343*). The ± 16 and ± 32 Da shifts between masses of quasi-molecular ions, caused by the different number of OH groups, as well as variable sequences and number of N-atoms in the polyamine backbone, could explain the diversity of indoleacetamide derivatives. It was possible to differentiate them only by MS/MS investigations.

In the group of indole derivatives, only one hexamine-containing α-agatoxin, **AG 505b** (*m*/*z* 506), was detected besides **AG 489** (*Table 1* and *2*)[4]). The ± 16 Da difference in the masses of the ions **h′**, **i′**, and **j′** confirmed the substitution of one of the N-atoms by another OH group besides R^2; as the fragment **f′** (*m*/*z* 288) was identical for both **AG 505b** and **AG 489**, it was possible to attribute this OH group to R^3. Furthermore, the same hexamine *PA33343* was established to be present in **AG 505b**

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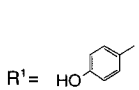
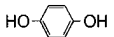
[4]) Systematic names of the natural products isolated and shown in *Tables 1* and *2:* **AG 452**, *N*-(20-Amino-4-hydroxy-4,8,12,17-tetraazaicosyl)-4-hydroxybenzamide; **AG 452a**, *N*-(20-Amino-4,8,12,17-tetraazaicosyl)-2,5-dihydroxybenzamide; **AG 468**, *N*-(20-Amino-4-hydroxy-4,8,12,17-tetraazaicosyl)-2,5-dihydroxybenzamide; **AG 489b**, *N*-(20-Amino-4,8,12,17-tetraazaicosyl)-4-hydroxy-1*H*-indole-3-acetamide; **AG 505**, *N*-(20-Amino-4-hydroxy-4,8,12,17-tetraazaicosyl)-4-hydroxy-1*H*-indole-3-acetamide; **AG 521**, *N*-(20-Amino-4,8-dihydroxy-4,8,12,17-tetraazaicosyl)-4-hydroxy-1*H*-indole-3-acetamide; **AG 489**, *N*-(20-Amino-4-hydroxy-4,8,12,17-tetraazaicosyl)-1*H*-indole-3-acetamide; **AG 505b**, *N*-(20-Amino-4,8-dihydroxy-4,8,12,17-tetraazaicosyl)-2-indole-3-acetamide.

Table 1. *MS/MS Fragment Ions* [%] *of Acylhexamine Derivatives from* Agelenopsis aperta. A dash indicates that the fragmentation was not observed. Both nomenclatures used in *Sect. 2* are given [a])



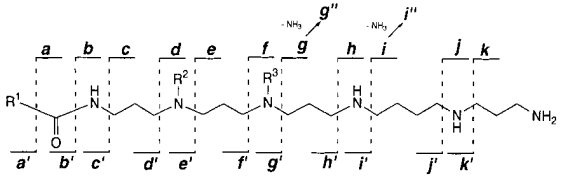
4-OH-Bz



2,5-$(OH)_2$*-Bz*

4-OH-IndAc

IndAc

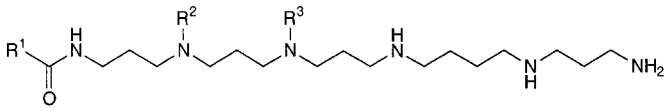


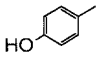
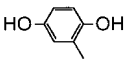
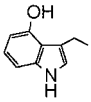
R^2 = H, OH
R^3 = H, OH

Type of the fragment	*4-OH-Bz-*	*2,5-(OH)$_2$-Bz-*		*4-OH-IndAc-*			*IndAc-*	
	-3(OH)3343 **AG 452**	*-33343* **AG 452a**	*-3(OH)3343* **AG 468**	*-33343* **AG 489b**	*-3(OH)3343* **AG 505**	*-3(OH)3(OH)343* **AG 521**	*-3(OH)3343* **AG 489**	*-3(OH)3(OH)343* **AG 505b**
a	–	–	–	–	–	–	–	–
a′	–	–	–	146 (5)	146 (11)	146 (7)	130 (7)	130 (13)
b	–	–	–	–	–	–	–	–
b′	–	–	–	–	–	174 (17)	–	–
c	–	–	–	–	–	–	–	–
c′	–	–	–	–	–	191 (12)	–	–
d	–	–	–	–	–	–	–	–
d′	178 (36)	194 (29)	194 (16)	231 (90)	231 (34)	231 (62)	215 (38)	215 (43)
e	–	243 (24)	259 (15)	243 (24)	243 (5)	–	243 (5)	–
e′	–	–	–	–	–	–	–	–
f	–	–	–	–	–	–	–	–
f′	251 (12)	251 (25)	267 (12)	288 (40)	304 (13)	304 (5)	288 (10)	288 (8)
g	186 (3)	186 (4)	186 (3)	186 (7)	186 (7)	186 (16)	186 (5)	186 (8)
g′	–	–	–	–	–	–	–	–
g″	169 (13)	169 (4)	169 (15)	169 (20)	169 (15)	–	169 (9)	169 (6)
h	–	–	–	–	–	–	–	–
h′	308 (20)	308 (35)	324 (12)	345 (41)	361 (28)	377 (13)	345 (20)	361 (16)
i	129 (100)	129 (100)	129 (100)	129 (90)	129 (100)	129 (100)	129 (100)	129 (100)
i′	325 (35)	325 (30)	341 (30)	362 (94)	378 (72)	394 (61)	362 (48)	378 (20)
i″	112 (97)	112 (96)	112 (100)	112 (100)	112 (76)	112 (32)	112 (66)	112 (55)
j	–	–	–	–	–	–	–	–
j′	379 (49)	379 (44)	395 (64)	416 (100)	432 (95)	448 (67)	416 (68)	432 (42)
k	–	–	–	–	–	–	–	–
k′	–	–	–	–	–	–	–	–

[a]) For systematic names, see *Footnote 4*.

Table 2. *Hexamine Derivatives Found in* Agelenopsis aperta *Spider Venom*



R^1	Name[a])	R^2	R^3	c [%]	t_R [min]	Parent quasi-molecular ion $[M+H]^+$
	**AG 452**[a])[b])	OH	H	17.7	23.0	453
	**AG 452a**[c])	H	H	1.0	27.0	453
	AG 468[b])	OH	H	4.8	27.2	469
	**AG 489b**	H	H	8.5	29.6	490
	AG 505[b])	OH	H	29.5	29.6	506
	AG 521[b])	OH	OH	6.3	30.5	522
	**AG 489**[b])	OH	H	100.0	34.0	490
	AG 505b	OH	OH	3.2	35.3	506

[a]) For systematic names, see *Footnote 4.* [b]) Compound found before in the venom of the spider *Agelenopsis aperta*. [c]) Compound found before in the venom of the spider *Hololena curta*.

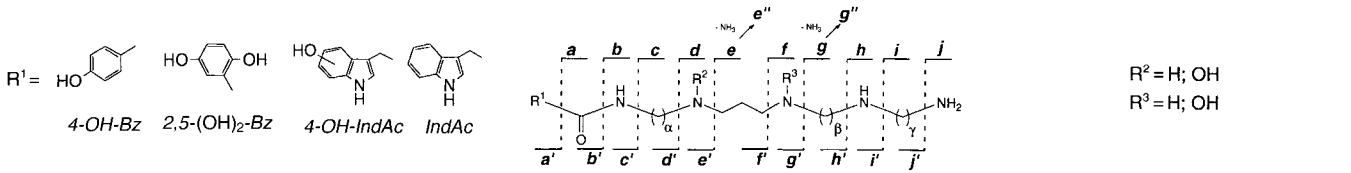
according to the characteristic fragments. Thus, the structure *IndAc-3(OH)3(OH)343* was deduced for **AG 505b**.

All the other quasi-molecular ions containing the indoleacetamide moiety (fragment m/z 130) had an odd mass number and should be considered to be pentamine derivatives.

2.4. *Indole Derivatives of Pentamines: Compounds* **AG 416**, **AG 416a**, **AG 416b**, **AG 432f**, **AG 432g**, **AG 432h**, **AG 432i**, *and* **AG 448c**. Eight different pentamine derivatives with an indoleacetamide moiety were found, and their structures were disclosed by HPLC-MS/MS experiments (*Tables 3* and *4*)[5]).

[5]) Systematic names of the natural products isolated and shown in *Tables 3* and *4:* **AG 379**, *N*-(16-Amino-4,8,12-triazahexadecyl)-4-hydroxybenzamide; **AG 379a**, *N*-(16-Amino-5,9,13-triazahexadecyl)-4-hydroxybenzamide; **AG 395**, *N*-(16-Amino-4-hydroxy-4,8,13-triazahexadecyl)-4-hydroxybenzamide; **AG 395a**, *N*-(16-Amino-4-hydroxy-4,8,12-triazahexadecyl)-4-hydroxybenzamide; **AG 395b**, *N*-(16-Amino-4,8,12-triazahexadecyl)-2,5-dihydroxybenzamide; **AG 395c**, *N*-(16-Amino-5,9,13-triazahexadecyl)-2,5-dihydroxybenzamide; **AG 432**, *N*-(16-Amino-4,8,13-triazahexadecyl)hydroxy-1*H*-indole-3-acetamide; **AG 432a**, *N*-(16-Amino-4,8,12-triazahexadecyl)hydroxy-1*H*-indole-3-acetamide; **AG 432b**, *N*-(16-Amino-4,8,13-triazahexadecyl)hydroxy-1*H*-indole-3-acetamide; **AG 432c**, *N*-(16-Amino-4,8,12-triazahexadecyl)hydroxy-1*H*-indole-3-acetamide; **AG 432d**, *N*-(16-Amino-5,9,13-triazahexadecyl)hydroxy-1*H*-indole-3-acetamide; **AG 432e**, *N*-(16-Amino-5,9,13-triazahexadecyl)hydroxy-1*H*-indole-3-acetamide; **AG 448**, *N*-(16-Amino-4-hydroxy-4,8,13-triazahexadecyl)-4-hydroxy-1*H*-indole-3-acetamide; **AG 448a**, *N*-(16-Amino-4-hydroxy-4,8,12-triazahexadecyl)-4-hydroxy-1*H*-indole-3-acetamide; **AG 448b**, *N*-(16-Amino-5-hydroxy-5,9,13-triazahexadecyl)-4-hydroxyindole-3-acetamide; **AG 464**, *N*-(16-Amino-5,9-dihydroxy-5,9,13-triazahexadecyl)-4-hydroxyindole-3-acetamide; **AG 464a**, *N*-(16-Amino-4,8-dihydroxy-4,8,13-triazahexadecyl)-4-hydroxy-1*H*-indole-3-acetamide; **AG 416**, *N*-(16-Amino-4,8,13-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 416a**, *N*-(16-Amino-4,8,12-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 416b**, *N*-(16-Amino-5,9,13-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 432f**, *N*-(16-Amino-4-hydroxy-4,8,13-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 432g**, *N*-(16-Amino-4-hydroxy-4,8,12-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 432h**, *N*-(16-Amino-5-hydroxy-5,9,13-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 432i**, *N*-(16-Amino-9-hydroxy-5,9,13-triazahexadecyl)-1*H*-indole-3-acetamide; **AG 448c**, *N*-(16-Amino-5,9-dihydroxy-5,9,13-triazahexadecyl)-1*H*-indole-3-acetamide.

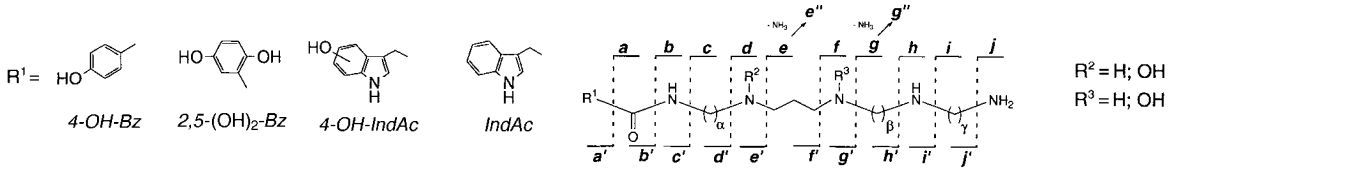
Table 3. *MS/MS Fragment Ions* [%] *of Acylpentamine Derivatives from* Agelenopsis aperta. A dash indicates that the fragmentation was not observed. Both nomenclatures used in *Sect. 2* are given [a])



Type of the fragment	*4-OH-Bz-*				*2,5-(OH)$_2$-Bz-*		*OH-IndAc-*					
	-3334 **AG 379**[a]) $\alpha=3$, $\beta=3$, $\gamma=4$	*-4333* **AG 379a** $\alpha=4$, $\beta=3$, $\gamma=3$	*-3(OH)343* **AG 395** $\alpha=3$, $\beta=4$, $\gamma=3$	*-3(OH)334* **AG 395a** $\alpha=3$, $\beta=3$, $\gamma=4$	*-3334* **AG 395b** $\alpha=3$, $\beta=3$, $\gamma=4$	*-4333* **AG 395c** $\alpha=4$, $\beta=3$, $\gamma=3$	*-3343* **AG 432** $\alpha=3$, $\beta=4$, $\gamma=3$	*-3334* **AG 432a** $\alpha=3$, $\beta=3$, $\gamma=4$	*-3343* **AG 432b** $\alpha=3$, $\beta=4$, $\gamma=3$	*-3334* **AG 432c** $\alpha=3$, $\beta=3$, $\gamma=4$	*-4333* **AG 432d** $\alpha=4$, $\beta=3$, $\gamma=3$	*-4333* **AG 432e** $\alpha=4$, $\beta=3$, $\gamma=3$
a	–	–	–		–	–	–	–			–	–
a′	–	–	–		–	–	146 (9)	146 (10)			146 (12)	146 (7)
b	260 (5)	260 (18)	276 (7)		260 (28)	260 (27)	260 (8)	260 (8)			260 (23)	260 (30)
b′	121 (14)	121 (17)	121 (11)		137 (8)	–	–	–			–	–
c	–	243 (5)	259 (5)		243 (17)	–	243 (8)	243 (8)			–	–
c′	–	–	–		–	–	–	–			–	–
d	–	189 (6)	205 (6)		–	–	203 (5)	203 (4)			189 (4)	189 (5)
d′	178 (100)	192 (30)	178 (100)		194 (100)	–	231 (100)	231 (100)			245 (27)	245 (21)
e	186 (7)	172 (30)	186 (9)		186 (15)	172 (27)	186 (12)	186 (11)			172 (47)	172 (51)
e′	–	–	–		–	–	–	–			–	–
e″	–	–	–		–	–	169 (4)	169 (5)			–	–
f	–	132 (8)	–		–	132 (18)	–	–			–	–
f′	235 (34)	249 (78)	251 (28)		251 (48)	265 (100)	288 (27)	288 (23)			302 (100)	302 (100)
g	129 (18)	115 (38)	129 (92)		129 (31)	115 (40)	129 (23)	129 (23)			115 (39)	115 (43)
g′	252 (5)	266 (12)	–		–	282 (21)	305 (11)	305 (10)			319 (43)	319 (61)
g″	112 (57)	98 (100)	112 (76)		112 (96)	98 (65)	112 (46)	112 (48)			98 (60)	98 (57)
h	–	–	–		–	–	–	–			–	–
h′	292 (12)	306 (50)	322 (50)	308 (19)	308 (30)	322 (31)	359 (15)	344 (10)	359 (10)	344 (10)	359 (38)	359 (51)
i	72 (10)	–	–	72 (18)	72 (31)	–	–	72 (5)	–	72 (5)	–	–
i′	309 (7)	–	–	325 (22)	325 (63)	–	–	361 (12)	–	361 (10)	376 (9)	376 (10)
j	–	–	–	–	–	–	–	–	–	–	–	
j′	363 (6)	363 (5)	379 (18)		379 (20)	379 (7)	416 (17)		–	416 (17)	416 (9)	416 (18)

[a]) For systematic names, see *Footnote 5*.

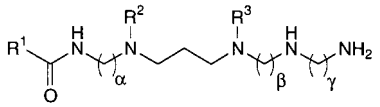
Table 3 (cont.)

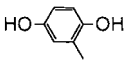
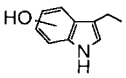
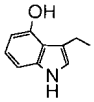


Type of the fragment	*4-OH-IndAc-*					*IndAc-*								
	-3(OH)343 **AG 448**[a]) $\alpha=3$, $\beta=4$, $\gamma=3$	*-3(OH)334* **AG 448a** $\alpha=3$, $\beta=3$, $\gamma=4$	*-4(OH)333* **AG 448b** $\alpha=4$, $\beta=3$, $\gamma=3$	*-4(OH)3(OH)33* **AG 464** $\alpha=4$, $\beta=3$, $\gamma=4$	*-3(OH)3(OH)43* **AG 464a** $\alpha=3$, $\beta=4$, $\gamma=3$	*-3343* **AG 416** $\alpha=3$, $\beta=4$, $\gamma=3$	*-3334* **AG 416a** $\alpha=3$, $\beta=3$, $\gamma=4$	*-4333* **AG 416b** $\alpha=4$, $\beta=3$, $\gamma=3$	*-3(OH)343* **AG 432f** $\alpha=3$, $\beta=4$, $\gamma=3$	*-3(OH)334* **AG 432g** $\alpha=3$, $\beta=3$, $\gamma=4$	*-4(OH)333* **AG 432h** $\alpha=4$, $\beta=3$, $\gamma=3$	*-43(OH)33* **AG 432i** $\alpha=4$, $\beta=3$, $\gamma=3$	*-4(OH)3(OH)33* **AG 448c** $\alpha=4$, $\beta=3$, $\gamma=3$
a	–		–	–	–	–		–	–		–		–	
a′	146 (19)		146 (17)	146 (18)	146 (14)	130 (5)		130 (5)	130 (22)		130 (23)		130 (37)	
b	276 (17)		276 (23)	292 (11)	292 (9)	260 (6)		260 (8)	276 (4)		–		–	
b′	–		–	–	–	–		–	–		–		–	
c	–		–	–	–	243 (4)		–	–		–		–	
c′	–		–	–	–	–		–	–		–		–	
d	–		205 (10)	221 (10)	–	–		–	–		205 (11)		221 (9)	
d′	231 (100)		245 (33)	245 (19)	231 (100)	215 (100)		229 (15)	215 (100)		229 (100)		229 (37)	
e	186 (15)		172 (28)	188 (30)	–	186 (20)		172 (37)	186 (6)		172 (56)	188 (31)	188 (48)	
e′	–		–	–	–	–		–	258 (5)		–	–	–	
e″	169 (10)		–	–	–	169 (9)		–	169 (5)		–	–	–	
f	–		–	–	–	–		–	–		–	–	–	
f′	304 (21)		318 (15)	318 (10)	304 (10)	272 (60)		286 (62)	288 (20)		302 (10)	–	302 (27)	
g	129 (80)		115 (39)	115 (16)	129 (15)	129 (32)		115 (27)	129 (78)		115 (84)		115 (35)	
g′	321 (8)		335 (19)	351 (9)	337 (5)	289 (16)		303 (20)	305 (7)		319 (23)		335 (16)	
g″	112 (73)		98 (30)	98 (12)	112 (22)	112 (75)		98 (34)	112 (82)		98 (57)		98 (26)	
h	–		–	–	–	–		–	–		–		–	
h′	375 (18)	361 (30)	375 (34)	391 (14)	377 (8)	343 (17)	329 (30)	343 (31)	359 (31)	345 (10)	359 (70)		375 (17)	
i	–	72 (19)	–	–	72 (6)	–	72 (10)	–	–	72 (7)	–		–	
i′	–	378 (30)	–	–	394 (18)	–	346 (34)	–	–	362 (16)	–		392 (5)	
j	–	–	–	–	–	–	–			–	–		–	
j′	432 (20)		432 (7)	448 (7)	448 (18)	400 (32)		400 (5)	416 (10)		416 (10)		432 (8)	

[a]) For systematic names, see *Footnote 5*.

Table 4. *Pentamine Derivatives Found in* Agelenopsis aperta *Spider Venom*



R^1	Name[a])	R^2	R^3	α	β	γ	c [%]	t_R [min]	Parent quasi-molecular ion $[M+H]^+$
	**AG 379**	H	H	3	3	4	2.9	20.6	380
	AG 379a	H	H	4	3	3	2.5	21.9	380
	AG 395	OH	H	3	4	3	2.1	22.0	396
	AG 395a	OH	H	3	3	4		22.0	396
	**AG 395b**	H	H	3	3	4	1.5	24.0	396
	AG 395c[b])	H	H	4	3	3	0.4	26.9	396
	**AG 432**	H	H	3	4	3	2.9	27.3	433
	AG 432a	H	H	3	3	4		27.3	433
	AG 432b	H	H	3	4	3	3.3	28.9	433
	AG 432c	H	H	3	3	4		28.9	433
	AG 432d	H	H	4	3	3	2.1	30.0	433
	AG 432e	H	H	4	3	3	0.5	32.4	433
	**AG 448**[c])	OH	H	3	4	3	14.8	28.9	449
	AG 448a	OH	H	3	3	4		28.9	449
	AG 448b	OH	H	4	3	3	2.5	32.4	449
	AG 464	OH	OH	4	3	3	7.2	32.4	465
	AG 464a[c])	OH	OH	3	4	3	0.6	29.6	465
	**AG 416**[c])	H	H	3	4	3	14.5	31.7	417
	AG 416a	H	H	3	3	4		31.7	417
	AG 416b[b])	H	H	4	3	3	6.3	33.9	417
	AG 432f	OH	H	3	4	3	9.9	33.6	433
	AG 432g	OH	H	3	3	4		33.6	433
	AG 432h	OH	H	4	3	3	1.1	36.5	433
	AG 432i	H	OH	4	3	3		36.5	433
	AG 448c	OH	OH	4	3	3	13.6	36.5	449

[a]) For systematic names, see *Footnote 5*. [b]) Compound found before in the venom of the spider *Hololena curta*. [c]) Compound found before in the venom of the spider *Agelenopsis aperta*.

Only one of these compounds has been characterized in *Agelenopsis aperta* spider venom (**AG 416**), and another one (**AG 416b**) in *Hololena curta* (Aranea: Agelenidae) spider venom (**HO 416b**) [7]. For the quasi-molecular ions at *m*/*z* 416, 432, and 448, the difference of ± 16 and ± 32 Da could be explained by a different number of OH substituents.

For the compounds **AG 416b**, **AG 432h**, **AG 432i**, and **AG 448c**, no signals were registered at *m*/*z* 129 (**g**) and 112 (**g″**), but corresponding ones appeared at *m*/*z* 115 (**g**) and 98 (**g″**). The difference of -14 Da testified that instead of a polyamine with 3 and 4 CH_2 groups distributed between the three N-atoms nearest to the right-hand terminus as in **AG 416**, these compounds have only propane-1,3-diyl groups between N(9), N(13), and the terminal N-atom. The fact that the signal at *m*/*z* 229 (**d′**) shaved the same difference of $+14$ Da compared to the signal at *m*/*z* 215 (**d′**) of **AG 416**, established that these compounds contained the pentamine *PA4333* as the basic backbone.

For the co-eluted compounds **AG 432h** and **AG 432i**, the two signals at *m*/*z* 172 and 188 certify that the OH group was present at different N-atoms (see *Table 3*), and could be the one of substituents R^2 and R^3, respectively.

Two other pairs of co-eluted compounds with the same molecular mass, **AG 416** and **AG 416a**, as well as **AG 432f** and **AG 432g**, showed isomerism in the pentamine backbone. The fragment ions at *m*/*z* 343 (**h′**) of **AG 416**, as well as those at *m*/*z* 329 (**h′**) and 346 (**i′**) of **AG 416a** allowed to differentiate structurally these two compounds. Compound **AG 416** with the pentamine *PA3343* is known [19] and does not possess fragment ions at *m*/*z* 346, 329, and 72. Therefore, **AG 416a** is a new isomer of **AG 416** with a polyamine backbone *PA3334*. The same type of isomerism was observed for the acylpentamines **AG 432f** and **AG 432g**. The corresponding signals were at *m*/*z* 359 (**h′**) for **AG 432f** as well as at *m*/*z* 345 (**h′**), 362 (**i′**), and 72 (**i**) for **AG 432g**, certifying this isomerism in the polyamine chain.

The structure of the toxin **AG 448c** consisted of a pentamine *PA4333* substituted with two OH groups at the same two N-atoms as in the hexamine **AG 505b** (see *Tables 2* and *4*). In the case of **AG 448c**, the signals at *m*/*z* 229, 115, and 98 testified the sequence *PA4333* of the polyamine chain.

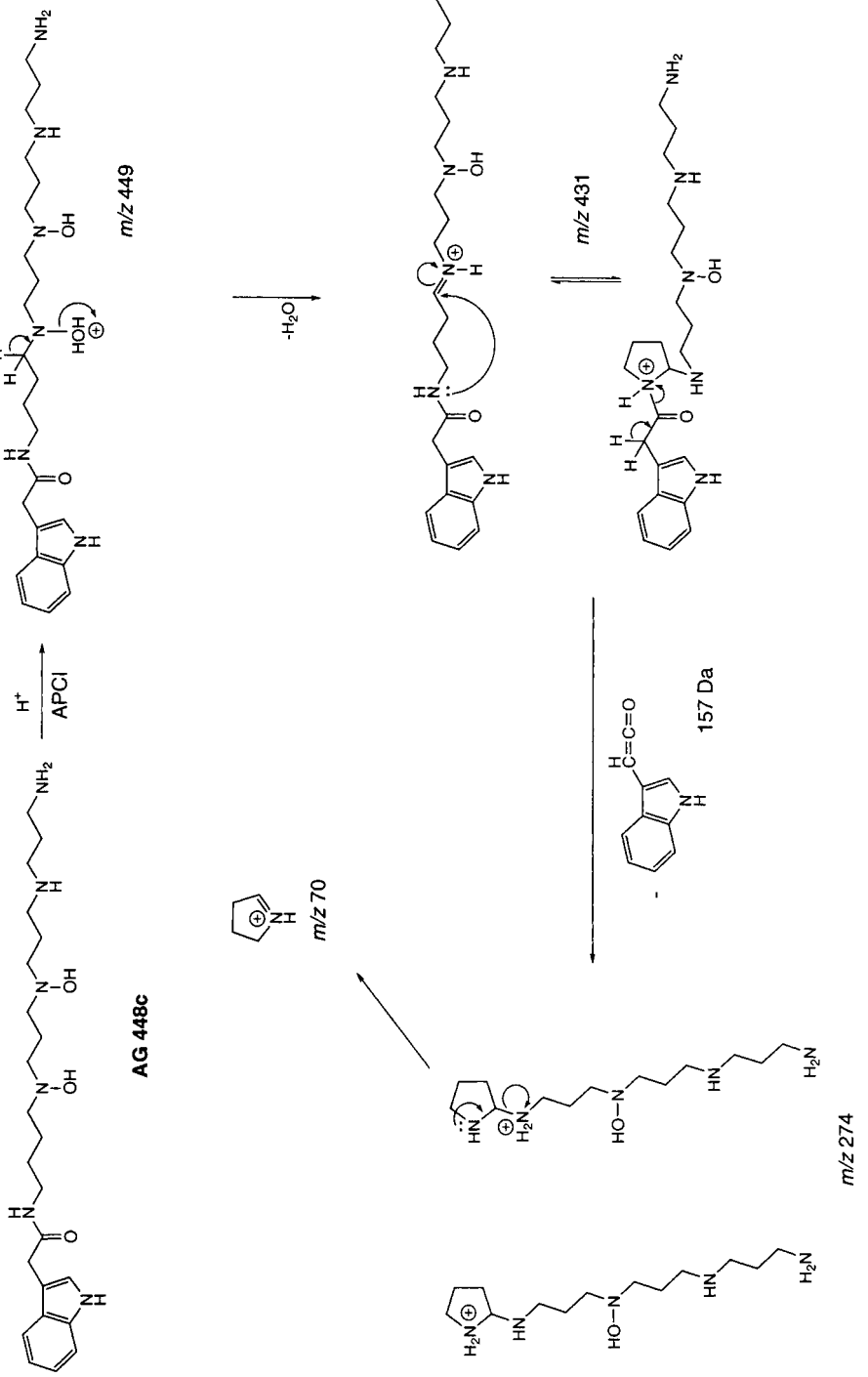
The signal *m*/*z 70* could be found only in MS/MS data of acylpolyamines with a *PA4333* pattern and substituted at N(5) with an OH group, *i.e.* for **AG 432h**, **AG 448c**, **AG 448b**, and **AG 464**. In the *Scheme*, a mechanism of the corresponding fragment-ion formation is proposed. In the ionization step, the OH group at N(5) is protonated, and then H_2O is eliminated. The resulting ion *m*/*z* 431 forms, by neighboring-group participation, a 2-substituted pyrrolidinium derivative. After elimination of both side chains, the pyrrolidinium ion with *m*/*z* 70 is generated. This ion signal is characteristic for all acylpolyamines *PA4333* carrying an HO−N(5) and might be some kind of fingerprint.

2.5. *Hydroxyindole Derivatives: Compounds* **AG 489b**, **AG 505**, **AG 521**, **AG 432**, **AG 432a**, **AG 432b**, **AG 432c**, **AG 432d**, **AG 432e**, **AG 448**, **AG 448a**, **AG 448b**, **AG 464**, *and* **AG 464a**. The group of hydroxyindole-containing polyamines is the largest one and consists of eleven pentamine and three hexamine toxins. Their MS/MS measurements revealed a signal at *m*/*z* 146, corresponding to the fragment ion **a′**, which was observed with a similar relative intensity for all compounds of this group. In the case of the major components possessing the typical absorption for the 4-hydroxy-1*H*-indole-3-acetamide moiety (**AG 448**, **AG 448a**, **AG 464**, **AG 505**, and **AG 521**), the position of the OH group was deduced from their UV spectra.

The three pairs of compounds **AG 432** and **AG 432b**, **AG 432a** and **AG 432c**, and **AG 432d** and **AG 432e**, with the same quasi-molecular ion at *m*/*z* 433 and close but, nevertheless, different t_R, had identical MS/MS data. This could be caused by the location of the OH group at the position 4 or at the positions 5 or 6 of the indole moiety. Because these constituents were the minor ones, the problem could not be solved by HPLC-NMR; it will be elucidated later by comparing the MS/MS data and t_R of these natural toxins with the corresponding synthetic analoga.

Fig. 5 illustrates the principle of structure elucidation by comparing MS/MS data of **AG 448c** (t_R 36.5 min) with that of the known **AG 448** (t_R 28.9 min). As it was mentioned before, the UV spectra for the fraction with t_R 28.9 min were typical for the 4-hydroxy-1*H*-indole-3-acetamide moiety and could be additionally confirmed by the

Scheme. *Mechanism of the formation of the ion* m/z *70 from acylpolyamines* PA4333, e.g., **AG 448c**



signal at *m*/*z* 146 (**a′**, *Table 3*) instead of that of a naked indole nucleus at *m*/*z* 130. The most intensive ion signal at *m*/*z* 231 (**d′**), as well as signals at *m*/*z* 129 (**g**) and 112 (**g″**), were in agreement with the pentamine backbones *PA3343* or *PA3334*. The signals at *m*/*z* 375 (**h′**) for **AG 448** as well as at *m*/*z* 378 (**i′**), 361 (**h′**), and 72 (**i**) for **AG 448a** established the presence of the co-eluted pentamine isomers containing in one case the *PA3343* (**AG 448**) and in the other the *PA3334* pattern (**AG 448a**). The structure *PA4333* for **AG 448c** was deduced from the signals at *m*/*z* 115 (**g**), 188 (**e**), 302 (**f′**), and 229 (**d′**). The relatively low intensity of the **d′** signal of **AG 448c** at *m*/*z* 229 compared to the **d′** signal of **AG 448** and **AG 448a** at *m*/*z* 231 could be explained by the more favorable formation of a six-member ring involving the C=O O-atom in the last cases.

Systematizing the data on the different types of fragmentation characteristic for acylpolyamines, it was noticed that for the known major component **AG 464**, the already proposed structure *4-OH-IndAc-3(OH)3(OH)43* seems to be wrong [19], and, therefore, an acylpentamine structure *4-OH-IndAc-4(OH)3(OH)33* was postulated. In *Fig. 6*, the MS/MS data of **AG 448c** and **AG 464**, as well as their structures, are compared. Both compounds showed the same signals at *m*/*z* 70, 98 (**g″**), 115 (**g**), 188 (**e**), and 221 (**d**), which means that they possess the same pentamine backbone *PA4333*. Additionally, the alignment of both quasimolecular ions revealed a correspondence between signal pairs at *m*/*z* 449 and 465 ($[M+H]^+$), 432 and 448 (**j′**), 375 and 391 (**h′**), 335 and 351 (**g′**), 302 and 318 (**f′**), as well as at *m*/*z* 229 and 245 (**d′**). The similarity of the two acylpolyamines was thus confirmed, their difference being due to the presence of one OH group at the lipophilic moiety. Additionally, the *PA3343* isomer **AG 464a** was detected in lower concentration at t_R 29.6 min than the *PA4333* isomer **AG 464** (t_R 32.4 min). The latter one, as reported in the literature [19], has a similar elution time as **AG 416** (31.7 min). Therefore, the structure of **AG 464** has to be revised to that presented in *Fig. 6*.

2.6. *Hydroxybenzene Derivatives: Compounds* **AG 452**, **AG 379**, **AG 379a**, **AG 395**, *and* **AG 395a**. *Dihydroxybenzene Derivatives: Compounds* **AG 452a**, **AG 468**, **AG 395b**, *and* **AG 395c**. Another class of polyamine derivatives possesses smaller t_R values (*Fig. 3*) and a different absorption curve showing only one λ_{max} at 251 nm. As mentioned above, this absorption is typical for 4-hydroxybenzoic acid derivatives. The only known compound of this type from *Agelenopsis aperta* is hexamine **AG 452** (*Table 1*) with t_R 23.0 min [19]. Analyzing the MS/MS data of the fraction with t_R 22.0 min (*Fig. 7,a*) and applying the principles of the above-discussed fragmentation mechanisms, two co-eluted pentamines with structures *4-OH-Bz-3(OH)343* (**AG 395**) and *4-OH-Bz-3(OH)334* (**AG 395a**) were disclosed. Two further fractions containing *m*/*z* 396 quasi-molecular ions were found (*Fig. 7,b* and *c*). For compound **AG 395b** with t_R 24.0 min, yielding the fragment ion **d′** at *m*/*z* 194 (100%) instead of *m*/*z* 178 (100%) for **AG 395**, an isomer containing the OH-group at the lypophilic moiety could be postulated. As the compound is the minor one in the fraction containing **AG 452**, no UV spectrum was available. Considering that the 2,5-dihydroxybenzoic moiety is that known for acylpolyamines from the spider *Agelenopsis aperta* (**AG 468**), as well as for those from the spider *Hololena curta* (**HO 395**, **HO 452**, and **HO 468**) [7], the structure *2,5-(OH)$_2$-Bz-3334* is proposed for **AG 395b**. Analogously, the fragment ions at *m*/*z* 322 (**h′**), 282 (**g′**), 265 (**f′**), 172 (**e**), and 115 (**g**) are consistent with the *2,5-(OH)$_2$-Bz-4333* structure for **AG 395c** (t_R 24.0 min). The structures of **AG 379**, **AG 379a**, and **AG 452a**

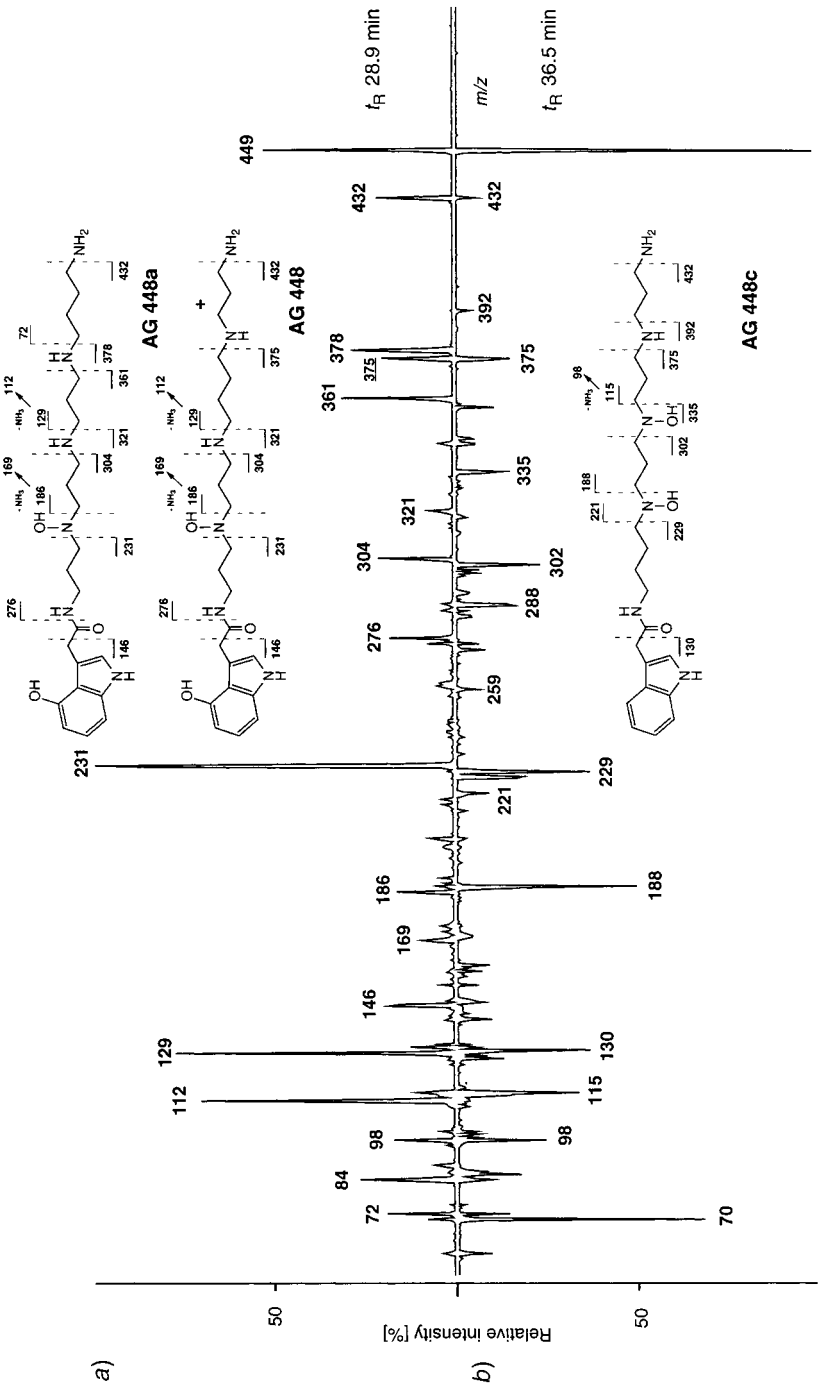


Fig. 5. *HPLC-APCI-MS/MS Experiments for the group of compounds with quasi-molecular ion* m/z *449*: a) *MS/MS analysis of* **AG 448** *and* **AG 448a** (both have the same t_R; the characteristic ion *m/z* 375 for **AG 448** is underlined); b) *MS/MS analysis of* **AG 448c**

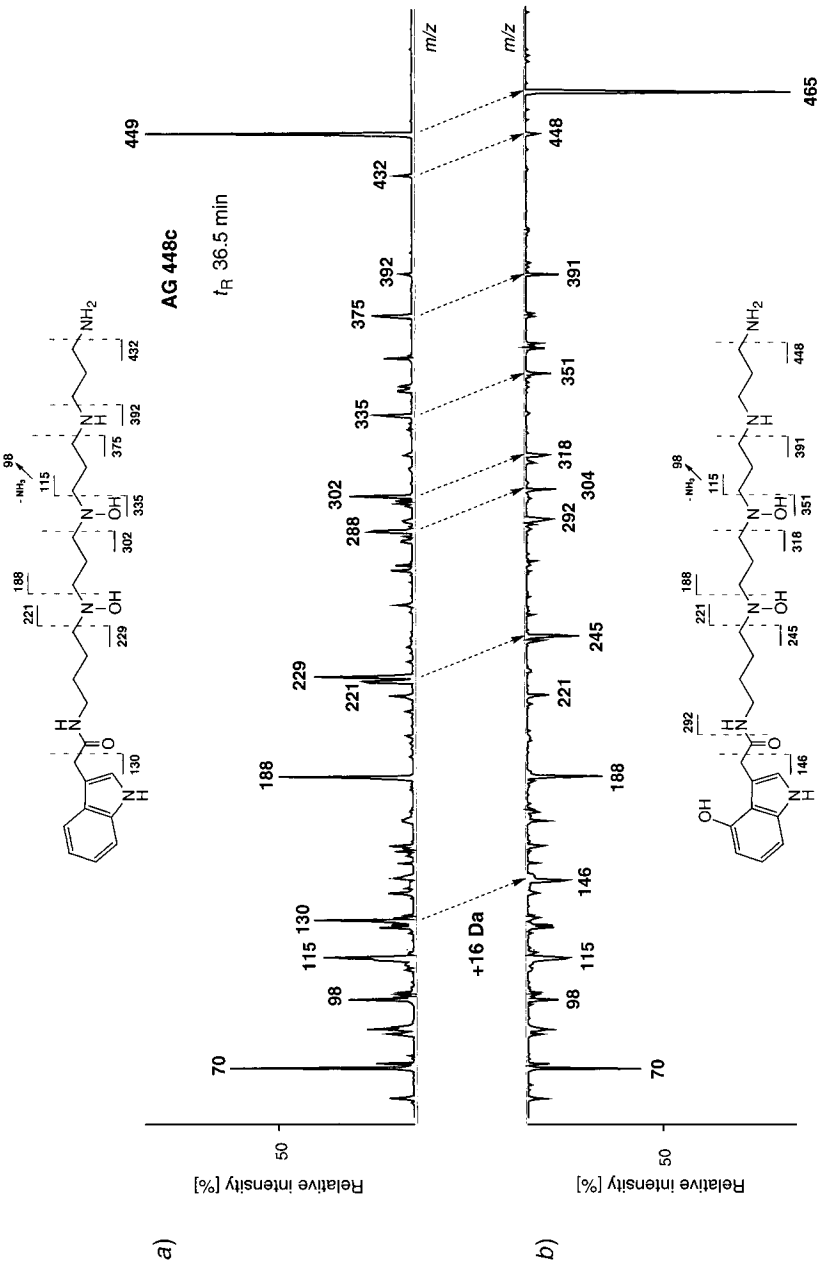


Fig. 6. *MS/MS Analysis of* a) **AG 448c** *and* b) **AG 464**. The signals connected with arrows are differing by 16 Da and correspond to the same type of fragmentation.

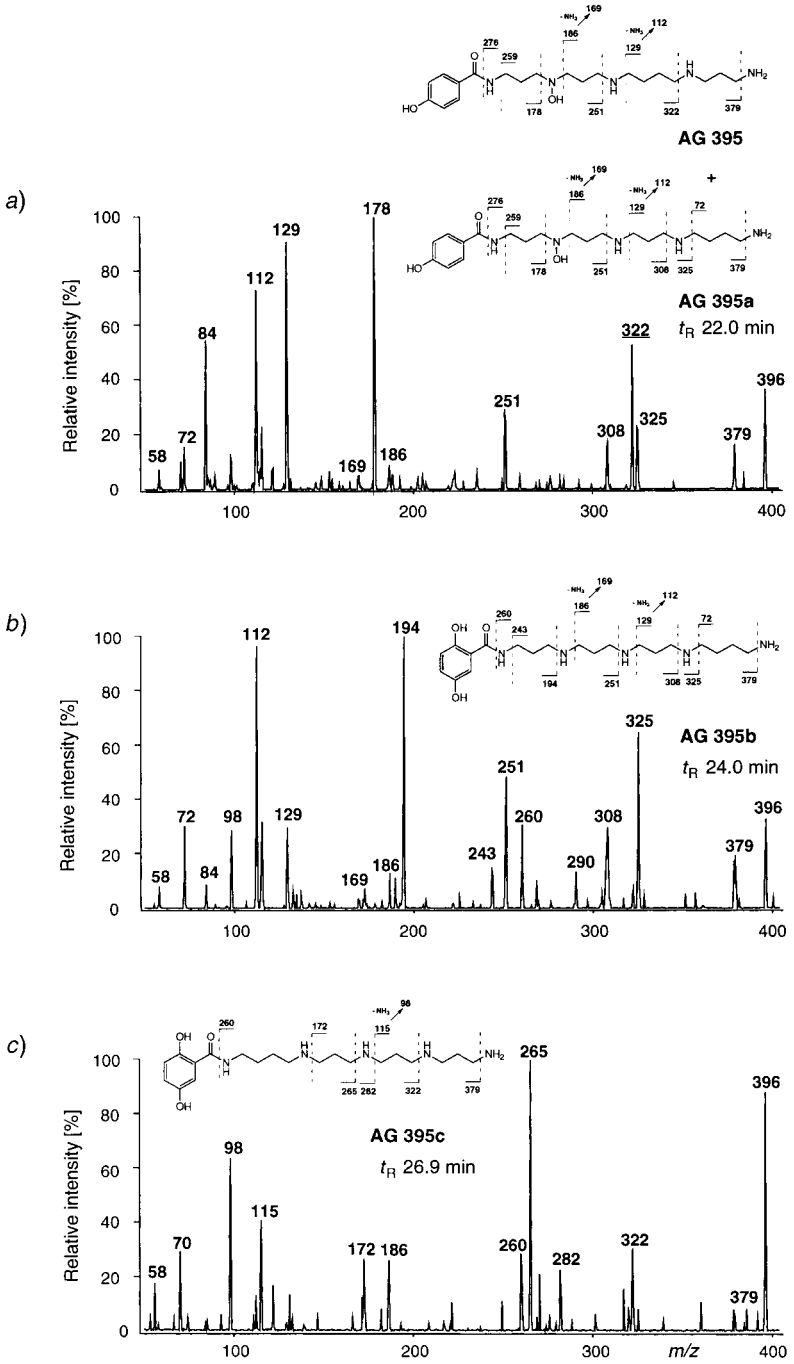


Fig. 7. *MS/MS Analysis of* a) **AG 395** *and* **AG 395a**, b) **AG 395b**, *and* c) **AG 395c**. Compounds **AG 395** and **AG 395a** have the same t_R and differ in three fragment ions at *m*/*z* 308, 322, and 325.

were elucidated as *4-OH-Bz-3334*, *4-OH-Bz-4333*, and *2,5-(OH)$_2$-Bz-33343*, respectively.

Conclusion. – The venom of the spider *Agelenopsis aperta* was re-analyzed by on-line coupled HPLC-APCI-MS/MS. Thirty-three polyamine-containing compounds (only eight were known for this venom before) were detected, and their structures were elucidated.

The results were obtained by UV spectroscopy as well as MS and MS/MS data. The structures of the seven known compounds were confirmed. In one case, the use of APCI-MS/MS even allowed the correction of the structure proposed for **AG 464**. The main advantage of the method is a reliable structure definition of even co-eluted isomers.

In the class of the acylpentamines, three different types of polyamines were found, and for the first time, polyamines *PA4333* and *PA3334* were characterized in *A. aperta* venom.

From the chemotaxonomical point of view, the above results establish that the *A. aperta* spider species is closer related to the *Hololena curta* and *Paracoelotes birulai* species, and they are a hint for further work in this domain.

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Experimental Part

General. Lyophilized *Agelenopsis aperta* venom was purchased from *Spider Pharm. Inc.*, Yarnell, AZ, U.S.A. During all experiments, solid material was stored at $-80°$ and the stock soln. of the venom at $-20°$. Solvents and reagents: MeCN (HPLC grade, *Scharlau*, E-Barcelona); CF_3COOH, *purum* (*Fluka*, Buchs, Switzerland). The H_2O was purified with a *Milli-Q$_{RG}$* apparatus (*Millipore*, Milford, MA, U.S.A.). Venom preparation: lyophilized venom (5 mg) was dissolved in 60 µl of 1% CF_3COOH soln. in H_2O/MeCN 3:2 at r.t., the soln. filtered through a 0.45 µm filter (*Eppendorf*, Hamburg, Germany), and the latter rinsed with 20 µl of H_2O. HPLC: The clear soln. (1–5 µl) was injected for every run and analyzed with HPLC-UV(DAD), HPLC-UV(DAD)-APCI-MS, and HPLC-UV(DAD)-APCI-MS/MS. All investigations were carried out with a *Waters-626-LC* system, fitted with a *996* photodiode-array detector, a *600S* controller, a *Millennium* chromatography manager *2010* v. 2.15 (*Waters Corp.*, Milford, MA, USA), and a *Rheodyne-Rotary-7725i* rotary valve, fitted with a 5-µl loop (*Rheodyne*, Cotati, CA, USA).

Chromatographic Conditions. Macherey-Nagel C$_{18}$ HD column (3 µm, 4.6 × 250 mm; *Macherey-Nagel*, F-Hoerdt); flow rate 0.5 ml min^{-1}. Mobile phase: step gradient during the first 5 min from 0 to 10% of solvent *B*, then 75 min from 10 to 45% of *B* and 20 min from 45 to 100% of *B* (solvent *A:* 0.1% soln. of CF_3COOH in H_2O; solvent *B:* 0.1% soln. of CF_3COOH in MeCN). MS: APCI-MS and APCI-MS/MS experiments were performed on a *Finnigan-TSQ-700* triple-stage quadrupole instrument equipped with an atmospheric-pressure chemical-ionization (APCI) ion source (*Finnigan*, San José, CA, USA). The APCI operating conditions in positive mode were: vaporizer temp. 450°, corona voltage 5 kV; heated capillary temp. 250°, sheath gas N_2 with an inlet pressure of 40 psi; conversion dynode -15 kV. For MS/MS experiments: collision gas Ar with a relative pressure of 2.5–3.3 mTorr; collision-induced dissociation offset (Coff) -27 eV.

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