Structure Elucidation of Polyamine Toxins in the Venom of the Spider *Larinioides folium*

Silvan Eichenberger§, Laurent Bigler, and Stefan Bienz*
§SCS Poster Prize Winner

Abstract: The lyophilized venom of the spider *Larinioides folium* (Araneidae) was analyzed by high-performance liquid chromatography, coupled on-line to electrospray ionization mass spectrometry (HPLC-ESI-MS) and tandem mass spectrometry (HPLC-ESI-MS/MS), as well as UV-DAD analysis. In combination with amino acid analysis, high-resolution mass spectrometry and on-column H/D-exchange experiments, the structures of 41 new acyl-polyamines contained in the complex venom of the spider *L. folium* were elucidated. Data interpretation in detail and the efficiency of this combined set of analytical method is exemplified by the structural elucidation of one of the toxins, namely of LF503a.

Keywords: Liquid chromatography · Polyamine alkaloids · Spider venom · Structure elucidation · Tandem mass spectrometry

Introduction

Polyamine alkaloids are widely distributed throughout the animal and plant kingdoms (as a review, see[1,2]), and they exhibit a variety of important and interesting biological activities.[3] It is not surprising thus, that new and efficient methods for their synthesis as well as more sensitive and selective analytical methods for the identification and structural elucidation of new examples from natural sources are searched for.

Spider venom is known to be an important source of polyamine alkaloids and has therefore attracted the attention of the scientific community.[2] Due to the complexity of the spider venom and its availability in smallest amounts only, only their major constituents could be revealed by classical methods, *e.g.* through isolation, purification and subsequent analysis of the pure compounds by NMR-, UV/VIS and mass spectrometry. Only the advent of newer analytical methods – in particular of the modern mass spectrometric approaches – has provided access also to minor constituents of spider venoms.

The method of choice to analyze acyl-polyamines in spider venoms became high-performance liquid chromatography (HPLC) on-line coupled with mass spectrometry (MS) and tandem mass spectrometry (MS/MS).[4] The power of this method was demonstrated by the structural elucidation of acylpolyamines from the venom of the spiders *Agelenopsis aperta* and *Paracoelotes birulai*.[5,6] The highly sophisticated method allowed the direct analysis of native lyophilized spider venoms without prior purification steps, revealing in addition to the formerly characterized major constituents also most of the minor components of the complex mixtures.

The investigation of a new venom, the venom of *Larinioides folium* (Araneidae) (Fig. 1), however, showed that the above-mentioned analytical combination is not sufficient for the unambiguous characterization of the structurally more complex polyamine toxins contained therein. The analytical setup had to be supplemented with on-column H/D exchange HPLC-MS, as well as with amino acid analysis and

Fig. 1: Picture of an adult female *Larinioides* spider. With permission of Dr. Chuck Kristensen, Spider Pharm, Inc.,Yarnell, AZ, USA

high-resolution mass spectrometry of venom fractions, as will be shown below.

Results and Discussion

Our investigation of the venom of *L. folium* started as usual with the HPLC-UV-ESI-MS analysis of the crude lyophilized natural sample. HPLC allowed the separation of an acylpolyamine fraction from fractions containing the other compound classes of the complex natural mixture. Within the acylpolyamine fraction, the several toxins were either completely or partially separated by the chromatography. Through coupling of the HPLC to a mass spectrometer, quasi-molecular ions of co-eluting components differing in molecular

Correspondence: Prof. Dr. S. Bienz
Universität Zürich
Organisch-chemisches Institut
Winterthurerstrasse 190
CH-8057 Zürich
Tel.: +41 44 635 42 45
Fax: +41 44 635 68 12
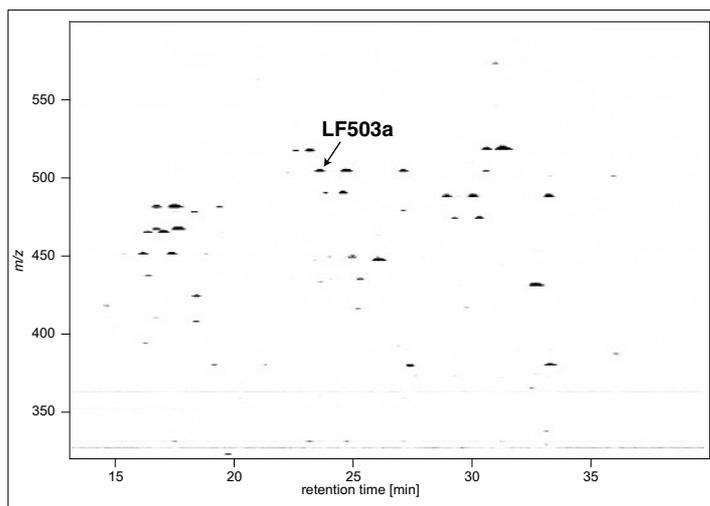E-Mail: sbienz@oci.unizh.ch

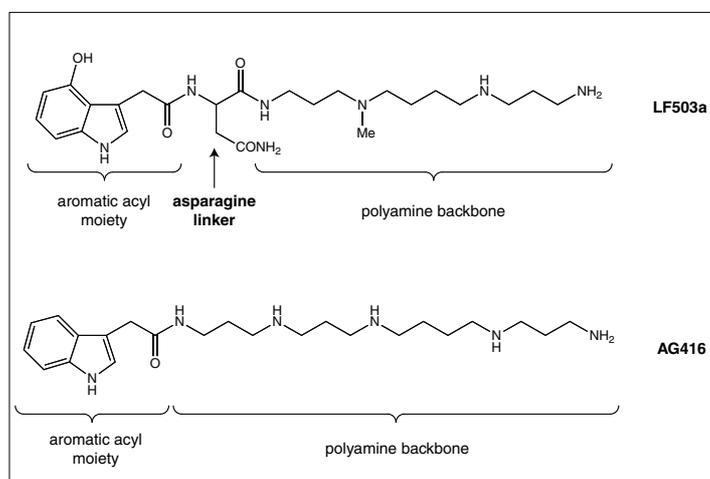Fig. 2. 2D-Plot of a single LC-ESI-MS analysis of the lyophilized venom of *L. folium*

Fig. 3. Structures of LF503a and AG416 contained in the venom of the spiders *L. folium* and *A. aperta*, respectively
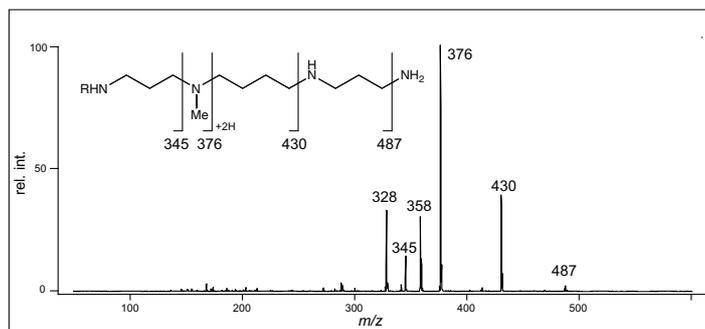

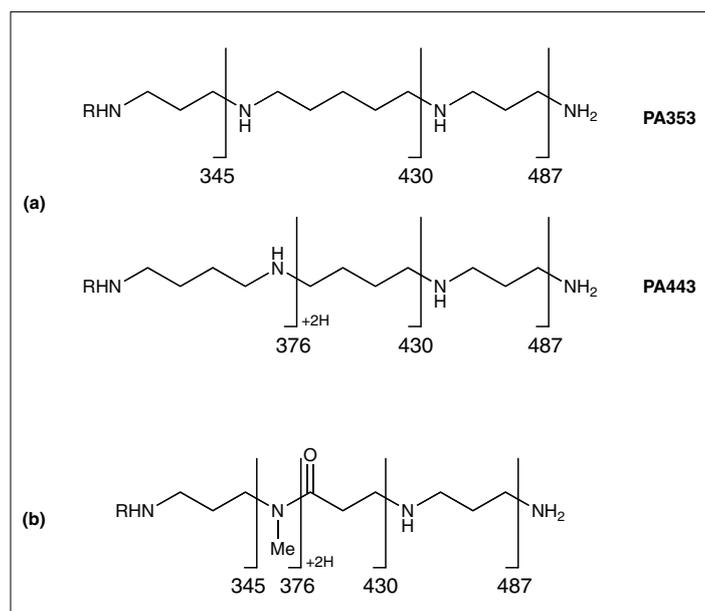Fig. 4. MS/MS pattern of [*M* + H]⁺ of LF503a at m/z 504 and interpretation of relevant fragment ions

Fig. 5. Structural alternatives for the polyamine backbone of LF503a consistent with the MS/MS pattern: a) Two co-eluting toxins with a PA353 and a PA443 unit, respectively. b) Compound containing a carbonyl group instead of a CH₂CH₂ portion.

masses were further separated by mass selection, thus adding a second dimension to the separation. The respective 2D-plot involving the retention time on the x-axis and m/z on the y-axis is shown in Fig. 2.

With regard to the on-line coupled UV/VIS spectroscopy, the spectra amenable from the several chromatographic peaks are solely meaningful when the eluting compounds are either pure or contain the same chromophore. A chromatographic peak that arises from a single compound only, namely from LF503a, was registered at t_R = 24 min with a quasi-molecular ion at m/z 504 (Fig. 2). This chromatographic fraction – and thus LF503a – was chosen to exemplify the new process of structure elucidation revealing finally a total of 41 new toxins.

The molecular structure of LF503a is shown in Fig. 3. The toxin contains, in addition to the parts also found in the earlier characterized acypolyamines of Agelenidae spiders, *e.g.* in Ag416 of *A. aperta*, an asparagine-linker in-between the polyamine backbone and the aromatic acyl moiety, as well as a methyl group attached to one of the N-atoms of the polyamine backbone. Due to this enhanced structural complexity, the analytical method used so far only revealed part of the structural information.

UV Spectrum

Since LF503a is not accompanied by other acylpolyamines in the HPLC, the UV spectrum for its chromatographic peak, obtained through the on-line coupled UV-DAD, was directly indicative for the aromatic acyl moiety of the compound. The observed λ_{max} at 268, 282, and 292 nm are characteristic for the 4-hydroxyindol-3-yl chromophore, indicating the presence of the (4-hydroxyindol-3-yl)acetyl group (4-OH-IndAc) as the head portion of the molecule.

On-Line ESI-MS/ESI-MS/MS

Electrospray ionization (ESI) of LF503a revealed a quasi-molecular ion [*M* + H]⁺ at m/z 504.3, indicating a molecular mass of 503.3 g mol⁻¹ for the compound and – by application of the nitrogen rule – an odd number of nitrogen atoms in its molecular formula. Selection of the quasi-molecular ion and its fragmentation by collision-induced dissociation (CID) afforded the MS/MS pattern shown in Fig. 4. Application of the fragmentation rules elaborated earlier[6,7] suggested the polyamine backbone PA3(1)43 for the compound, also shown in Fig. 4. The MS/MS-information, however, was not unambiguous for the structural elucidation of this polyamine portion. While the signal at m/z 430 can be regarded as definite proof for the terminal aminopropyl moiety of the compound, the left side of the molecule remained more or less in the dark. Despite the fact that the signals at m/z 376 and 345 fit nicely to the fragmentation pattern expected for PA3(1)43 contained in LF503a, they could also have arisen from a) a mixture of two isomeric compounds possessing PA353 and PA443 backbones or b) a compound containing a CO group instead of a CH₂CH₂ portion (Fig. 5). The latter structural component is known from acylpolyamines of other Araneidae spiders.[2]

In addition to the uncertainty with respect to the polyamine portion, the MS/MS analysis left also a lack of information regarding the structural moieties positioned in-between the polyamine portion and the chromophoric acyl group. Since a number of toxins of Araneidae spiders contain amino acids as linkers in-between the polyamine tail and the acyl head, such a structural unit was regarded as a possible moiety of LF503a. Thus, the remaining questions to be answered were a) is there an amino acid linker in-between the polyamine portion of LF503a and its acyl head moiety, b) is the polyamine portion a purely aliphatic polyamine or does it contain a carbonyl or carboxyl group (CO instead of CH_2CH_2), and c) is in fact one N-atom of the polyamine backbone methylated or does the observed fragmentation pattern in MS/MS derive from a mixture of isomeric polyamine derivatives? The first question can be answered by hydrolysis of LF503a, followed by amino acid analysis, the second by high-resolution MS (HR-MS), and the last by exchange of the acidic hydrogens of the compound by deuteriums.

Amino Acid Analysis

For the amino acid analysis, the lyophilized venom was fractionated by HPLC-UV using the same conditions as applied for the HPLC-MS experiments. The fraction containing LF503a was isolated and, after its vapor-hydrolysis in 6 M aqueous HCl, standard amino acid analysis through derivatization with *ortho*-phthalaldehyde and HPLC/fluorescence detection revealed Asp/Asn as possible components of the natural product. Taking the nitrogen rule into account, aspartic acid could be excluded, leaving asparagine as the amino acid linker contained in the toxin. In fact, asparagine is a common amino acid linker found in a number of other spider toxins as well[2].

HR-MS

After the amino acid linker was identified, the structure elucidation of the polyamine backbone could be brought forward. To obtain the molecular formula of LF503a, highly accurate HR-MS was performed with the fractionated toxin on a MAT900 double focusing mass spectrometer (maximal relative error of ±3 ppm). This delivered the accurate mass of $[M + H]^+$ as 504.3293 u ($\Delta m = -0.5$ mDa corresponding to 1 ppm). In combination with the structural moieties already known, the molecular formula $C_{24}H_{42}N_7O_4$ (exact mass: 504.3298 u) for the toxin LF503a, or $C_{11}H_{27}N_4$ for the polyamine backbone was deduced. Hence, the polyamine backbone is a purely saturated aminoalkane and does not contain any CO moiety.

H/D-Exchange

To secure finally the presence of the methylated amine in the structure of LF503a, an on-column H/D-exchange HPLC/MS experiment was performed. When D_2O instead of H_2O was used as the aqueous mobile phase for the LC-MS, a mass shift of +10 amu for $[M + H]^+$ of LF503a was observed, indicating nine exchangeable protons in the natural product. The respective ESI-MS measured in the presence of D_2O and H_2O are shown in Fig. 6. The observed nine exchangeable protons in the molecule confirm the proposed structure of the N-methylated polyamine backbone of the toxin. The alternative structures, containing secondary amines only, could be excluded, since they should have exposed ten exchangeable protons (difference of 11 amu of the corresponding ionized molecules). Together with the MS/MS-pattern observed for the toxin, solely methylation of the N-atom next to the asparagine linker is possible, giving the overall structure of PA3(1)43 for the polyamine portion of LF503a as shown in Fig. 3.

Analogous to the analytical scheme exemplified above, a total of 41 new acylpolyamines were structurally elucidated. All these compounds share the same type of structural composition. They all posses a linear polyamine backbone attached at one end to an aromatic acyl moiety through asparagine as an amino acid linker (see Fig. 3).

Overall, we found nine different aromatic acyl moieties and seven different polyamine portions in the toxins of *L. folium*. The chromophoric head groups are 4-hydroxyindole-3-acetyl (4-OH-IndAc), 4-hydroxyphenylacetyl (4-OH-PhAc) and 2,4-dihydroxyphenylacetyl (2,4-(OH)$_2$-PhAc) – identified directly by their characteristic UV spectra – as well as indole-3-acetyl (IndAc), indole-3-lactyl (IndLac), tryptophan (Trp), phenylacetyl (PhAc), phenyllactyl (PhLac) and phenylalanine (Phe), structurally determined by their UV spectra in combination with the MS data (Fig. 7).

The seven polyamine backbones are the tetraamines PA3(1)43, PA353, PA533, PA343, and PA433 as well as the triamines PA35 and PA53 (Fig. 8). In contrast to the toxin derivatives of the tetraamines, the derivatives of the triamines were found as co-eluting isomers in the HPLC only.

By variation of the several acyl head and polyamine tail portions shown in Fig. 7 and 8, a total of 63 toxins could theoretically be expected to be found. Such complete combinatorial libraries were in fact located in
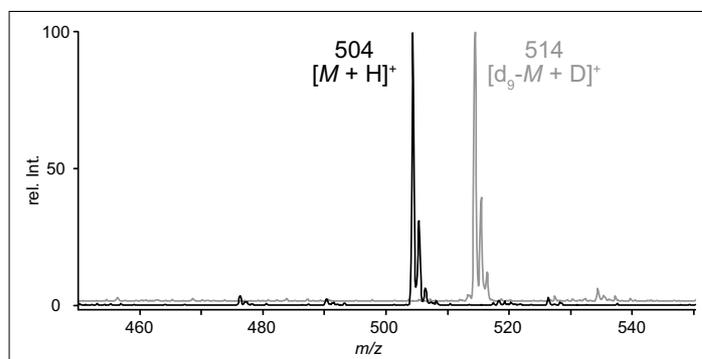

Fig. 6. ESI-MS of LF503a of the on-column H/D-exchange-MS experiment performed with H$_2$O/MeCN and D$_2$O/MeCN, respectively
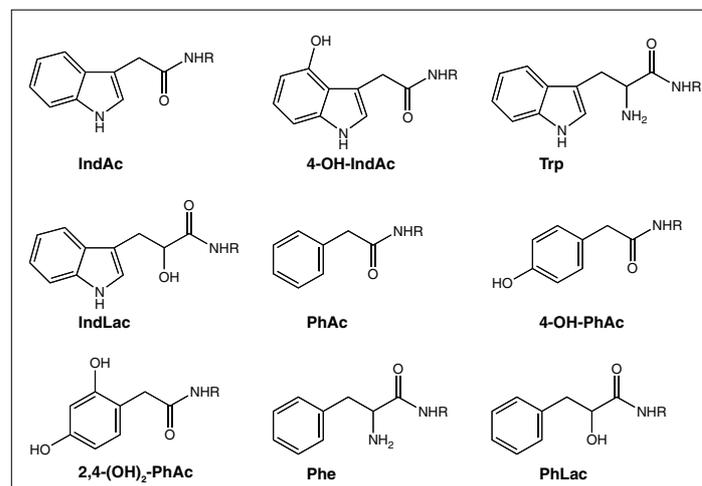

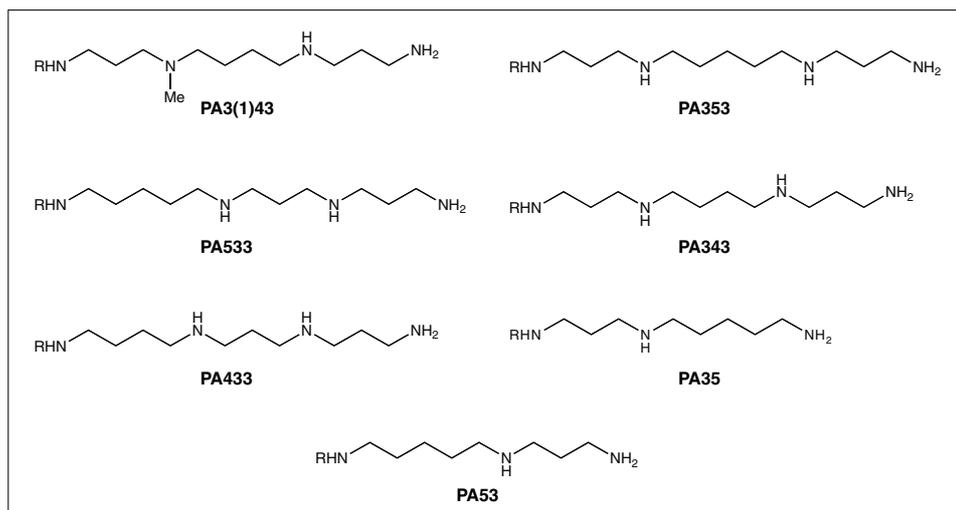Fig. 7. Aromatic acyl moieties found in the polyamine toxins of *L. folium*

Fig. 8. Polyamine backbones found in the polyamine toxins of *L. folium*

the venom of other spiders.[6] It seems that in the case of *L. folium*, the sensitivity of the applied method is not sufficient to detect all compounds. While the combinations of the several acyl head groups with the most abundant polyamine skeleton PA353 were all found and characterized, the combinations of the lesser abundant polyamines, particularly with the lesser abundant acyl moieties, evaded detection. With regard to the toxins with the newly found head groups IndLac and Trp, however, it is possible that the biosynthesis is limited to specific polyamines only. It is striking to see that all toxins containing these head groups start with a PA3 unit in the backbone.

Conclusions and Perspectives

Although HPLC-UV-MS and -MS/MS was proven to be a powerful tool for the structural elucidation of minor components in complex mixtures of polyamine derivatives, this analytical setup turned out to be insufficient for the structural determination of polyamine alkaloids of the spider *L. folium*. Due to the more complex structures of these compounds, it was necessary to supplement the analytical setup with high-resolution mass spectrometry, amino acid analysis, and on-column H/D exchange. It is our goal to learn more about the fragmentation behavior of the newly found toxins to be able to refine the analytical setup – preferentially to regain a completely on-line analytical tool.

[1] S. Bienz, P. Bisegger, A. Guggisberg, M. Hesse, *Nat. Prod. Rep.* **2005**, *22*, 647; S. Bienz, R. Detterbeck, C. Ensch, A. Guggisberg, U. Häusermann, C. Meisterhans, B. Wendt, C. Werner, M. Hesse, in 'The Alkaloids', Ed. G. A. Cordell, Academic Press, New York, **2002**, Vol. 58, p. 83.

[2] A. Schäfer, H. Benz, W. Fiedler, A. Guggisberg, S. Bienz, M. Hesse, in 'The Alkaloids', Ed. G. A. Cordell, Academic Press, New York, **1994**, Vol. 45, p. 1.

[3] G. Karigiannis, D. Papaioannou, *Eur. J. Org. Chem.* **2000**, 1841; A. L. Mueller, R. Roeloffs, H. Jackson, in 'The Alkaloids', Ed. G. A. Cordell, Academic Press, New York, **1995**, Vol. 46, p. 63.

[4] S. Chesnov, L. Bigler, M. Hesse, *Eur. J. Mass Spectrom.* **2002**, *8*, 1.

[5] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2000**, *83*, 3295; S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2001**, *84*, 2178.

[6] N. Manov, M. Tzouros, S. Chesnov, L. Bigler, S. Bienz, *Helv. Chim. Acta* **2002**, *85*, 2827.

[7] M. Tzouros, N. Manov, S. Bienz, L. Bigler, J. *Am. Soc. Mass Spectrom.* **2004**, *15*, 1636.