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Solution conformation of leiurotoxin I (scyllatoxin) by ^1H nuclear magnetic resonance

Resonance assignment and secondary structure*

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A proton NMR study at 500 MHz of leiurotoxin I in water is presented. Nearly complete sequence-specific assignments of the individual backbone and side-chain proton resonances were achieved using through-bond and through-space connectivities obtained from standard two-dimensional NMR techniques. The secondary structure of this toxin is inferred from a combination of short-range nuclear Overhauser enhancements, scalar couplings and proton/deuteron exchange rates. Three disulfide bridges locate the N-terminal part (that is α-helical from residue 6 to 16) on one side of a C-terminal two stranded antiparallel β sheet (from Leu^{18} to Val^{29}). The latter features a tight turn at Gly^{23}-Asp^{24}.

Scorpion toxin; NMR, 2-dimensional; Structure, secondary; Apamin

1. INTRODUCTION

Several groups have recently reported that the venom of the scorpion *Leiurus quinquestriatus hebraeus* contains a fraction having binding and physiological properties similar to those found for apamin, an octadecapeptide from been venom. Leiurotoxin I (also named scyllatoxin) is able to inhibit apamin binding to its receptor [1-4]. Like apamin [5], leiurotoxin acts as a blocker for Ca^{2+}-activated K^+ channels in different cellular types [1-4]. Leiurotoxin I has been isolated and sequenced [3]. It consists of a single chain 31 amino acids long peptide containing 3 disulfide bridges. Remarkably, although their biological activities are very similar, there is no sequence homology between apamin and leiurotoxin I (fig.1). However, the fact that a reduced and S-alkylated form of the toxin is totally inactive [4] suggests that the spatial arrangement is of crucial importance for its biological activity. The determination of the three-dimensional structure of leiurotoxin I is thus essential for a better understanding of the structural features involved in the binding to this particular class of ion-channels.

In recent years NMR spectroscopy provided powerfull two-dimensional (2D) techniques that allow the determination of the structure of small proteins in 1-10 mM solutions [6,7]. As the total amount of leiurotoxin I represents only 0.02% of the proteins in the crude venom, a synthetic peptide corresponding to its sequence has been prepared by solid-phase synthesis. The binding and the physiological properties of the synthetic peptide were shown to be totally identical to those of the natural toxin [4].

In this paper we report the nearly complete resonance assignment and the secondary structure of leiurotoxin I in water, using standard ^1H 2D NMR correlation techniques (DQF COSY, HOHAHA, NOESY, ROESY). We show that leiurotoxin I adopts a compact globular conformation and contains the major types of secondary structure of proteins. In addition, a structural basis to explain the similarity in biological activities of leiurotoxin I and apamin is postulated.

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apamin
1     5     10    15
C N C K A P E T A L C A R R C Q Q H - NH2

leiurotoxin I
1     5     10    15    20    25    30
A F C N L R M C Q L S C R S L G L L G K C I G D K C E C V K H - NH2
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Fig.1. Amino acid sequences of apamin and leiurotoxin I.

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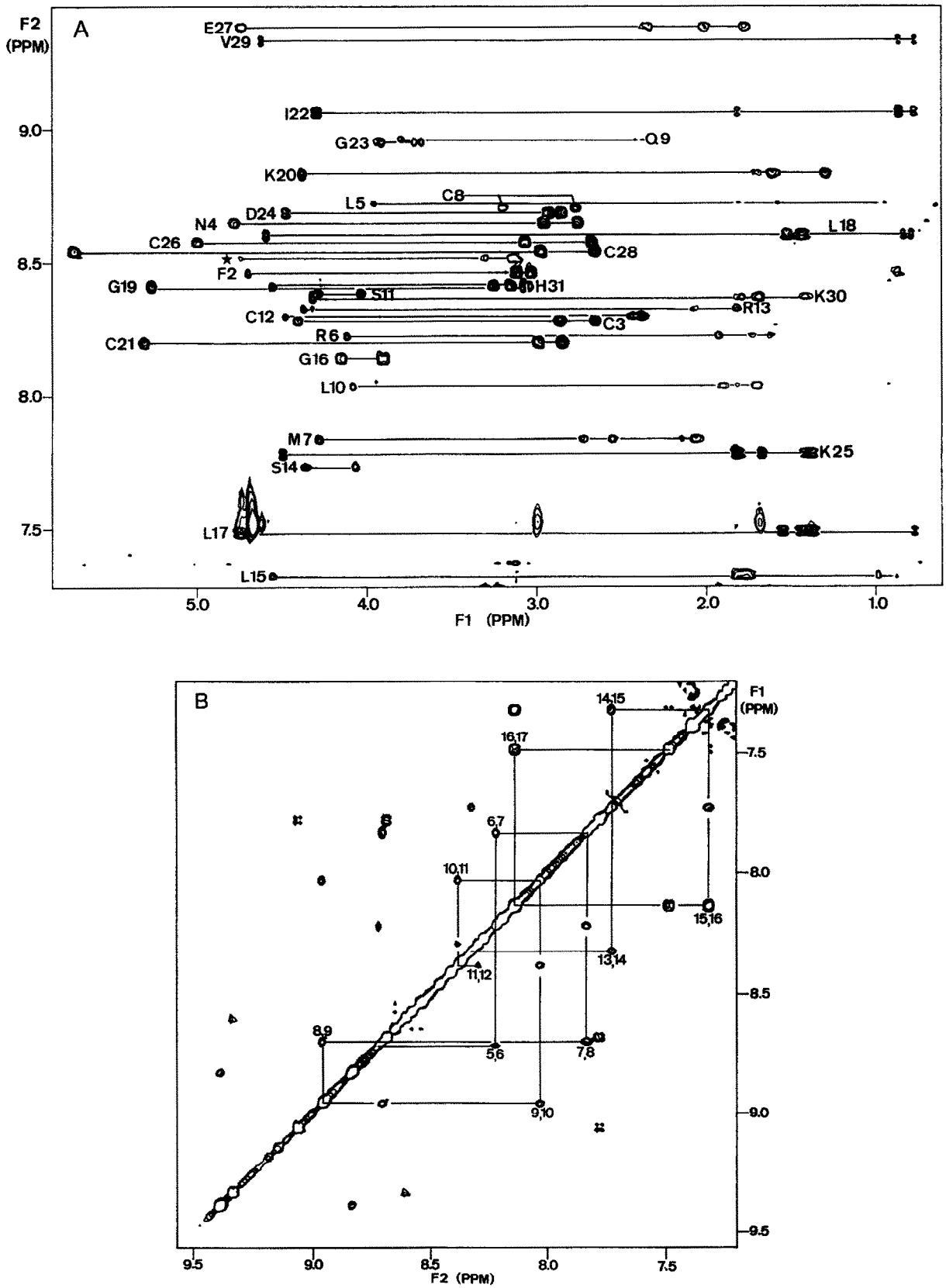

Fig.2. Contour plots of 500 MHz 2D absorption mode spectra of 1.5 mM leiurotoxin I in water, pH 2.72, 40°C. (A) HOHAHA spectrum showing intra-residue scalar connectivities (* is an impurity). (B) NH region of NOESY spectrum. The sequential connectivity in the Leu[5]-Leu[17] segment is traced.

2. MATERIALS AND METHODS

Leiurotoxin I was prepared by solid-phase synthesis. A two step purification scheme yielded the highly purified toxin in 8% overall yield. The details of the synthesis, cyclisation, purification and biological characterization have been reported elsewhere [4].

All spectra were recorded at 500.13 MHz on a Bruker AM spectrometer, equipped with an Aspect 3000 computer. Data were processed on a Bruker X32 workstation. A 1.5 mM solution of leiurotoxin I was prepared in 90% H_2O/10% D_2O. Chemical shifts are quoted relative to internal DSS. Standard pulse sequences were applied for the following 2D techniques: DQF-COSY [8,9], HOHAHA [10–12], NOESY [13,14] and ROESY [15,16]. All measurements were performed at 40°C and pH 2.72. For pure phase absorption line shapes, phase-sensitive acquisition using time-proportional phase incrementation [17] was used in all 2D spectra. The spectral width was 6024 Hz resulting in a digital resolution of 5.88 Hz/pt in both dimensions, with zero filling in the t_1 dimension only. For the DQF-COSY 1024 FID's of size 2 K, 48 scans each were recorded, while all others were obtained with 750 FID's of size 2 K, 64 scans each. Prior to Fourier transformation the data matrix was multiplied by a sine bell function, with a phase shift of $\pi/8$ in the t_2 dimension and $\pi/4$ in t_1. A polynomial baseline correction was performed in the F_2 dimension using standard Bruker software.

The water signal was suppressed by selective irradiation during the relaxation delay (1.3 s in all experiments) and the t_1 period for the DQF-COSY and carried on during the 300 ms mixing time in the NOESY experiment. No water presaturation was used in HOHAHA and ROESY. Instead, a pulse scheme proposed by Bax [18] was used which allows a selective excitation by means of a '1-1 echo' read sequence [19] after spin-locking. Maximum excitation was set at 8 ppm using $\tau = 148.6\ \mu s$.

The HOHAHA spectrum was obtained through 77 ms MLEV17 mixing with a field strength of 6.41 kHz (39 μs $\pi/2$ pulse) flanked by 2.5 ms trim pulses. The ROESY spectrum was recorded with a 300 ms continuous irradiation of 4.63 kHz (54 μs $\pi/2$ pulse) for spin locking. In both experiments acquisition was followed by a 4 ms long pulse to ensure identical amounts of z-magnetisation at the start of each sequence.

Exchange rates of amide protons in D_2O were measured from 1D 32 K spectra with a spectral width of 6024 Hz, using a linear least squares fit of the data. $^3J(NH,\alpha H)$ coupling constants were obtained from a 64 K 1D spectrum of 6024 Hz using the standard NOESY pulse sequence with a 5.1 μs $\pi/2$ pulse. The water resonance was saturated during the 1.3 s relaxation delay, 2 μs t_1 period and 50 ms mixing time.

3. RESULTS AND DISCUSSION

Sequence specific assignment of the resonances comprises two steps [6]. First, the spin systems of all amino acid residues are identified by their through-bond connectivities. Fig.2A shows part of a HOHAHA spectrum in H_2O, with all NH side-chain correlations indicated, except for Ala[1] whose terminal amino group is protonated and in fast exchange with water. DQF-COSY (not shown) was used to distinguish direct from relayed connectivities in the aliphatic side-chains.

Second, the position of these spin systems in the sequence is obtained using through-space connectivities between neighbouring residues from NOESY and ROESY spectra. Three unique spin systems Ala[1], Ile[22] and Val[29] occur only once in the sequence and are used as starting points for the sequential assignment process. Beginning at Ile[22], the Gly[19]-Asp[24] segment is assigned by αH_i-NH_{i+1} NOESY cross-peaks. Similarly the Lys[25]-His[31] part is defined from Val[29]. The train of αN NOE connectivities is interrupted between Asp[24] and Lys[25], where the connection is formed by a NH_i-NH_{i+1} NOE, indicating a change in the local secondary structure.

Starting from Gly[16], as the remaining Gly spin system, the Leu[5]-Leu[17] segment is sequentially assigned by a continuous series of NH_i-NH_{i+1} NOE's, as il-

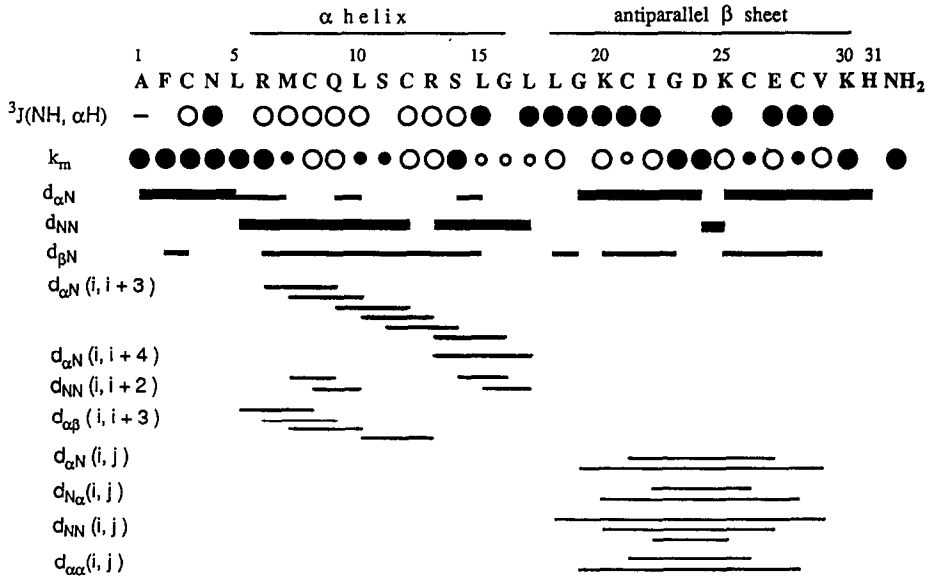

Fig.3. Overview of NMR constraints of leiurotoxin I. $^3J(NH,\alpha H) > 8.5$ Hz and < 5.3 Hz are presented as filled (●) and open (○) circles respectively. Amide H/D exchange rates (k_m) are classified as fast (●), moderately fast (•), moderately slow (○) and slow (○). NOE distance constraints (d) are denoted as usual, e.g. $d_{NN}(i,i+3)$ represents NOE between NH protons of residues i and $i+3$ (default indices are $i,i+1$). Regular secondary structure elements are indicated on top.

Table I

Resonance assignments for leiurotoxin I protons

Residue	NH	Hα	Chemical shift Hβ	Others	^3J(NH,αH)	100 × k_m (min^{-1})
Ala 1	(a)	4.07	1.51		(a)	>10
Phe 2	8.44	4.68	3.12, 3.02	δH 7.24; εH 7.37; ζH 7.34	7.6	>10
Cys 3	8.28	4.38	2.87, 2.65		(b)	>10
Asn 4	8.64	4.77	2.95, 2.75	δNH_2 7.80; 6.97	8.9	>10
Leu 5	8.72	3.95	1.79, 1.56	γCH_2 1.47; δCH_3 0.94	(b)	>10
Arg 6	8.22	4.12	1.92	γCH_2 1.74, 1.61; δCH_2 3.30, 3.22; εNH 7.26	3.9	>10
Met 7	7.83	4.28	2.16, 2.06	γCH_2 2.73, 2.55; εCH_3 2.12	5.5	3.2
Cys 8	8.70	4.55	3.18, 2.76		4.1	0.2
Gln 9	8.96	3.80	2.40, 1.99	γCH_2 2.62; εNH_2 7.34; 6.79	3.9	0.3
Leu 10	8.03	4.09	1.90, 1.72	γCH_2 1.81; δCH_3 0.93	4.3	5.6
Ser 11	8.37	4.30	4.04		(b)	1.7
Cys 12	8.29	4.48	2.43, ~2.38		3.9	0.2
Arg 13	8.32	4.36	2.17, 2.07	γCH_2 1.81; δCH_2 3.25; εNH 7.21	3.27 4.07	0.1
Ser 14	7.73	4.36	4.07		3.8	>10
Leu 15	7.31	4.55	~1.76	γCH_2 ~1.76; δCH_3 0.98, 0.88	9.2	1.2
Gly 16	8.13	4.15, 3.90			6.2; 6.6	0.7
Leu 17	7.49	4.74	1.47, 1.39	γCH_2 1.55; δCH_3 0.78	10.0	2.4
Leu 18	8.60	4.59	1.52	γCH_2 1.43; δCH_3 0.83, 0.78	9.0	0.3
Gly 19	8.41	5.26, 3.06			8.6	(b)
Lys 20	8.83	4.38	1.56	γCH_2 1.29; δCH_2 1.70; εCH_2 2.85	8.7	0.001
Cys 21	8.17	5.32	2.99, 2.85		9.2	2.0
Ile 22	9.06	4.31	1.81	γCH_3 0.86; δCH_3 0.76	9.8	0.3
Gly 23	8.95	3.93, 3.69			Σ11.6	>10
Asp 24	8.68	4.47	2.92, 2.84		8.2	>10
Lys 25	7.77	4.50	1.82	γCH_2 1.39; δCH_2 1.67; εCH_2 3.00; ζNH 7.52	9.5	0.3
Cys 26	8.57	5.00	3.07, 2.67		7.6	~10
Glu 27	9.38	4.71	2.01, 1.78	γCH_2 2.33	9.6	0.007
Cys 28	8.54	5.73	2.97, 2.66		9.2	10
Val 29	9.33	4.62	2.20	γCH_3 0.87, 0.77	9.6	0.3
Lys 30	8.36	4.32	1.795	γCH_2 1.41; δCH_2 1.69; εCH_2 2.961	(b)	>10
His 31	8.41	4.56	3.25, 3.15	δ2H 8.64; ε1H 7.37; βNH_2 7.57, 7.10	(b)	(b)

^1H chemical shifts δ (in p.p.m. relative to internal DSS) in H_2O solution at 40°C and pH 2.72, vicinal coupling constants ^3J(NH,αH) (in Hz) and amide hydrogen/deuterium exchange rates k_m (in min^{-1})
(a) Fast exchange with H_2O; (b) Not evaluated, overlapping resonances

lustrated in fig.2B. The NH_{12}-NH_{13} NOE is not observed possibly due to the identical chemical shifts of the amide protons of Cys[12] and Arg[13]. These assignments are supported by a set of weak β_i-NH_{i+1} NOE's. Leu[17], Leu[18] and Gly[19] are linked by αH_{17}-NH_{18} and βH_{18}-NH_{19} NOE's. Finally, the two AMX spin systems of Phe[2] and Asn[4] can be recognized by the intra-residue NOE from βH to the aromatic ring and the side chain protons respectively [6]. The remaining AMX spin system is Cys 3. An α_i-NH_{i+1} 'walk' from Ala[1] to Leu[5] confirms this. Table 1 summarizes the chemical shifts of the assigned proton resonances of leiurotoxin I.

The delineation of regular secondary structure of proteins from NMR data [6,20] is based on characteristic sets of specific and short interproton distances (i.e. sequential, medium- and long-range NOE's), ^3J(αH,NH) coupling constants and amide proton exchange rates. The relevant data for leiurotoxin I are presented in table 1 and fig.3. Several distinct zones of regular secondary structure can be recognized. A chain of relatively intense NH_i-NH_{i+1} NOE's and

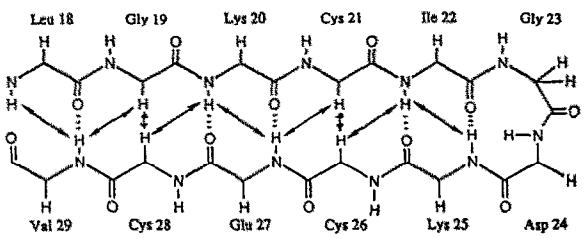

Fig.4. The antiparallel β sheet is leiurotoxin I. Observed interstrand NOE's are indicated by arrows.

$^3J(NH,\alpha H)$ values smaller than 4 Hz (except 5.3 Hz for Met^7) indicates the presence of an α-helix extending from Arg^6 till Gly^{16}. The appearance of a series of NOE's characteristic for a helix confirms this: αH_i-NH_{i+3} (for i = 6, 7, 9, 10, 11, 13), αH_i-βH_{i+3} (i = 5, 6, 7, 10), NH_i-NH_{i+2} (i = 7, 8, 14, 15) and αH_i-NH_{i+4} (i = 13). Typically, in this region αH_i-NH_{i+1} NOE's are weak or absent and amide proton exchange rates are small as compared to the first five N-terminal residues. The latter show k_m values $>10^{-1}$ min^{-1} and strong αN sequential NOE's indicating a solvent exposed rather extended conformation.

An antiparallel β sheet can be located in the Leu^{18}-Val^{29} segment, with a tight turn at Gly^{23}-Asp^{24}. The large $^3J(NH,\alpha H)$ values and the strong αH_i-NH_{i+1} NOE's indicate an extended conformation. A large number of interstrand connectivities of the type α-H_i-αH_j (i-j = 19-28; 21-26), NH_i-NH_j (18-29; 20-27; 22-25); NH_i-αH_j (20-28; 22-26) and αH_i-NH_j (19-29; 21-27) imply the tight turn at residues 23, 24 and the antiparallel nature of the β-sheet (see fig.4). The slow exchange observed for the amide protons of residues 18, 22 and 25 ($k_m \sim 3 \times 10^{-3}$ min^{-1}) and especially for Lys^{20} and Glu^{27} near the center of the sheet ($k_m = 10^{-5}$ and 7×10^{-4} min^{-1} respectively) reflect the interstrand hydrogen bonding. The k_m-values typical of solvent exposed NH's appear alternately in the two strands. The β turn is marked by the two successive large k_m values ($>10^{-1}$ min^{-1}) for Gly^{23} and Asp^{24}, and by short αH_{23}-NH_{24} and $NH_{24}NH_{25}$ distances.

The side chains of Cys^{21}, Cys^{26} and Cys^{28} point to one side of the β sheet. At the same time Cys^8 and Cys^{12} are located on the same side of the α helix. When the local NOE and $^3J(NH,\alpha H)$ constraints concerning the Leu^{15}-Leu^{18} fragment are implemented in a 3D mechanical model, it follows that the Leu^{17} side chain is located in the pocket between the α helix, the β sheet and the Cys^{12}-Cys^{28} disulfide bridge. The observed NOE's between the butyl side chain of Leu^{17} and the β protons of both Cys^{12} and Cys^{28} prove this. The two disulfide bridge arrangements 12-28, 3-26, 8-21 and 12-28, 3-21, 8-26 cannot be distinguished at the present level of structural analysis.

In conclusion, this paper presents the sequence specific resonance assignments and the secondary structure of leiurotoxin I. A quantitative treatment of NMR constraints using distance geometry techniques and restrained molecular dynamics in order to determine its three-dimensional structure, is in progress.

In spite of their different chemical structures, apamin and leiurotoxin I probably have identical binding sites [4]. In apamin, the two arginine residues, Arg^{13} and Arg^{14}, which are known to be essential for the toxin activity [21], have been shown to be located on an α helix [22]. There are two arginine residues in leiurotoxin I, Arg^6 and Arg^{13} which are also located on an α helix. They are not sequential but are found two turns of the helix apart from each other on the same side of an amphiphilic structure. In both cases, the length of the side chain of arginine allows the two charged guanidinium groups to be located in close vicinity. Although further work will be necessary to determine the exact arrangement of the His-$CONH_2$ C-terminus with respect to these residues, this result provides a structural basis to explain the similarity in biological activities between the two toxins.

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