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A NATURAL COMBINATORIAL CHEMISTRY STRATEGY IN ACYLPOLYAMINE TOXINS FROM NEPHILINAE ORB-WEB SPIDERS

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The present study shows how nature combined a small number of chemical building blocks to synthesize the acylpolyamine toxins in the venoms of Nephilinae orb-web spiders. Considering these structures in four parts, it was possible to rationalize a way to represent the natural combinatorial chemistry involved in the synthesis of these toxins: an aromatic moiety is connected through a linker amino acid to a polyamine chain, which in turn may be connected to an optional tail. The polyamine chains were classified into seven subtypes (from A to G) depending on the way the small chemical blocks are combined. These polyamine chains may be connected to one of the three possible chromophore moieties: 2,4-dihydroxyphenyl acetic acid, or 4-hydroxyindole acetic acid, or even with the indole acetic group. The connectivity between the aryl moiety and the polyamine chain is usually made through an asparagine residue; optionally a tail may be attached to the polyamine chain; nine different types of tails were identified among the 72 known acylpolyamine toxin structures. The combinations of three chromophores, two types of amino acid linkers, seven sub-types of polyamine backbone, and nine options of tails results in 378 different structural possibilities. However, we detected only 91 different toxin structures, which may represent the most successful structural trials in terms of efficiency of prey paralysis/death.

Keywords: Acylpolyamine toxins, Orb-web spiders, Spider toxins, Combinatorial chemistry.

I. Introduction

Spider venoms are complex mixtures of peptides, proteins, and low molecular weight organic molecules, many of which act on

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neurons, particularly on ion channels or receptors (Escoubas et al., 2000). Among the low molecular weight organic compounds present in spider venoms are: free acids such as citric and lactic; glucose, free amino acids; biogenic amines such as: diaminopropane, putrescine, cadaverine, spermine, and spermidine; and neurotransmitters such as aspartate, glutamate, serotonin, histamine, γ-butyric acid, dopamine, and epinephrine (Welsh and Batty, 1963; Schambacher et al., 1973; Chan et al., 1975; Aramaki et al., 1986; Early and Michaelis, 1987; Frew et al., 1994). Spider venoms also may contain at least four types of relatively low molecular weight toxins well characterized at less than 1 kDa: bis-(agmatine)-oxamide isolated from the venom of *Plectreurys tristis* (Quistad et al., 1993); a gluconucleoside disulfate isolated from the venom of *Hololena curta* (McCormick and Mainwald, 1993); a series of tetrahydro-β-carbolines isolated from the venom of the social spider *Parawixia bistriata* (Cesar, 2000), and the most well understood toxin type in this class: the acylpolyamines isolated from the venoms of several different spider species (Aramaki et al., 1986; McCormick and Mainwald, 1993; Kawai, 1991; Usherwood et al., 1984; Grishin et al., 1986).

Most of the known acylpolyamine toxins are neurotoxic compounds, acting as antagonists of different subtypes of ionotropic glutamate receptors (Parks et al., 1991), while some of these toxins also may act on nicotinic acetylcholine receptors (Willians, 1997). Some chemical structures of acylpolyamines have been elucidated, constituting a family of closely related toxins. The amino acid–containing polyamine toxins generally act on neuromuscular junctions of invertebrates, causing reversible or irreversible noncompetitive inhibition of quisqualate sensitive glutamate receptors. However, there are different selectivities and specificities for different subtypes of glutamate receptors (Kawai, 1991; Jackson et al., 1988; Priestley et al., 1989). The nonamino acid–containing acylpolyamine toxins, generally present in the venom of funnel-web spiders, trap door spiders, and some tarantulas seem to be selective and reversible, noncompetitive inhibitors of NMDA glutamate receptors from mammalian brain (Parks et al., 1991).

The best characterized chemical structures of acylpolyamine toxins among the Araneidae spiders are those from the orb-web spiders belonging to the Nephilinae subfamily (Aramaki et al., 1986; Aramaki et al., 1987; Toki et al., 1988; Chiba et al., 1994;

Fujita et al., 1997; Palma et al., 1997; Palma et al., 1998; Itagaki et al., 1997). Until the 1990s the standard procedures for elucidating the structure of these toxins required an extensive purification from a huge amount of venom, followed by the use of traditional chemical protocols (hydrolysis and derivatization, amino acid analysis by Edman Degradation chemistry, and ^{1}H- and ^{13}C-NMR). Between 1985 and 1990 about 17 different structures were elucidated with this experimental approach from the venom of the spider *Nephila clavata* (Aramaki et al., 1986; Toki et al., 1988).

A very sensitive methodology for the direct detection of these toxins in venom extracts was developed by using on-line microcolumn HPLC continuous flow (FRIT) FAB LC/MS and high energy CID methods with sodium-attached molecular ions, to produce very effective information about the structures of this class of toxins in *Nephila clavipes* and *Nephilengys borbonica* (Fujita et al., 1997; Itagaki et al., 1997). The venom of the Brazilian garden spider*Nephilengys cruentata* was characterized by using a combination of HPLC/MS, MALDI-TOF, and MALDI-sector type mass spectrometry (Palma et al., 1997; Palma et al., 1998).

The general structures of the acylpolyamine toxins may be separated into four parts (Schambacher et al., 1973) as represented in Figure 1: a lipophylic aromatic acyl moiety (part I); a linker amino acid residue (part II); the polyamine backbone chain

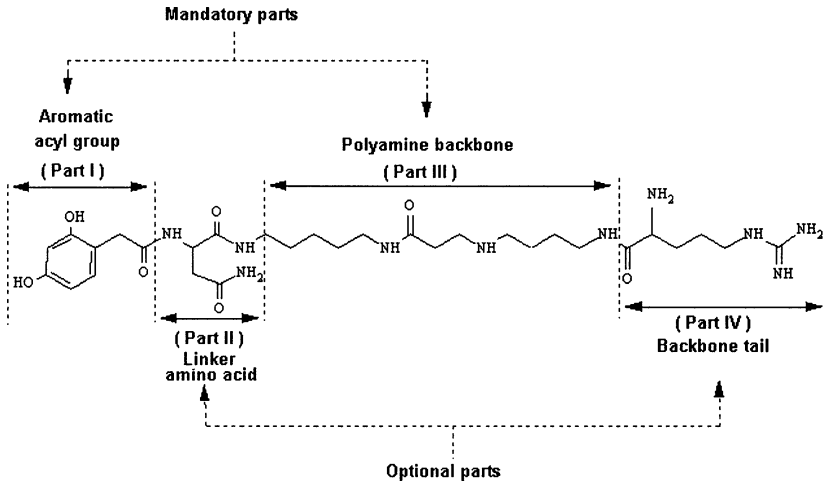

FIGURE 1 Structural parts of the acylpolyamine toxins from spider venoms.

(part III), and the backbone tail (part IV). The aromatic acyl group and the polyamine backbone constitute mandatory parts of these compounds, shared by all known toxins of this class, whereas the linker amino acid(s) and the tail constitute optional parts, present only in some toxins. The Araneidae orb-web spiders biosynthesize acylpolyamine toxins containing both the mandatory and optional structural parts, while the toxins from funnel-web, trap door and tarantula spiders generally present only the two mandatory structural parts (Schambacher et al., 1973).

Each of these structural parts is built from simple chemical blocks. The way these chemical blocks are connected to each other represents a natural combinatorial chemistry strategy played by the Nephilinae spiders, based on millions of years of evolution. As a result they maximize the efficiency of insect preying, reflecting the plasticity of this group of spiders to diversify their venom arsenal according to the different prey availability during each season and in different ecological niches.

II. The Structure of the Nephilinae Acylpolyamine Toxins

The structural parts of Nephilinae acylpolyamine toxins are constituted by chemical blocks described as follows: I) the aromatic lipophylic head part from: indole acetic acid or 4-hydroxyindole acetic acid or 2,4-dihydroxy phenyl acetic acid; II) the linker amino acid part, which is asparagine residue or a dipeptide asparaginyl-ornithine; III) the polyamine backbone part, which may be constituted by simple chemical blocks, such as: polyamines—cadaverine, putrescine and diamine propane; or by amino acid residues—glycine, alanine, asparagine, and ornithine; IV) the backbone tail part, which is either putreanine or arginine and/or ornithine; sometimes even a glycine residue may take part in this tail. Depending on the different combinations of these chemical blocks, the polyamine backbone chains were classified into seven different subtypes A to G (Fujita et al., 1997; Hisada et al., 1998).

The aim of the present work is to organize the literature data, rationalizing the known chemical structures to a common representation for the most investigated Nephilinae spider venoms (Ne*hila clavata, Nephila madagascariensis, Nephilengys cruentata, and Nephilengys borbonica*) in order to demonstrate how nature may have adopted a combinatorial chemistry strategy for the biosynthesis of

the acylpolyamine toxins in this group of orb-web spiders. Thus, we reviewed the literature about the structural elucidations of the acylpolyamine toxins and reorganized the data according to the four structural parts mentioned previously.

III. The Relationship Between the Structure of the Toxins and the Mass Chromatogram

Since the structural elucidations of acylpolyamine toxins were performed in different experiments and published in a series of different papers, it was necessary to represent all data obtained from four different species of Nephilinae spiders in a simple and uniform way in order to provide a general view of all toxins. Thus, the 2-D mass chromatograms of protonated molecular ions $[M + H]^+$ obtained for all spider venoms investigated were plotted in the same scale of representation and overlapped to each other. In order to simplify the final display, the irregular spots corresponding to the assignment of each toxin position, according to their individual profile of elution, were replaced by a simple dot followed by the representation of the characteristic value of the molecular mass in the monoprotonated form.

To date, 91 structures (66 completely elucidated and 15 partially assigned) of acylpolyamine toxins are known from the venom of Nephilinae spiders. The common feature of these toxins is a polyamine chain modified by the introduction of a lipophylic moiety in one end and a tail at the other end.

Due to both the large number of structurally related chemical structures of acylpolyamine toxins of Nephilinae spiders, and the complex nature of the combinatorial system in the structural organization of these toxins, the pattern of distribution of each of the four toxin parts in the 2-D representation of the mass chromatogram profiles was analyzed. Thus, the low-molecular mass fraction of the four Nephilinae spider venom extracts were chromatographed under reversed phase conditions by using a linear gradient from 5% to 60% (v/v) MeCN in a C-18 column, as described in previous publications (Palma et al., 1997; Palma et al., 1998; Fujita et al., 1995). Under these conditions the chromatographic resolution is very sensitive to each one of the four structural parts. The fractionation of low-molecular weight components from Nephilinae spider venom is strongly influenced by the type

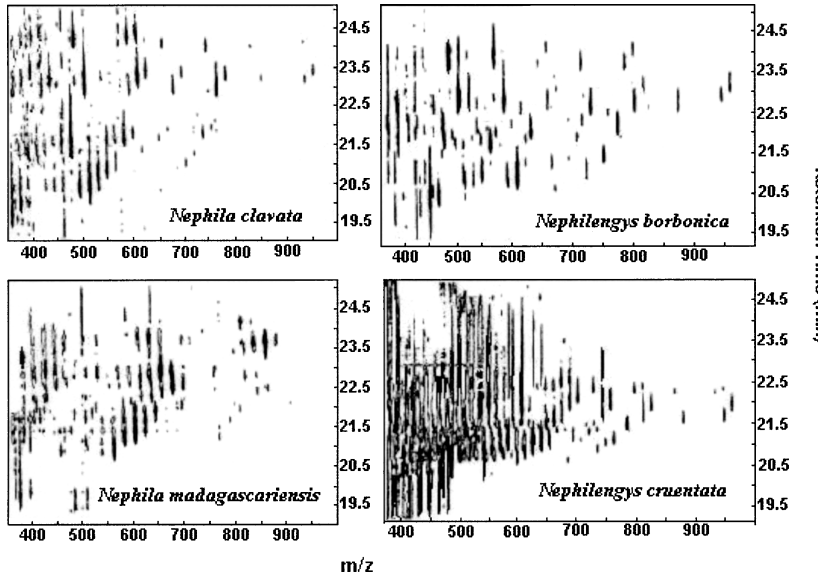

FIGURE 2 2-D representations of the mass chromatograms of the low molecular mass fractions from the venoms of four different species of Nephilinae spiders. The chromatographic profiles were obtained by using a RP-microcolumn Develosil ODS-HG-5 (0.3 × 150 mm) coupled to a HPLC and eluted under gradient of MeCN from 0% to 80% (v/v) in 20 min.

of lipophylic chromophore. The LC-MS chromatographic profile showing the distribution of the acylpolyamine toxins in a 2-D format for each Nephilinae venom investigated is shown in Figure 2, where each toxin eluted is represented by a black spot. At first, a direct comparison of these profiles seems to be too complex to permit a rationalization. However, the 2-D mass chromatogram displays of protonated molecular ions $[M + H]^+$ obtained for all spider venom extracts were overlapped with each other. In order to simplify the final display, the spots corresponding to the individual position of each toxin in the profile of elution were replaced by a simple dot, followed by the representation of the characteristic m/z value (Figure 3). All known structures of acylpolyamine toxins (Figures 6 to 12) were positioned in the 2-D mass chromatogram display (Figure 3). When the type of chromophore moieties (structural part I) is positioned in the 2-D mass chromatogram display, it may be clearly observed that the profile of distribution is as shown in Figure 3.

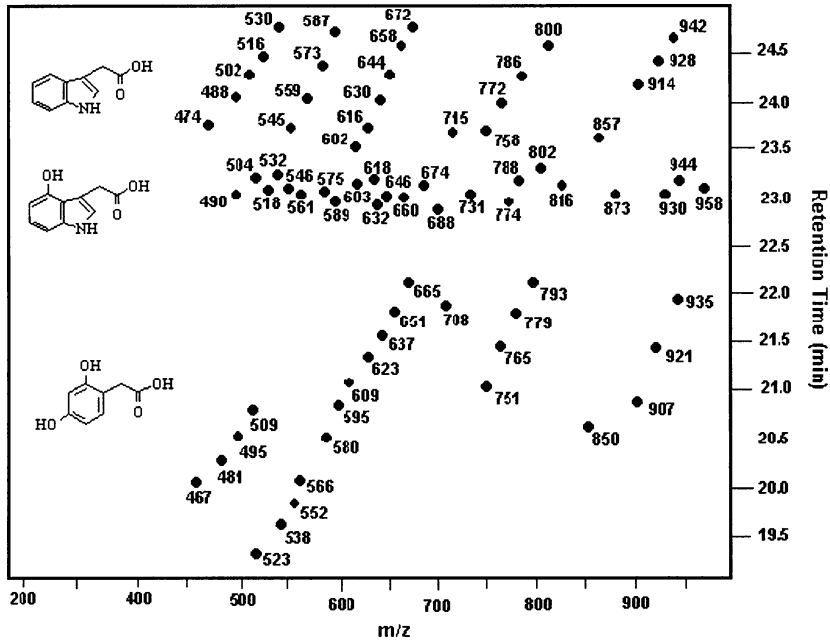

FIGURE 3 Schematic representation of the overlapping of all the 2-D mass chromatograms from the low molecular mass fractions of the Nephilinae spider venoms. The black spots were placed just on the retention time of each eluting peak, obtained by using a RP-microcolumn Develosil ODS-HG-5 (0.3 × 150 mm) coupled to a HPLC under gradient of MeCN from 0% to 80% (v/v) in 20 min. The numbers represent the molecular masses of each toxin in the monoprotonated form. The schedule also shows the distribution of the aromatic moieties (structural part I) along the resulting overlapped chromatographic profile of acylpolyamine toxins.

The structures bearing the 2,4-dihydroxyphenyl acetic group are all situated in the inferior part of the mass chromatogram, eluted at retention times between 19 and 22 min. Those toxins presenting 4-hydroxyindole acetic acid as aromatic moiety are localized in the center of the mass chromatogram, which eluted between the retention times 22.5 and 23.5 min. Meanwhile, the acylpolyamine toxins presenting indole acetic acid as chromophore were eluted after 23.5 min. Thus, the position of each spot in the 2-D representation of the mass chromatogram is enough to provide information about the nature of the lipophylic head of each acylpolyamine toxin. Concerning this structural aspect, an exception was detected: the toxin Joroamine (Figure 10) was described in venom of *N. clavata* as presenting 4-hydroxyphenyl

acetic acid as the chromophore group (Chiba et al., 1994), while the usual structural pattern of chromophore among the Araneidae spiders is the 2,4-dihydroxyphenyl acetic group. Figure 3 also shows the pattern of distribution of the toxins presenting different alignments over the total mass chromatogram, where the mass difference between two consecutively eluted components is always 14 u.m.a. The meaning of these alignments and mass differences will be discussed in the following paragraphs. The only exception was the compound representing m/z 523.

Comparing the structure of each one of the known polyamine backbones, it is possible to identify 24 different polyamine chains among all the toxins. Each one of these polyamine backbones (structural parts II, III, and IV, combined) are commonly shared among three toxins, among whom the only structural difference is the replacement of the aromatic moiety, like the example in Figure 4. The groups of toxins presenting a common polyamine backbone are represented in Figure 5, in which the arrays of the combined structural parts II, III, and IV are depicted. Those structures presenting the same array are connected by a continuous line. Thus, one of the ways nature has used to optimize the chemical

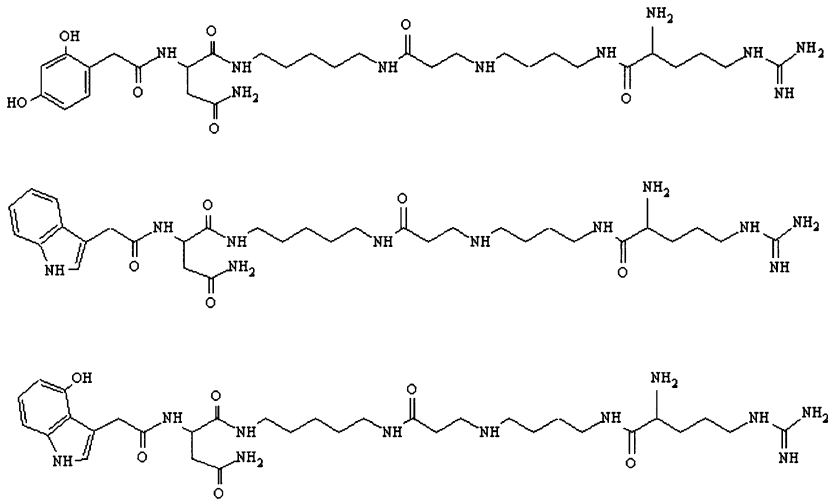

FIGURE 4 Example of three different acylpolyamine toxins from Nephilinae spider venoms sharing a common polyamine backbone chain and backbone tail (combined structural parts II, III, and IV). These toxins are differentiated from each other by the presence of different aromatic moieties (structural part I).

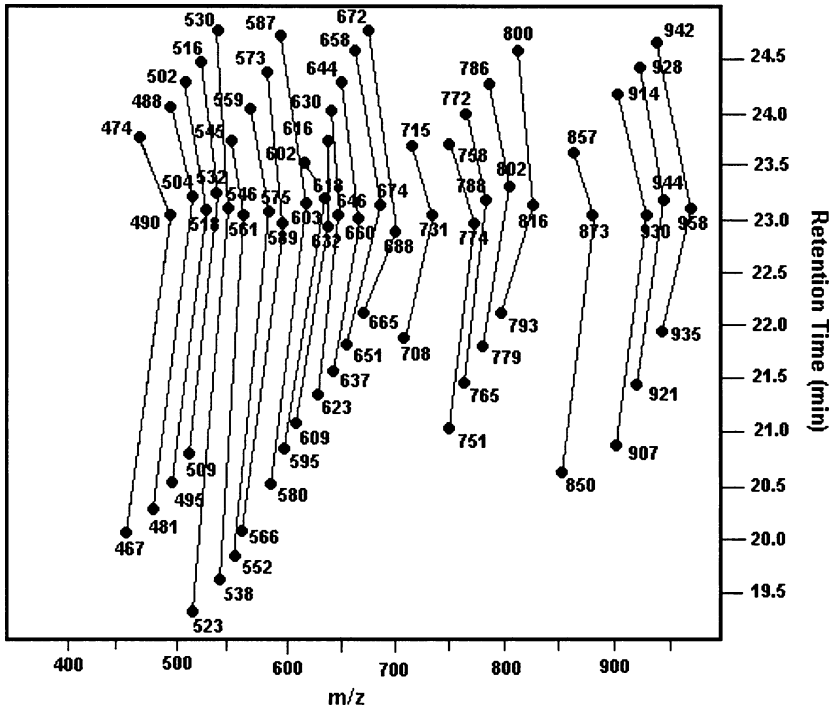

FIGURE 5 Schematic representation of the overlapping of all the 2-D mass chromatograms from the low molecular mass fractions of the Nephilinae spider venoms. The black spots were placed just on the retention time of each eluting peak, obtained by using a RP-microcolumn Develosil ODS-HG-5 (0.3 × 150 mm) coupled to a HPLC under gradient of MeCN from 0% to 80% (v/v) in 20 min. The numbers represent the molecular masses of each toxin in the monoprotonated form. Each set of three different compounds connected by continuous lines represent a group of toxins sharing exactly the same polyamine backbone and tail (combined structural parts II, III, and IV).

variability of these toxins was the use of a common polyamine backbone combined different times, with three different aromatic moieties.

A careful examination of the most frequently found chemical structures (Figures 6 to 12) of the Nephilinae acylpolyamine toxins reveals the following other interesting aspects about the polyamine backbone chains (structural part III): i) they are composed of subtypes; ii) these backbone chain subtypes are basically produced by the conjugation of five building blocks: three types of polyamines—cadaverine, putrescine, and diaminopropane; two

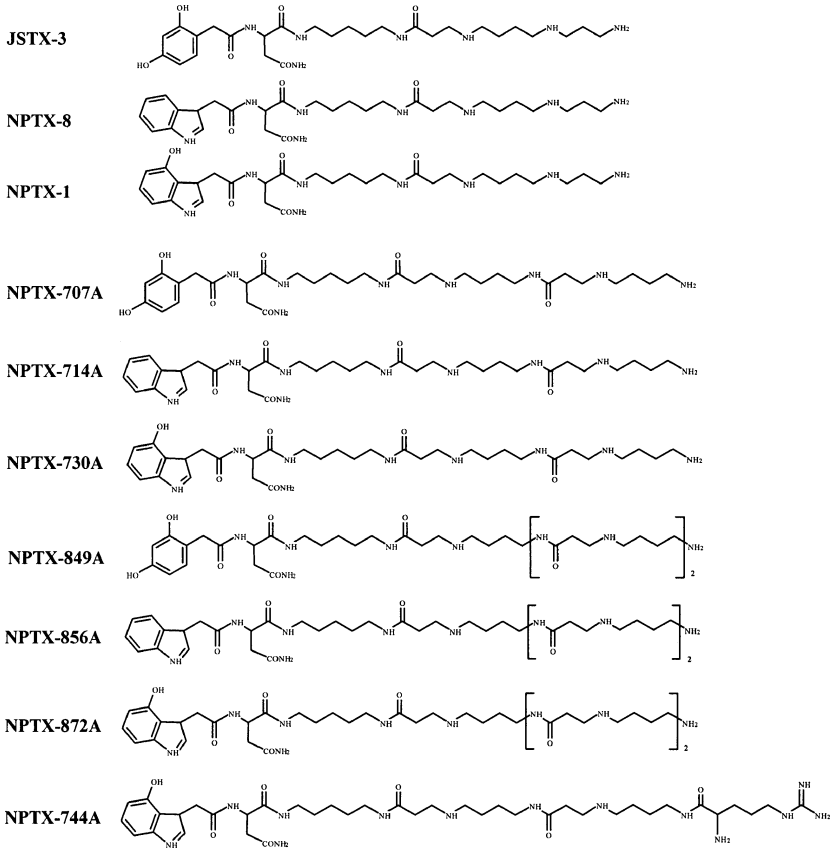

FIGURE 6 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype A.

types of amino acid residues—glycine and alanine; and iii) the combination of putrescine and alanine produces a putreanine unit, which frequently appears as building blocks in polyamine backbone chain. Taking into account these structural features, the polyamine backbones (part III) were previously classified into six subtypes, from A to F (Hisada et al., 1998; Fujita et al., 1995). After a detailed comparative analysis of all known structures we propose a new subtype, G. The subtypes were proposed as follows:

Subtype A—contains a cadaveryl-putreanyl diaminopropyl moiety (Figure 6);

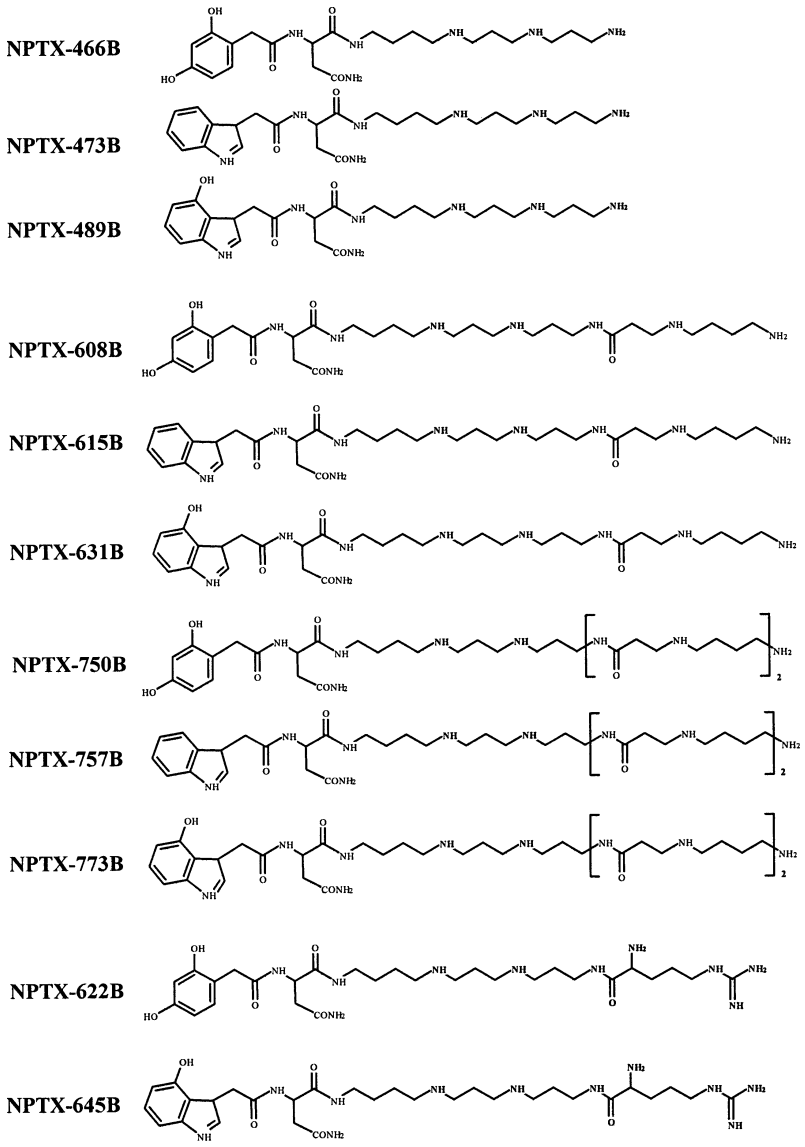

FIGURE 7 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype B.

Subtype B—has a putrescyl-diaminopropyl-diaminopropyl moiety (Figure 7);
Subtype C—has a cadaveryl-glycyl-putrescyl moiety (Figure 8);
Subtype D—has a cadaveryl-putreanyl moiety (Figure 9);

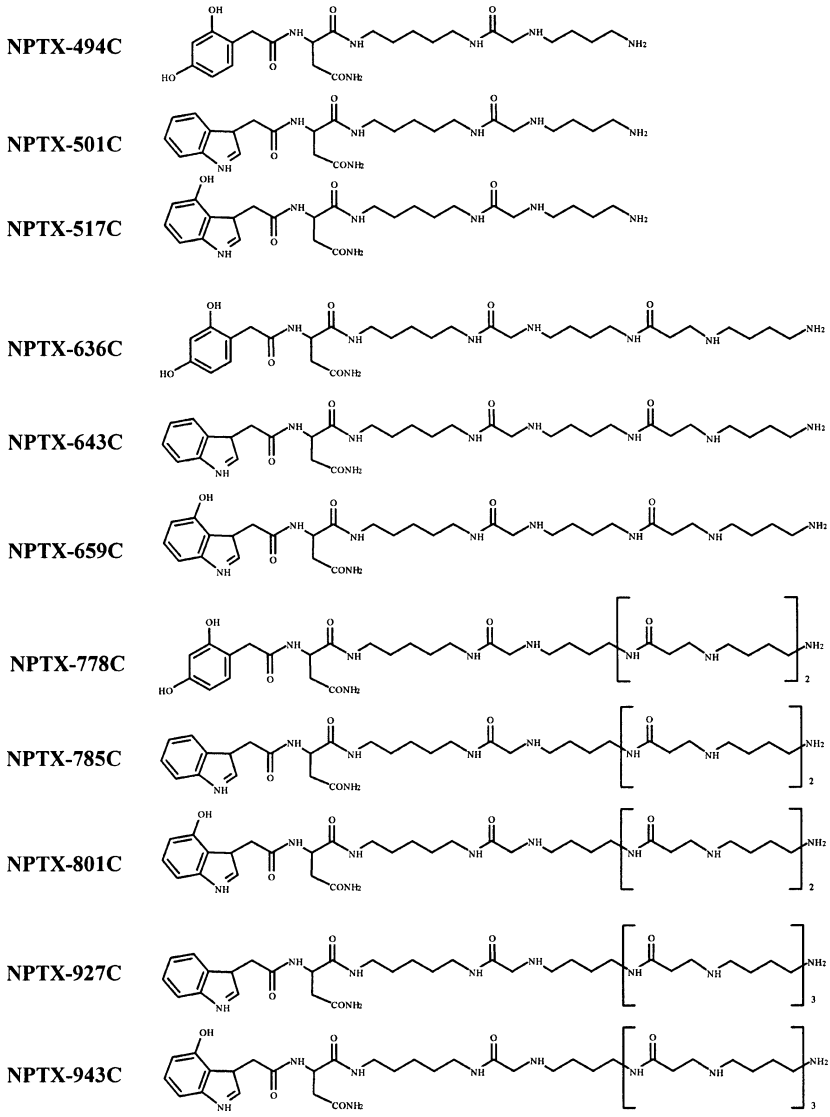

FIGURE 8 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype C.

Subtype E—has a cadaveryl-alanyl-diaminopropyl moiety (Figure 10);

Subtype F—has a cadaveryl-diaminopropyl-diaminopropyl moiety (Figure 11);

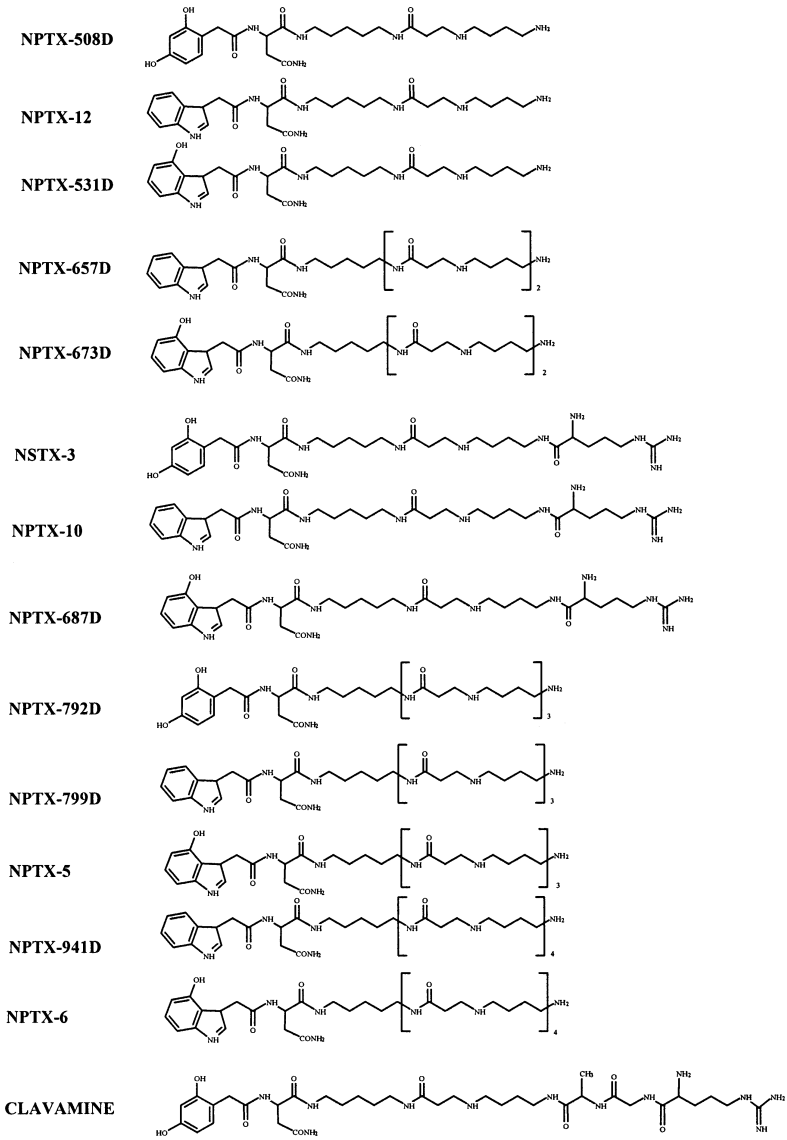

FIGURE 9 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype D.

Subtype G—may be considered as a pseudo subtype since it is contains only a cadaveryl moiety (Figure 12).

This classification became important to identify the toxins, not only due to their structural differences, but also to distinguish

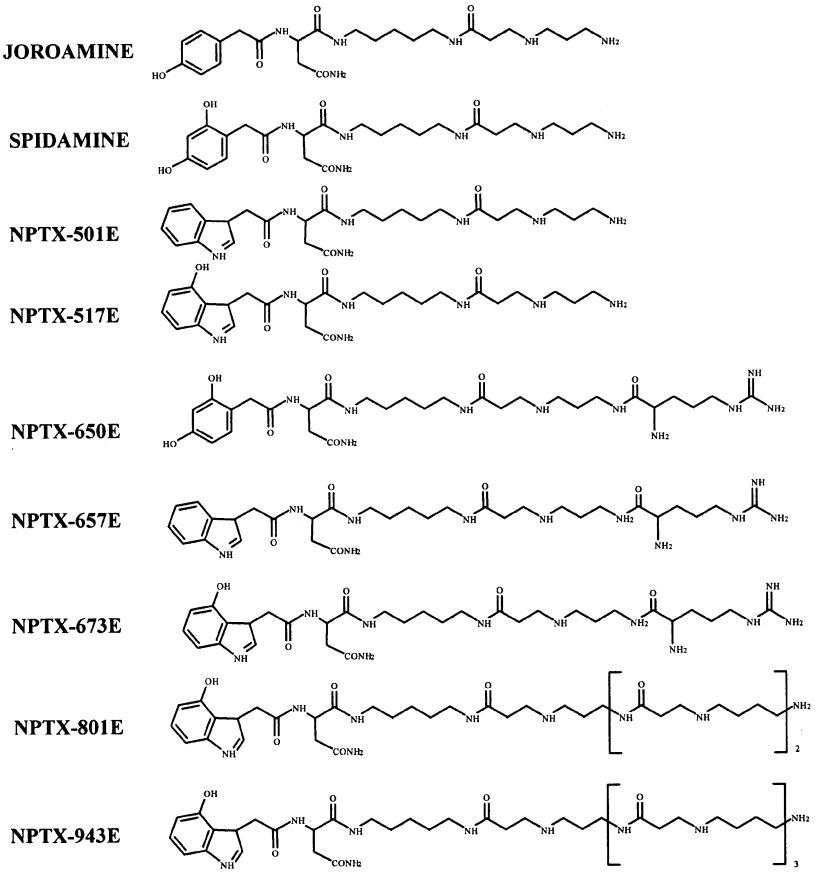

FIGURE 10 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype E.

their isomeric forms. Thus, it was suggested to include both the molecular mass and the backbone chain subtype in the nomenclature of novel acylpolyamine toxins, maintaining the already existing nomenclature for the previously known toxins (Itagaki and Nakajima, 2000). Among the 67 most commonly observed

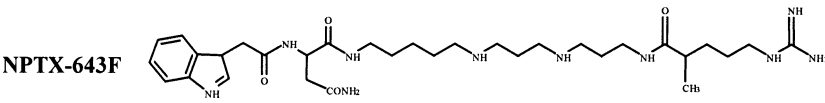

FIGURE 11 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype F.

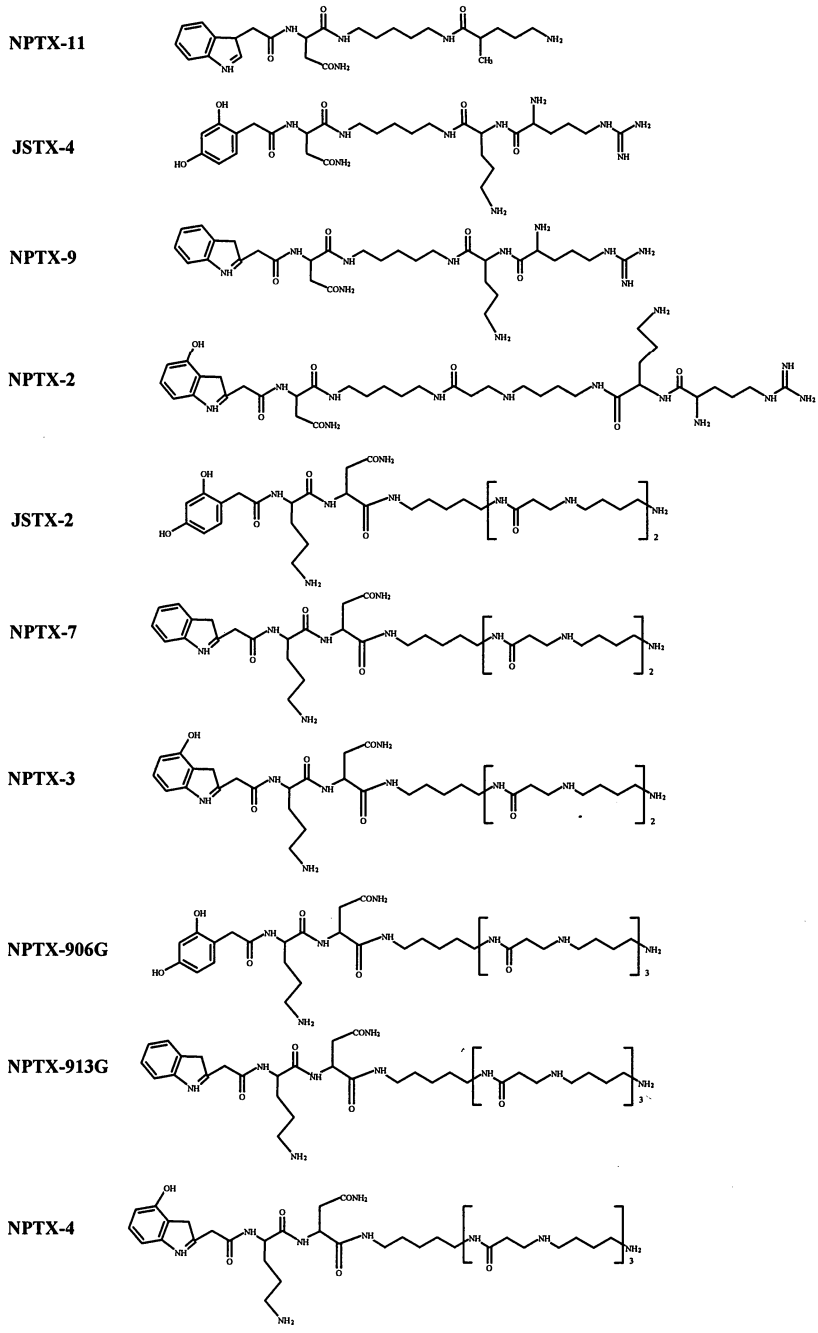

FIGURE 12 Chemical structures of the amino acid–containing acylpolyamine toxins from Nephilinae spiders presenting backbone subtype G.

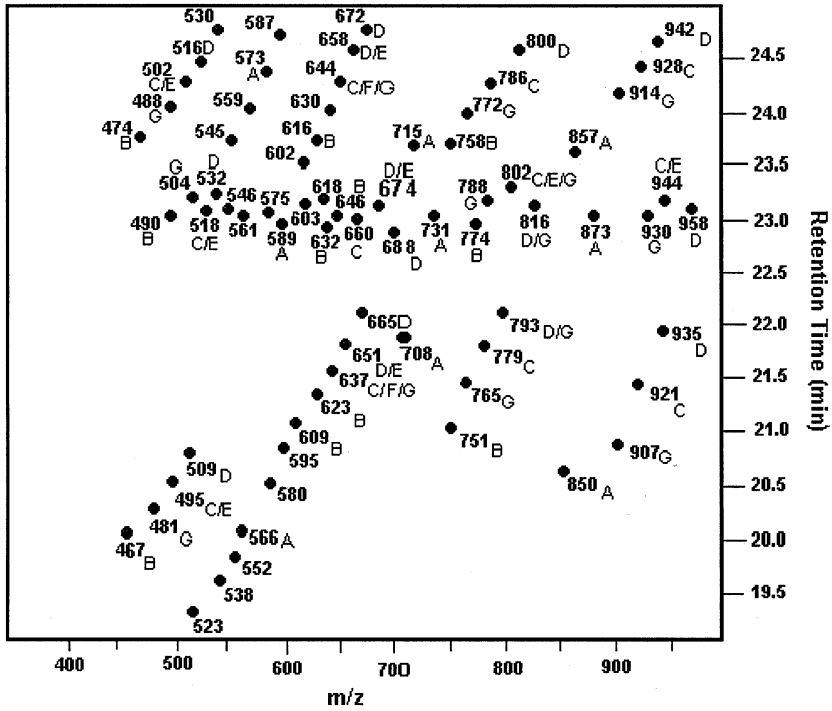

FIGURE 13 Schematic representation for the overlapping of all the 2-D mass chromatograms from the low molecular mass fractions of the Nephilinae spider venoms. The black spots were placed just on the retention time of each eluting peak, obtained by using a RP-microcolumn Develosil ODS-HG-5 (0.3 × 150 mm) coupled to a HPLC under gradient of MeCN from 0% to 80% (v/v) in 20 min. The numbers represent the molecular masses of each toxin in the monoprotonated form. The schedule represents the distribution of the polyamine backbones subtypes from A to G (structural part III) along the resulting overlapped chromatographic profile of acylpolyamine toxins.

chemical structures of acylpolyamine toxins from Nephilinae spiders, 10 belong to subtype A, 10 belong to subtype B, 12 belong to subtype C, 14 belong to subtype D, 9 belong to subtype E, 1 belongs to subtype F, and 10 belong to subtype G (Figures 6 to 12).

Figure 13 depicts the pattern of distribution of the backbone subtypes (structural part III) in the 2-D representation of the mass chromatogram. It is possible to observe that there is no specific grouping, i.e., all the subtypes of polyamine backbones are spread over the entire mass range. It is important to emphasize the occurrence of multiple backbone subtypes concentrated

in some groups of toxins: I) the toxins presenting m/z 495, 502, 518, and 944 as $[M + H]^+$ were detected as C- and E- subtypes; II) the toxins presenting m/z 637 and 644 as $[M + H]^+$ were detected as C-, F-, and G-, subtypes; III) the toxins presenting m/z 651, 658, and 674 as $[M + H]^+$ were detected as D- and E- subtypes; IV) the toxin presenting m/z 802 was detected with chemical structures of types C-, E-, and G- (NPTX-2); and V) the toxins presenting m/z 793 and 816 were detected as D- and G-subtypes. Another important aspect that must be considered is the rare occurrence of the toxin JSTX-1 in the Japanese spider *Nephila clavata,* with a diaminopropyl-putrescyl-diaminopropyl moiety as the polyamine backbone, while the toxin NPTX-466B with putrescyl-diaminopropyl-diaminopropyl moiety was the most common isomeric form of this toxin among the Nephilinae spiders (Figure 7). In fact, the polyamine backbone of JSTX-1 does not fit into any other known subtype. The occurrence of polyamine chain backbones reorganized into different subtypes, enlarges the structural variability of toxins, possibly increasing the killing/paralytic arsenal of the Nephilinae spiders. Those toxins, which are represented in Figure 13 without any letter assigned tor the m/z values, represent structures that were detected, but have their chemical structure only partially elucidated. Thus, it was not possible to classify them according to the criteria discussed earlier.

If the backbone tails (structural part IV) of known structures of the acylpolyamine toxins (Figures 6 to 12) are represented through the 2-D chart of mass chromatograms, it is possible to get their pattern of distribution over all the mass range of known toxins (Figure 14). This representation clearly shows that the toxins with m/z values from 467 to 603 as $[M + H]^+$ do not present any backbone tail. The toxins presenting their molecular ion in the monoprotonated form in the region from m/z 595 to 731 may contain either an arginine residue or a putreanine unit located at the backbone tail. From m/z 751 to 873 all the toxins have two putreanine units connected to each other in tandem at their tails, while all the toxins presenting m/z values higher than 907 as $[M + H]^+$ have three consecutives putreanine units in tandem at the end of their backbones. A detailed examination of the known structures reveals that the attachment of putreanine units at the tail of polyamine chains is a common way to elongate the polyamine backbones,

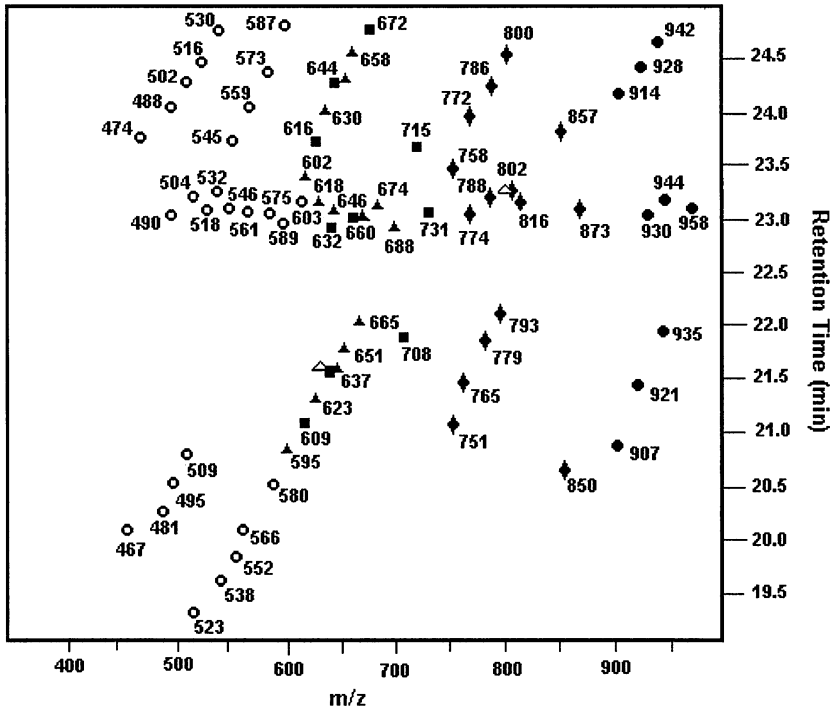

FIGURE 14 Schematic representation for the overlapping of all the 2-D mass chromatograms from the low molecular mass fractions of the Nephilinae spider venoms. The black spots were placed just on the retention time of each eluting peak, obtained by using a RP-microcolumn Develosil ODS-HG-5 (0.3 × 150 mm) coupled to a HPLC under gradient of MeCN from 0% to 80% (v/v) in 20 min. The numbers represent the molecular masses of each toxin in the monoprotonated form. The schedule represents the distribution of the polyamine backbones tail (structural part IV) along the resulting overlapped chromatographic profile of acylpolyamine toxins: (○) represents those toxins not presenting a tail; (▲) represents the toxins presenting an arginine residue as backbone tail; (■) represent the toxins presenting a single putreanine unit as backbone tail; (△) represent the toxins presenting two putreanine units conjugated in tandem as backbone tail; (●) represent the toxins presenting three putreanine units conjugated in tandem as backbone tail; (♦) represent the toxins presenting the dipeptide ornithyl-arginine as backbone tail.

creating more structural variability. This strategy is present in about 50% of all known acylpolyamine toxin structures from Nephilinae spider venoms.

The toxins presenting the m/z values 488, 637, 644, and 802 were described as bearing the dipeptide ornithyl-arginine

at their tail. In addition to these examples, ornithine was also used as a chemical building block, forming the dipeptide ornithyl-asparagine as a very rare linker group as depicted in Figure 12. Curiously, all these structural examples were observed only for "Joro Spider" (*Nephila clavata*) (Table 1), whose occurrence became limited to Japan by end of 1980s and early 1990's (Itagaki et al., 1997; Yoshioka et al., 1990; Teshima et al., 1990). Three different toxins presenting MW 801 Da were elucidated: NPTX-801C, NPTX-801E, and NPTX-2 (subtype G). In spite of having the same molecular masses, these molecules are not isomers, and individually each one has a different polyamine chain subtype (C, E, and G) (Aramaki et al., 1986; Palma et al., 1997; Palma et al., 1998). Both NPTX-801C and NPTX-801E have in the tail position two putreanine units attached in tandem; while the toxin NPTX-2 has the rare tripeptide putreanyl-ornithyl-arginine as polyamine chain tail.

Thus, once more nature used simple chemical building blocks to create nine options of tails among the acylpolyamine toxins from Nephilinae spiders, summarized as follows:

- no tail attachment, i.e., just a reduction of the last chemical building group of the polyamine chain sub-type;
- a single putreanyl unit;
- a di-putreanyl in tandem;
- a tri-putreanyl in tandem;
- a single arginine residue;
- a single ornithine residue;
- a dipeptide ornithyl-arginine;
- a tripeptide putreanyl-ornithyl-arginine;
- a tripeptide alanyl-glycyl-arginine.

All these observations may be used as evidence to corroborate the plasticity of the natural combinatorial chemistry of Nephilinae spiders, operating to create more effective acypolyamine toxin structures as paralyzing/killing tools.

A careful examination of Table 1 reveals some interesting aspects that come out of the prior discussion, which must be emphasized:

- *Nephila madagascariensis* does not use the polyamine backbone subtypes A and D very frequently. At first it could be thought

TABLE 1 Distribution of the Acylpolyamine Toxins Among the Nephilinae Spiders

Subtype	Toxin name	N. clavata	N. madagascariensis	N. cruentata	N. borbonica
A	JSTX-3	+	+	+	+
	NPTX-8	+	+	+	+
	NPTX-1	+	nd	+	+
	NPTX-707A	+	nd	+	+
	NPTX-714A	+	nd	+	+
	NPTX-730A	+	+	+	+
	NPTX-849A	+	nd	+	+
	NPTX-856A	+	nd	+	+
	NPTX-872A	+	nd	+	+
	NPTX-744A	+	nd	+	+
B	NPTX-466B	+	+	+	+
	NPTX-473B	nd	nd	+	+
	NPTX-489B	nd	+	+	+
	NPTX-608B	+	+	+	+
	NPTX-615B	nd	+	+	+
	NPTX-631B	nd	+	+	+
	NPTX-750B	+	+	+	+
	NPTX-773B	nd	+	+	+
	NPTX-622B	+	+	+	nd
	NPTX645B	nd	+	+	+
C	NPTX-494C	+	+	+	+
	NPTX-501C	+	+	+	+
	NPTX-517C	+	+	+	+
	NPTX-636C	+	+	+	nd
	NPTX-643C	+	+	+	+
	NPTX-659C	+	+	+	+
	NPTX-778C	+	nd	+	+
	NPTX-785C	+	+	+	+
	NPTX-801C	+	+	+	+
	NPTX-927C	nd	nd	+	+
	NPTX943C	+	nd	+	+
D	NPTX-508D	+	+	+	+
	NPTX-12	+	+	+	+
	NPTX-531D	+	+	+	+
	NPTX-657D	+	+	+	+
	NPTX-673D	+	+	+	+
	NSTX-3	+	nd	nd	+
	NPTX-10	+	nd	+	nd
	NPTX-687D	+	+	nd	nd
	NPTX-792D	+	nd	nd	+
	NPTX-799D	+	+	+	+

(Continued on next page)

TABLE 1 Distribution of the Acylpolyamine Toxins Among the Nephilinae Spiders *(Continued)*

Subtype	Toxin name	N. clavata	N. madagascariensis	N. cruentata	N. borbonica
	NPTX-5	+	nd	+	+
	NPTX-941D	+	nd	+	+
	NPTX-6	+	nd	+	+
	CLAVAMINE	+	nd	nd	+
E	JOROAMINE	+	nd	nd	nd
	SPIDAMINE	+	+	+	+
	NPTX-501E	+	+	+	+
	NPTX-517E	+	+	+	+
	NPTX-650E	+	+	+	nd
	NPTX-657E	+	+	+	+
	NPTX-673E	+	+	+	+
	NPTX-801E	+	+	+	+
	NPTX-943E	+	nd	+	+
F	NPTX-643F	+	+	+	+
G	NPTX-11	+	nd	nd	nd
	JSTX-4	+	nd	nd	nd
	NPTX-9	+	nd	nd	nd
	NPTX-2	+	nd	nd	nd
	JSTX-2	+	nd	nd	nd
	NPTX-7	+	nd	nd	nd
	NPTX-3	+	nd	nd	nd
	NPTX-906G	+	nd	nd	nd
	NPTX-913G	+	nd	nd	nd
	NPTX-4	+	nd	nd	nd

that it would be related to a local adaptative strategy to combine the use of the building blocks cadaverine and putreanine, since this spider synthesizes other toxins that use other arrays of the same chemical building blocks.

- *Nephila clavata* does not seem to use frequently the backbone subtype B. At first, this observation could suggest that the spider has some difficulty in producing the chemical building blocks putrescine and diaminopropane. However, this spider biosynthesizes other toxins bearing these moieties, such as those of subtype C. Another aspect about *N. clavata* that must be emphasized is related to the occurrence of all known toxins of subtype G. The amino acid ornithine is used in this subtype both to form a linker group with the residue of asparagine and as part of the

backbone tail. Among the Nephilinae spiders investigated up to now, only *N. clavata* used ornithine as a chemical building block.
- *N. cruentata* and *N. borbonica* present a similar profile of toxins, in which those structures bearing a backbone subtype D are relatively less common than the other subtypes.

Thus, during evolution these spiders already may have tried successfully and/or unsuccessfully, many different arrays of chemical building blocks to be used as paralyzing/killing tools for the types of preys existing in each natural ecosystem from which each spider came. The toxins that were detected may just represent the result from the successful trials during evolution and were being used as part of the spiders' arsenal to improve the efficiency of their forage at the moment of sample collection. Orb-web spiders are polyfagous animals, which prey on a large number of different species of flying Arthropods of different Orders, in which the venom constitutes an important part of the strategy for prey capture. Thus, the venom must contain a large number of different chemical structures of acylpolyamine toxins in order to enable the spiders to adapt to catching most of potential prey existing in each ecological situation.

IV. Summary

The large number of spider toxin structures presenting the same chemical nature (acylpolyamine amides), elucidated in the same subfamily of spiders, offered a very interesting opportunity to understand the way that nature has combined a small number of chemical blocks, in a combinatorial array, to create a wide weaponry to kill/paralyze the many different types of prey. In spite of the spiders being well known predators, the orb-web spiders are almost sedentary animals that use their webs as nests, as prey-traps, and as interface to interact with the surrounding world (Quicke, 1988). This way, in order to optimize the spider foraging, the venom composition of these spiders must include a wide number of closely related, but structurally different, acylpolyamine toxins to act on the large spectrum of potential preys.

A total of 67 different structures of acylpolyamine toxins have been structurally solved in the venoms of four different species of Nephilinae spiders. This large number of structurally related

compounds seems to reflect both the overall successful attempts of nature to produce effective toxins and the wide variety of preys captured by the orb-web spiders.

Considering the polyamine chain (part III) as structural reference to start the biosynthesis of these toxins, is possible to select one of its seven subtypes (from A to G) to combine with the linker group (part II) to produce the polyamine backbone. The asparagine residue is the most commonly used linker group in this backbone. However, nature has already tried the dipeptide ornithyl-asparagine, instead of the single amino acid residue as linker group. This backbone, in turn, may be connected to one of the three known chromophores, i.e., either with 2,4-dihydroxyphenyl acetic acid, 4-hydroxyindole acetic acid, or even with indole acetic moiety, creating molecules with different hydrophobicity. Optionally a tail (part IV) may be attached to the polyamine chain in a single unit, or sometimes in tandem mode.

Most of structures of these toxins were repeatedly observed in all species investigated at different times. The fortuitous findings in Japan of the uncommon chromophore 4-hydroxyindole acetic acid in the toxin Joroamine, or the observations of the dipeptide Orn-Arg and the tripeptide alanyl-glycyl-arginine at the polyamine backbone tail of some toxins, are not observed anymore in other parts of world. These specific structures were not observed again, even in Japan, in a more recent investigation with the same spider species (*N. clavata*) for which these structural features were originally described (Hisada et al., 1998). These observations may be used to speculate about the attempts of nature to apply a chemical combinatorial array to biosynthesize a few polyamine toxin structures in the spider, *N. clavata,* by using simple chemical building blocks. Probably these were unsuccessful trials (just-in-time detected) still ongoing by the end of 1980s and/or early 1990s, when the spiders *Nephila clavata* were collected in Japan.

Potentially the combinations of three chromophores, two types of amino acid linkers, seven polyamine backbone subtypes, and nine options of tails offer 378 different structural possibilities. However, we detected only 91 toxin structures, which may represent only the most successful structural trials in terms of efficiency of prey paralysis/death. This means that there potentially still remain many other structures to be discovered, if they were not already tried and discarded earlier in the course of evolution

of the Nephilinae spiders. The knowledge both of a wide number of chemical structures and the basic rules used by nature to produce these toxins based on a combinatorial strategy, may help us to develop new laboratory routes to new drugs.

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