The Spider *Paracoelotes birulai:* Detection and Structure Elucidation of New Acylpolyamines by On-Line Coupled HPLC-APCI-MS and HPLC-APCI-MS/MS

by **Sergiy Chesnov**[1]), **Laurent Bigler**, and **Manfred Hesse***

Organisch-chemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich

The lyophilized venom of the spider *Paracoelotes birulai* (Araneidae: Amaurobiidae) has been analyzed. A number of acylpolyamines were found and separated. By on-line coupled high-performance liquid chromatography and atmospheric-pressure chemical-ionization mass spectrometry, the structures of the three most abundant compounds **PB 490** (*N*-(16-guanidino-4-hydroxy-4,8,12-triazahexadecyl)-2-(4-hydroxyindol-3-yl)acetamide; *Fig. 3,b*), **PB 421** (*N*-(16-guanidino-4-hydroxy-4,8,12-triazahexadecyl)-2-(4-hydroxybenzamide; *Fig. 4,a*), and **PB 448** (*N*-(16-amino-4-hydroxy-4,8,12-triazahexadecyl)-2-(4-hydroxyindol-3-yl)acetamide; *Fig. 5,b*) were elucidated. Two different types of polyamines were found in the α-palutoxins and compared with acylpolyamines from the Agelenidae spider family. The results of this investigation will initiate further chemotaxonomical studies on spiders.

Introduction. – Spider venoms attract the undivided attention of scientists interested in these unique natural mixtures of different classes of biologically active compounds. Chemists elucidate the structures of the constituents of spider venoms, biologists try to derive the mechanisms of the interactions of venoms with the spiders' prey and create new specific insecticides, while pharmacists search for new drugs. The continuing discovery of new spider species also stimulates these investigations.

Depending on the family and species, spider venoms contain, in diverse ratios, various natural compounds like amino acids, polyamines, purine bases, acylpolyamines, peptides, polypeptides or proteins; sometimes just some of these compounds, sometimes all of them together are present. In the last two decades, the assumption that only proteins are responsible for the toxicity of spider venoms was revised, and the activities of the compounds present in the venoms have been investigated. At the present time, several classes of natural products are believed to be the toxic constituents of spider venoms. Acylpolyamines, peptides, polypeptides, proteins, either by themselves or co-acting with one another, are responsible for the toxic action of the venom.

Being available in only milligram amounts, spider venoms are quite difficult to investigate by classical methods, and the successful application of NMR spectroscopy or elemental analysis for structure elucidation is more of an exception than a generally applied procedure.

Paracoelotes birulai, known from several sources as *Paracoelotes luctuosus* (Araneae: Amaurobiidae), is found in Kazakhstan, China, Korea, and Japan. It was

[1]) Part of the Ph.D. thesis of *S. C.*

reclassified from the family Agelenidae to the family Amaurobiidae on the basis of morphological criteria.

The structures of almost all known acylpolyamines isolated from the family Agelenidae were elucidated by mass spectroscopy (normally: fast-atom-bombardment (FAB); matrix-assisted laser-desorption ionization (MALDI); chemical ionization (CI); electrospray ionization (ESI)) or NMR (for the most abundant compounds) and verified by total synthesis [1–3]. In contrast, no data could be found in the literature concerning studies on acylpolyamines isolated from the venoms of spiders in the Amaurobiidae family.

As a continuation of our work on low-molecular-weight compounds present in spider venom [4–6], the venom of *Paracoelotes birulai* has been analyzed. For us, it was interesting to establish the difference between the structures of the acylpolyamines present in the venoms of spiders in the family Agelenidae (*Agelenopsis aperta*, *Hololena curta*) and those obtained from the venom of *Paracoelotes birulae*. Reversed-phase liquid chromatography (RP-HPLC), coupled on-line with atmospheric-pressure chemical-ionization mass spectroscopy (APCI-MS), has been used successfully for the isolation and structure elucidation of polyamines contained in plants, and this method was also employed during these studies. Recently, the peptide fraction of the venom of *Paracoelotes birulae* was analyzed [7], and, according to the nomenclature proposed by the authors (the peptides were assigned the name δ-palutoxins), acylpolyamines from this species will be called α-palutoxins.

Herewith, several novel polyamine-containing toxins from *Paracoelotes birulae* venom are reported.

Results and Discussion. – *Sample Preparation*. Lyophilized *Paracoelotes birulae* venom was dissolved in a H_2O/MeCN solution of CF_3COOH (TFA) and filtered, which resulted in a clear solution that was further analyzed without additional handling.

HPLC Analysis. The alteration of several available columns had shown that a *RP18* column with a particle size 3 μm and the mobile phase H_2O/MeCN containing 0.1% of TFA leads to the best separation of the venom constituents. It was possible to separate several UV-active compounds by HPLC (*Fig. 1,a*), but the small amount of available venom makes the collection of α-toxin-containing fractions and their further investigation (*e.g.*, by NMR) quite laborious. Therefore, this problem could best be tackled with special experiments.

HPLC-UV(DAD)-APCI-MS. It is known that spider venoms are complex natural mixtures of amino acids, purine bases, acylpolyamines, peptides, polypeptides, proteins and even free polyamines, as we know from our recent studies [6]. All these classes of compounds can be separated and detected by means of the HPLC-UV(DAD)-APCI-MS technique.

A preliminary APCI-MS gave a number of quasi-molecular ions $[M+H]^+$ at *m/z* 422, 449, 491, and several others. To get the correspondence between the molecular weights of these compounds and their UV signals, the HPLC-UV(DAD) coupled on-line with an APCI mass spectrometer was applied. All experiments were performed in full-scan mode (FS).

The UV chromatogram (254-nm detection wavelength) is very similar to the mass spectrum reconstructed ion chromatogram (RIC; see *Fig. 1,b*).

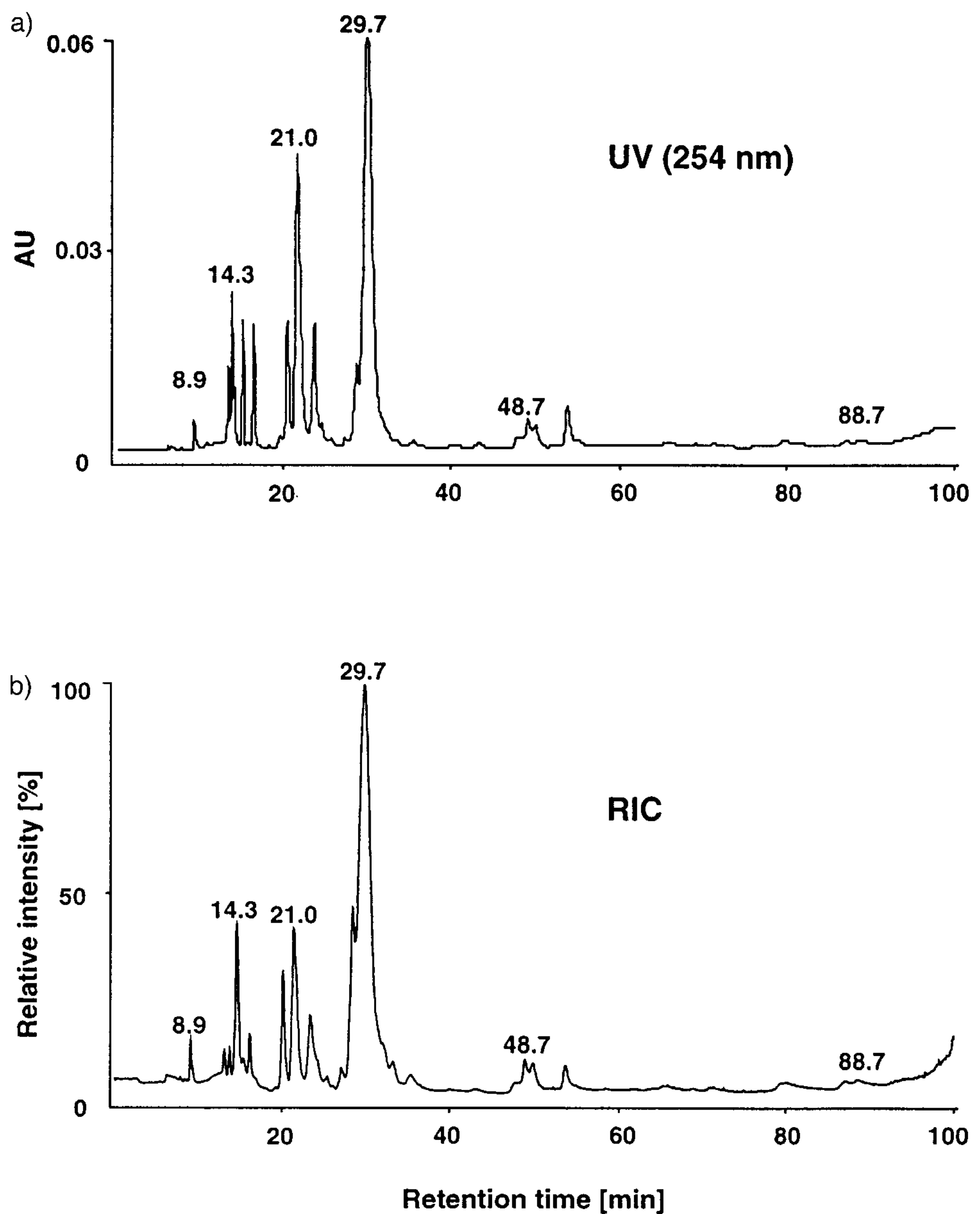


Fig. 1. *HPLC-UV(DAD)/APCI-MS Chromatograms of* Paracoelotes birulai *venom. a*) UV Detection at 254 nm; in absorbance units (AU). *b*) RIC Detection. For HPLC and MS conditions, see *Exper. Part.*

The fraction selected for further investigations was located between those containing the purine bases and those containing peptides (18–40 min). Several well-separated signals in this time domain of the UV chromatogram could be attributed to acylpolyamines.

Fig. 2 shows more-detailed views of the RIC (*Fig. 2,d*) and extracted-ion chromatograms for the most abundant $[M+H]^+$ signals with m/z 422 (*Fig. 2,a*), 449 (*Fig. 2,b*), and 491 (*Fig. 2,c*). The corresponding compounds were selected for further

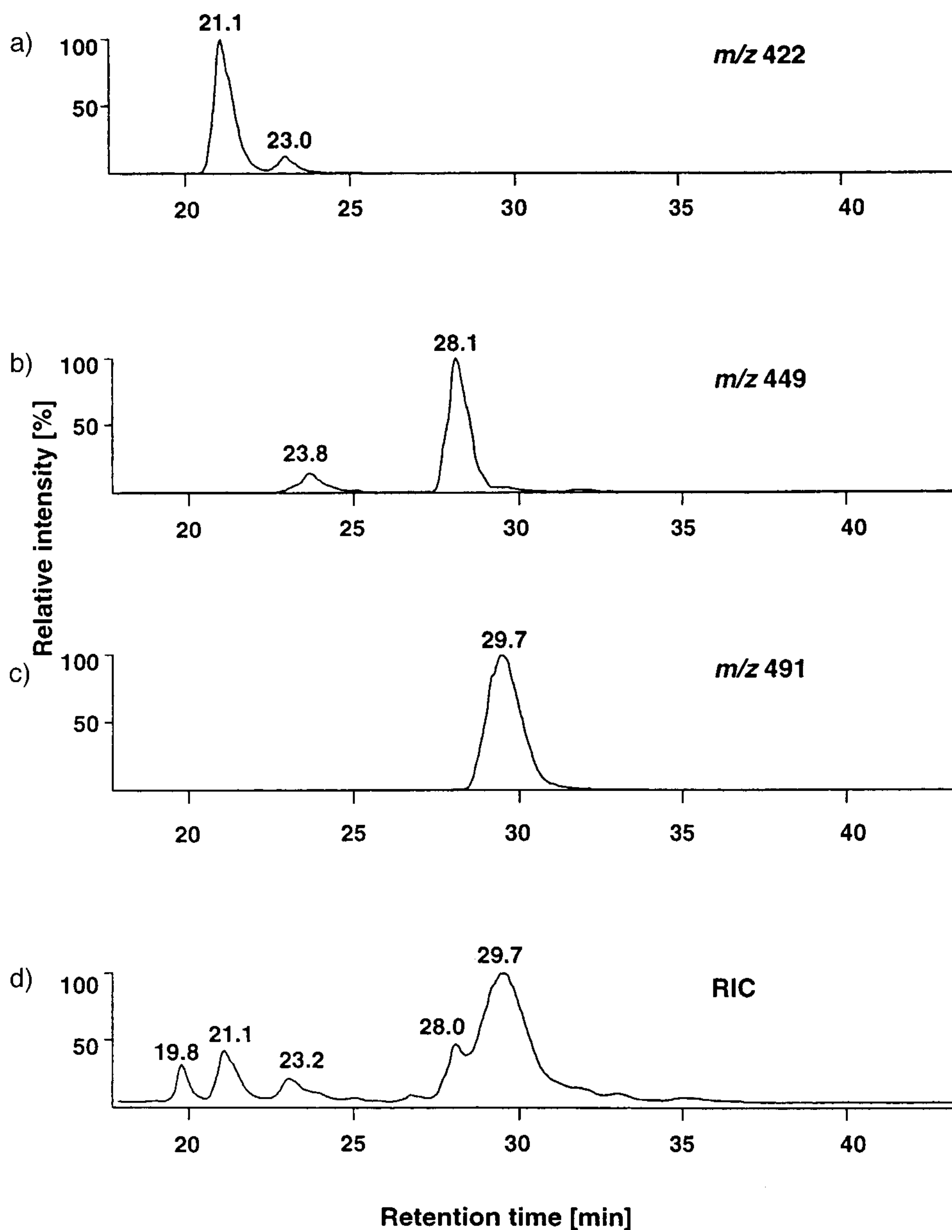


Fig. 2. *Acylpolyamine-containing fraction analyzed by HPLC-UV(DAD)/APCI-MS. a)–c)* Extracted-ion chromatograms of quasi-molecular ions m/z 422, 449, 491. *d*) RIC Chromatogram. For HPLC and MS conditions, see *Exper. Part.*

investigation. That almost all of the extracted ion chromatograms contain more than one quasi-molecular-ion peak establishes the isometric nature of compounds in this group. This paper reports only the results obtained for the main compounds; details of the minor components will be published later.

Analysis of the UV spectra shows that the compound with *m*/*z* 422 for the quasi-molecular ion and with a retention time (t_R) of 21.0 min has a UV maximum at 251 nm (*Fig. 2*,*a*). This could mean that it contains a hydroxybenzoyl chromophore. In the case of the compounds with *m*/*z* 449 (28.1 min) and *m*/*z* 491 (29.7 min) for the quasi-molecular ion, the UV spectra could correspond to the presence of a (hydroxyindol-3-yl)acetamide moiety. Comparing those absorptions with the spectra of the already known acylpolyamines from *Agelenopsis aperta* venom [6], acquired in our laboratory, we can conclude that **PB 421**[2]) could contain the 4-hydroxybenzoyl chromophore, while **PB 448** and **PB 490** possess the (4-hydroxyindol-3-yl)acetamide moiety.

HPLC-APCI-MS/MS. The MS/MS techniques provide valuable structural information, and they are very important for substance identification. The selectivity of a triple-stage quadruple mass spectrometer enables one to obtain not only collision-induced dissociation (CID) spectra of pure samples, but also to get independent CID spectra of two or more co-eluted substances (provided 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. To obtain product-ion spectra, the first quadrupole is fixed to the *m*/*z* value of the selected quasi-molecular ions, and the third quadrupole is scanned over a particular *m*/*z* range [8].

In the literature, one can find the data concerning structure elucidation of the spider acylpolyamines by CI-MS, MALDI-MS, FAB-MS, HPLC-ESI-MS, and continuous-flow (FRIT) FAB LC/MS methods [9–11]. We attempted to study this class of compounds by coupling HPLC on-line with an APCI mass spectrometer. This method has not been employed before for the investigation of spider toxin, but has been used effectively for the structure elucidation of plant alkaloids [8]. The main attractivity of this method is its relative simplicity compared with other on-line combined LC/MS methods (*e.g.*, continuous flow (FRIT) FAB LC/MS).

HPLC-APCI-MS/MS Experiments were performed on the three ions with *m*/*z* 422, 449, and 491, presented before. In different cases, optimal HPLC and MS conditions were used, but all of the data shown and discussed here were obtained under the same conditions. For analysis of the new substances, it is important to note that the already known toxins from *Agelenopsis aperta*, **AG 489** and **AG 448a**, were used as the references. **AG 448a** is a new compound found during our re-investigation of the venom from *Agelenopsis aperta* [6]. In *Fig. 3*, the APCI-MS/MS spectra of **AG 489** and **PB 490**, as well as their structures, are presented.

N-*(16-Guanidino-4-hydroxy-4,8,12-triazahexadecyl)-2-(4-hydroxyindol-3-yl)acetamide* (**PB 490**; $[M+H]^+$ *m*/*z* 491). As mentioned above, the UV spectrum of the compound with the t_R value of 29.7 min testifies to the presence of a (4-hydroxyindol-3-yl)acetamide moiety (λ_{max} 218, 266, 281, 291 nm). According to the 'nitrogen rule', one should expect five or seven N-atoms in the polyamine chain of **PB 490**, instead of the

---

[2]) According to the accepted procedure in the literature, we propose to use the abbreviation PB for *Paracoelotes birulai*.

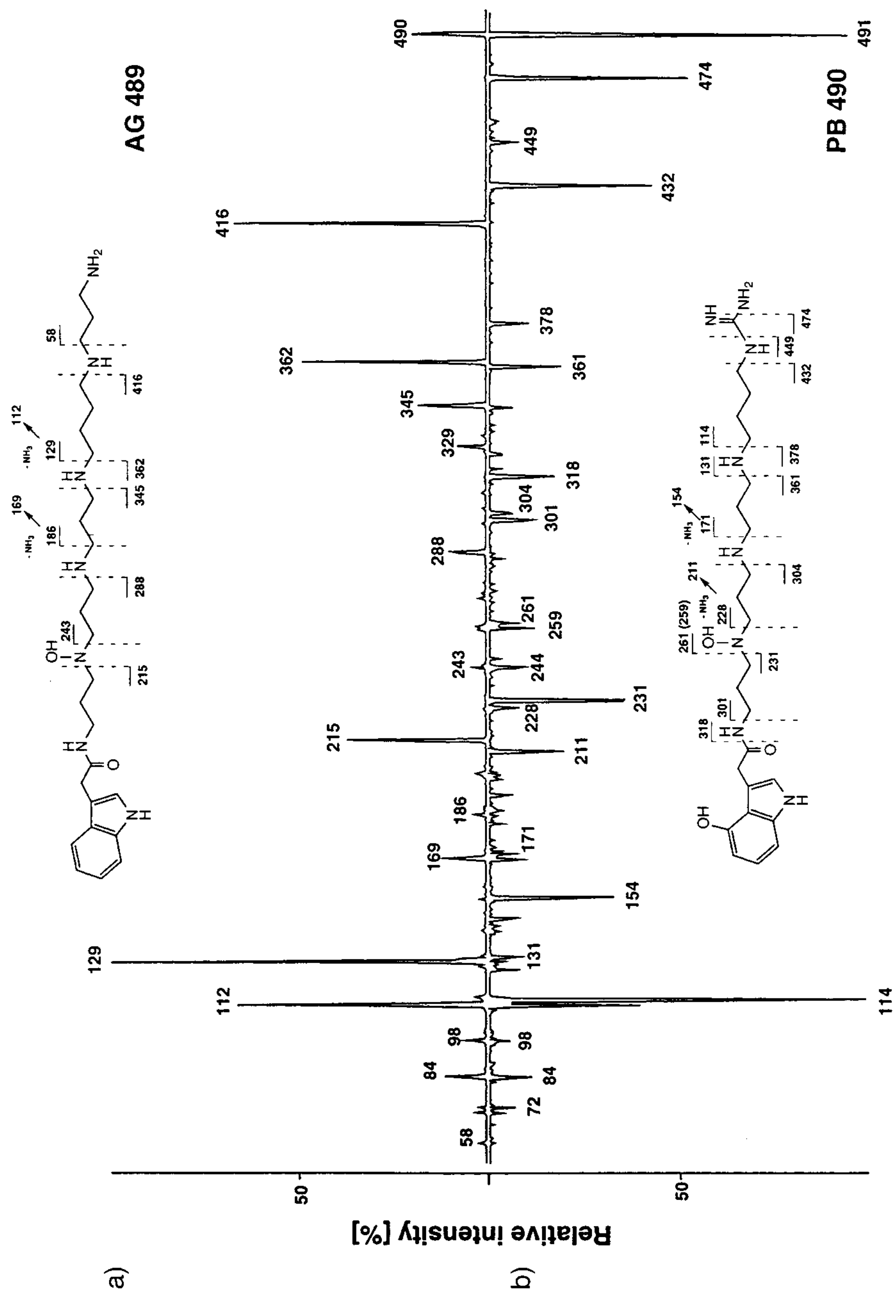
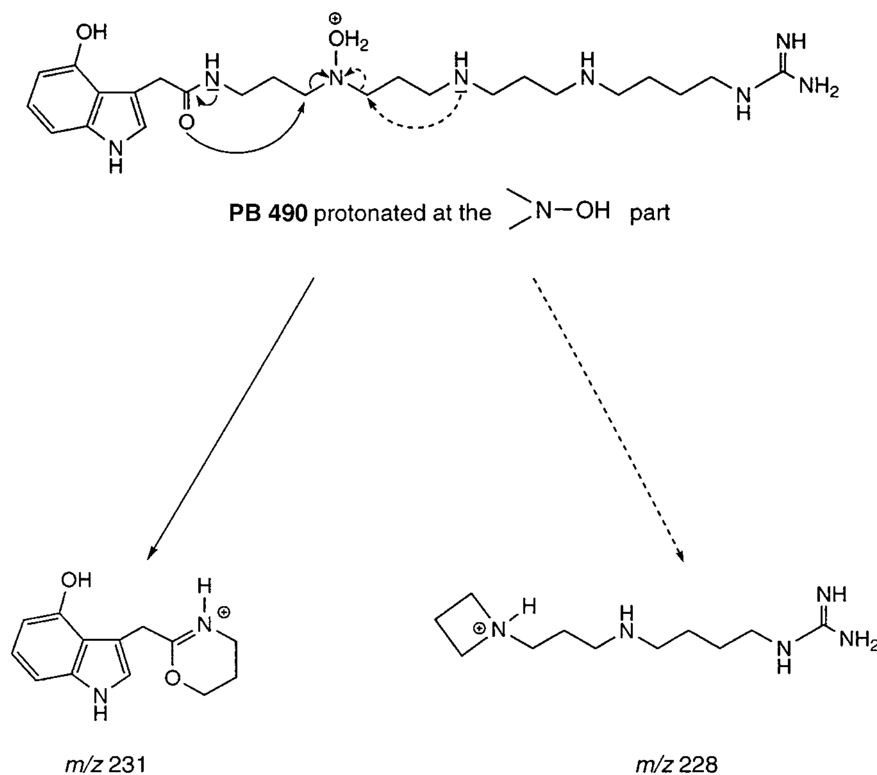


Fig. 3. *MS/MS Spectra of* a) **AG 489** *and* b) **PB 490**

six N-atoms found in the polyamine backbone of **AG 489**. The signal corresponding to the fragment of m/z 231 proves the existence of the (4-hydroxyindol-3-yl)acetamide moiety in **PB 490**, while the signal corresponding to the fragment of m/z 215 testifies to the presence of the (indol-3-yl)acetamide group in **AG 489**. Most interesting are the signals of the fragments at m/z 114, 432, 449, and 474, which establish that there is a guanidine tail in **PB 490**. The signals of the fragments at m/z 261, 231, and 228 certify the presence of a OH group at N(4). The principal fragmentation pattern for this kind of acylpolyamine decomposition is shown in the *Scheme* for m/z 231 and 228. The other fragments can be explained analogously by way of the quasi-molecular ions protonated at different N-atoms.

Scheme. *The Principal Fragmentation of Acylpolyamine* **PB 490**. There exist several species of molecular ions monoprotonated at different N-atoms.



From the above mentioned details, we can propose that **PB 490** has an acylpolyamine structure, containing (4-hydroxyindol-3-yl)acetamide as the lypophilic moiety and a OH group at N(4), as well as a guanidine tail. This type of structure (*Fig. 3,b*) partly combines the structure of compounds isolated from the Agelenidae family ((4-hydroxyindol-3-yl)acetamide moiety and the tetraamine in the polyamine backbone) with that from compounds of the Araneidae family that incorporate the guanidine tail of the acylpolyamines. The combination of both types of structural elements is not

known in either spider families, but this combination seems to be important for the specific character of the *Paracoelotes birulae* venom.

N-*(16-Guanidino-4-hydroxy-4,8,12-triazahexadecyl)-4-hydroxybenzamide* (**PB 421**; $[M+H]^+$ *m*/*z* 422). The main compound of this group of α-palutoxines contains a hydroxybenzoyl chromophore and has a t_R value of 21.0 min. *Fig. 4* shows the MS/MS spectra of **PB 421** (*Fig. 4,a*) and **PB 490** (*Fig. 4,b*). The signals of the fragment ions at *m*/*z* 178 and 235 confirm the presence of the hydroxybenzoyl moiety, and, according to the 'nitrogen rule', five or seven N-atoms should be present in **PB 421**. To make the task easier, we aligned the quasi-molecular ions in both MS/MS spectra. The absolute correspondence of the pairs of the signals: *m*/*z* 405 and 474, 388 and 457, 380 and 449, 363 and 432, 309 and 378, and 292 and 361 allows us to conclude that both acylpolyamines **PB 421** and **PB 490** have the same structural elements.

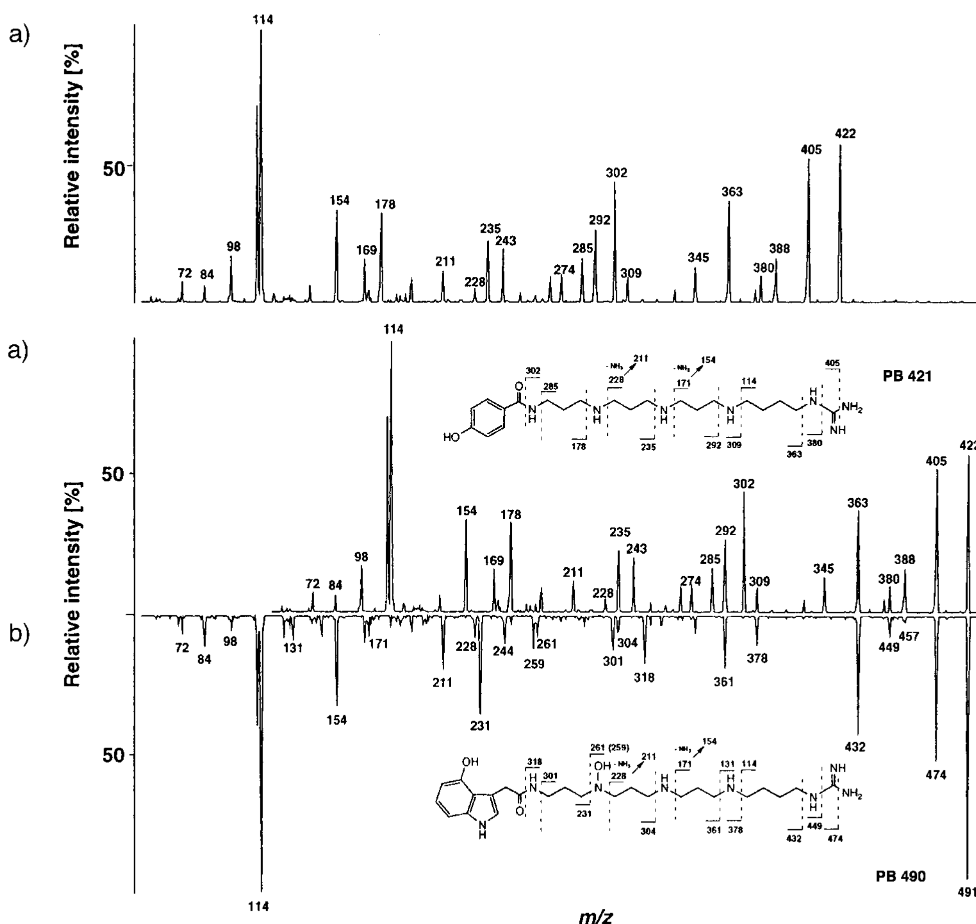


Fig. 4. *MS/MS Spectra of* a) **PB 421** *and* b) **PB 490**. Spectra demonstrate the similarity in fragmentation of the compounds at the guanidine tail.

N-*(16-Amino-4-hydroxy-4,8,12-triazahexadecyl)-2-(4-hydroxyindol-3-yl)acetamide* (**PB 448**; $[M+H]^+$ *m/z* 449). Analysis of the UV spectrum corresponding to the compound with the quasi-molecular ion at *m/z* 449 (28.1 min) revealed the presence of a chromophore that could be attributed to the (4-hydroxyindol-3-yl)acetamide moiety. *Fig. 5* compares the MS/MS analyses of **PB 448** (*Fig. 5,b*) and **AG 448a** (*Fig. 5,a*) that has a t_R value of 28.9 min for the same experimental conditions. The absolute coincidence of both spectra and the matching of the t_R values for these compounds makes it possible to conclude that **AG 448a** and **PB 448** are the same compounds.

The structures of the acylpolyamines present in lower concentrations will be reported later and compared with those found in the venoms of *Agelenopsis aperta* and *Hololena curta* (Agelenidae).

Conclusion. – For the first time, atmospheric-pressure chemical ionization (APCI) was used for the structure elucidation of the low-molecular-weight compounds present in natural spider venom. In spite of small amounts of material, it was possible to tackle the problem by means of on-line coupling of APCI-MS/MS with HPLC.

The results of the investigation of the venom from the spider *Paracoelotes birulai* were interpreted using UV, MS, and MS/MS data, and the structures of the three main compounds have been proposed.

Two types of polyamines were detected in the α-palutoxins. One of them is usual for the Agelenidae family pentaamine **PA 3334** and the second one is a completely different class of polyamines with a guanidine tail, as in the toxins of the Araneidae family.

This 'dualism' is in good agreement with the fact that *Paracoelotes birulai* was initially assigned to the Agelenidae family and then moved to the Amaurobiidae family. It is a hint for future chemotaxonomical studies.

This work was supported by the *Swiss National Science Foundation* and the *Dr. Helmut Legerlotz Stiftung* des Organisch-chemischen Institutes der Universität Zürich. We thank Dr. *Anthony Linden* for linguistic help.

Experimental Part

General. Material: Lyophilized *Paracoelotes birulai* venom was purchased from *Spider Pharm Inc.*, Yarnell, AZ, USA, and collected in Kazakhstan (*Fauna Ltd.*, Kazakhstan) in 1999. 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); TFA (*Fluka*, purum, Switzerland). The water was purified with an *MILLI-Q$_{RG}$* apparatus (*Millipore*, Milford, MA, USA). Venom preparation: lyophilized venom (5 mg) was dissolved in 60 µl of the 1% H_2O/MeCN soln. (3:2) of TFA at r.t., filtered through a 0.45-µm filter (*Eppendorf*, Germany) and rinsed with 20 µl of H_2O. 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. HPLC: All investigations were carried out with a *Waters 626 LC* system, fitted with a *996* photodiode-array detector, and a *600S* controller and a *Millennium Chromatography Manager 2010* v. 2.15 (Waters Corp., Milford, MA, USA), with a *Rheodyne 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*, Hoerdt, France); flow rate 0.5 ml min^{-1}. Mobile phase: step gradient during 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 TFA in H_2O, solvent *B:* 0.1% solution of TFA in MeCN). MS: APCI-MS and APCI-MS/MS experiments were performed on a *Finnigan TSQ-700* triple-stage quadrupole instrument equipped with a 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;

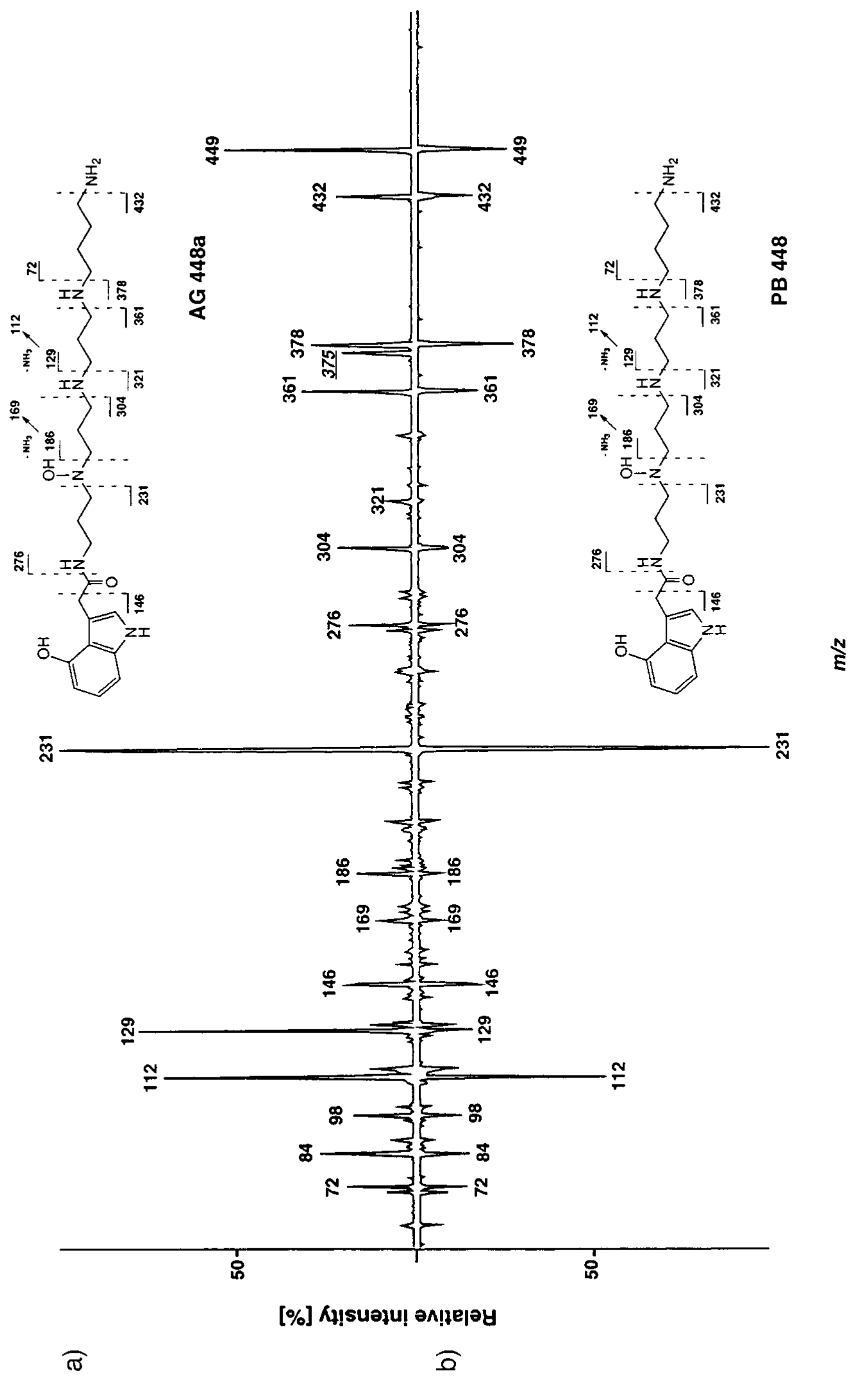


Fig. 5. *MS/MS Spectra of* a) **AG 448a** *and* b) **PB 448**. The signal at *m/z* 375 corresponds to a compound that co-eluted with the acylpolyamine **AG 448a**. The corresponding acylpolyamine **AG 448** contains the polyamine backbone **PA 3343** instead of **PA 3334** (**AG 448a**).

conversion dynode: −15 kV. For MS/MS experiments: collision gas Ar with a relative pressure 2.5–3.3 mTorr; collision-induced dissociation offset (Coff): −27 eV.

REFERENCES

[1] V. J. Jasys, P. R. Kelbaugh, D. M. Nason, D. Phillips, K. J. Rosnack, N. A. Saccomano, J. G. Stroh, R. A. Volkmann, *J. Am. Chem. Soc.* **1990**, *112*, 6696.
[2] V. J. Jasys, P. R. Kelbaugh, D. M. Nason, D. Phillips, K. J. Rosnack, J. T. Forman, N. A. Saccomano, J. G. Stroh, R. A. Volkmann, *J. Org. Chem.* **1992**, *57*, 1814.
[3] G. B. Quistad, S. Suwanrumpha, M. A. Jarema, M. J. Shapiro, W. S. Skinner, G. C. Jamieson, A. Lui, E. W. Fu, *Biochem. Biophys. Res. Commun.* **1990**, *169*, 51.
[4] A. Schäfer, H. Benz, W. Fiedler, A. Guggisberg, S. Bienz, M. Hesse, *The Alkaloids* **1994**, *45*, 1.
[5] A. Horni, D. Weickmann, M. Hesse, *Toxicon* **2001**, *39*, 425.
[6] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta*, in preparation.
[7] G. Corzo, P. Escoubas, M. Stankievicz, M. Pelhate, C. Kristensen, T. Nakajima, *Eur. J. Biochem.* **2000**, *267*, 5783.
[8] N. Youhnovski, L. Bigler, C. Werner, M. Hesse, *Helv. Chim. Acta* **1998**, *81*, 1654.
[9] Y. Itagaki, T. Fujita, H. Naoki, T. Yasuhara, M. Andriantsiferana, T. Nakajima, *Nat. Toxins* **1997**, *5*, 1.
[10] M. S. Palma, Y. Itagaki, T. Fujita, M. Hisada, H. Naoki, T. Nakajima, *Nat. Toxins* **1997**, *5*, 47.
[11] M. Hisada, T. Fujita, H. Naoki, Y. Itagaki, H. Irie, M. Miyashita, T. Nakajima, *Toxicon* **1998**, *36*, 1115.

Received November 9, 2000