SEQUENCE-SPECIFIC ASSIGNMENT OF ¹H-NMR RESONANCE AND DETERMINATION OF THE SECONDARY STRUCTURE OF HAINANTOXIN-Ⅰ

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Abstract:　Hainantoxin-Ⅰ (HNTX-Ⅰ) is an insect blocker of voltage-gated sodium channel isolated from the venom of the Chinese bird spider *Ornithoctonus hainana* (early named *Selenocosmia hainana*).　The three-dimensional conformation of HNTX-Ⅰ in aqueous solution was determined using two-dimensional ¹H NMR techniques.　The complete sequence-specific assignments of proton resonance in the ¹H-NMR spectra of HNTX-Ⅰ were obtained by analyzing a series of 2D spectra,　including double-quantum-filtered correlation spectroscopy (DQF-COSY),　total correlated spectroscopy (TOCSY) and nuclear overhauser effect spectroscopy (NOESY) in H_2O and D_2O. All the backbone protons and more than 96% of the side-chain protons were identified by $d_{\alpha N}$, $d_{\beta N}$, d_{NN} and $d_{\alpha\delta}$ connectivities in NOESY spectrum. Furthermore, it was found that the main element of the secondary structure of HNTX-Ⅰ is a short triple-stranded antiparallel β-sheet with Lys7-Cys9, Tyr20-Asn23 and Trp28-Val31, based on $^3J_{NH\text{-}C\alpha H}$ coupling constants, sequential NOE connectivities and slowly exchanging amide protons. These characters of the secondary structure of HNTX-Ⅰ are similar to those of HWTX-Ⅰ, SHL-Ⅰ and HWTX-Ⅳ, which have known solution structures.　These results provide a basis for the further determination of the solution conformation of HNTX-Ⅰ.

Key Words:　HNTX-Ⅰ; 2D-NMR; Sequence-specific assignment; Secondary structure

1　Introduction

Many peptide toxins isolated from different animal phyla (e.g. spiders, scorpions, sea anemones and snails) have been studied intensively and were found to target insect and mammalian neuronal ion channels[1–3]. Due to their high affinity and specificity, a variety of peptide toxins have been extensively used as structural and pharmacological tools for studying ion channels[4,5].　It is significant to determine the structures of toxins,　which act on different ion channels or different subtype of ion channels,　for investigating the molecular mechanism and the relationship of the structure-function of ion channels.

Recently,　a novel peptide neurotoxin, Hainantoxin-Ⅰ (HNTX-Ⅰ) from the venom of the Chinese bird spider *Ornithoctonus hainana* (early named *Selenocosmia hainana*), has been identified[6].

It contains 33 amino acid residues crosslinked by three conversed intramolecular disulfide bonds and has the amino acid sequence of NH2-ECKGFGKSCVPGKNECCSGYACNSRDKW-CKVLL-CONH2.　The linkage pattern of disulfide bridges is Ⅰ-Ⅳ, Ⅱ-Ⅴ and Ⅲ-Ⅵ (Cys2-Cys17, Cys9-Cys22 and Cys16-Cys29), as assigned by a chemical strategy including partial reduction and sequence analysis[6].　The toxin has been shown to inhibit insect sodium channels[6]. In order to study the structure-activity relationships of HNTX-Ⅰ, we determine the solution structure of HNTX-Ⅰ using

2D ^{1}H-NMR spectroscopy.

In the present study, the complete sequence-specific assignments of proton resonances and the secondary structure of HNTX‐Ⅰ were determined.

2 Materials and methods

2.1 Sample preparation

HNTX‐Ⅰ was isolated and purified from the venom of *O. hainana* by ion exchange chromatography and reverse-phase HPLC as described by Li. The purity of the peptide was confirmed by N-terminal sequence and mass spectrometry analysis. The sample was prepared by dissolving the lyophilized powder of HNTX‐Ⅰ in 500 μl of 20 mmol/L deuterium sodium acetate buffer (H_2O/D_2O, 9/1, v/v) containing 2 mg/L NaN_3 and 0.1 mmol/L ethylene diamine tetra aceticacid (EDTA), with a final concentration of 4.5 mmol/L HNTX‐Ⅰ and pH 4.0. Sodium 3- (trimethyl-silyl) propionate-2, 2, 3, 3-D4 (TSP) was added to a final concentration of 200 μmol/L as an internal chemical shift reference. For experiments in D_2O, the sample used in H_2O experiments was lyophilized, redissolved in 99.8% D_2O, and then allowing it to stand at room temperature for 24 h. After lyophilization, the peptide powder was redissolved in 500 μl of 99.996% D_2O (Cambridge Isotope Laboratories).

2.2 NMR spectroscopy

All NMR spectra were observed on a Bruker DRX-500 spectrometer operating at a proton frequency of 500 MHz with a sample temperature of 298 K. Two-dimensional DQF-COSY, TOCSY and NOESY measurements were recorded in a phase-sensitive mode by the time-proportional phase incrementation (TPPI) method following standard pulse sequences and phase cycling. TOCSY spectra were obtained with a mixing time of 85 ms. NOESY spectra were recorded in D_2O with a mixing time of 200 ms and in H_2O with mixing times of 100, 200 and 400 ms. Solvent suppression was achieved by the presaturation method. All 2D measurements were recorded with 1024 ×512 frequency data points and

were zero-filled to yield 2048 ×1024 data matrices except for the high resolution DQF-COSY spectrum. DQF-COSY spectrum was recorded with 2048 ×512 data points in the t2 and t1 dimensions, respectively, and zero-filled to 4096 ×1024 points to measure the coupling constants. All spectra were processed and analyzed using Felix 98.0 (Biosym Technologies) software running on a Silicon Graphics O$_2$ workstation. The signal was multiplied by a sine bell or sine bell square window functions with a π/2 phase shift, in both dimensions prior to Fourier transformation. Base-line correction was applied using a fifth order polynomial.

2.3 H-D exchange experiment

For the slowly exchanging backbone amide protons, the sample lyophilized from H_2O was redissolved in D_2O and was identified by analyzing a series of one-dimensional (1D) spectra recorded at time scalars of 10 min, 20 min, 40 min, 1.5 h, 3 h, 5 h, 9 h and 24 h. A TOCSY spectrum was recorded after 2 h of exchanging.

3 Results and discussion

Sequence-specific resonance assignments were performed according to the standard procedures established by Wuthrich [7]. It was done in two steps: (i)identification of the spin systems and (ii) sequential assignment of resonances.

3.1 Spin system assignments

The 33 residues of HNTX‐Ⅰ were divided into four groups according to their structural characteristics and spin systems. In short, AX spin systems including four Gly; Five methyl-containing residues including Ala21, two Val and two Leu; Fifteen AMX spin systems including six Cys, three Ser, two Asn, Phe5, Tyr20, Asp26 and Trp28; Nine long side-chain spin systems including five Lys, two Glu, Pro11 and Arg25. The proton resonances of HNTX‐Ⅰ were assigned to the spin systems of specific residue types by analyzing scalar coupling patterns observed in TOCSY and DQF-COSY spectra. Residue types that were immediately

identified were Gly, Ala, Val and Leu.

According to the amino acid sequence of HNTX-I, 35 NH$_i$-C$_\alpha$H$_i$ cross-peaks are expected in the fingerprint region of DQF-COSY spectrum because Glu1 and Pro11 do not exhibit cross-peaks, and four Gly exhibit eight cross-peaks. Figure 1

shows 32 cross-peaks in DQF-COSY spectrum. The other peaks were too weak (Trp28) or overlapped by H$_2$O (Ser8 and Cys29) ("■"), but they could be observed in other spectra. In TOCSY spectrum with a mixing of time 85 ms, all the spin systems of HNTX-I except for Glu1 and Pro11 are shown in Figure 2.
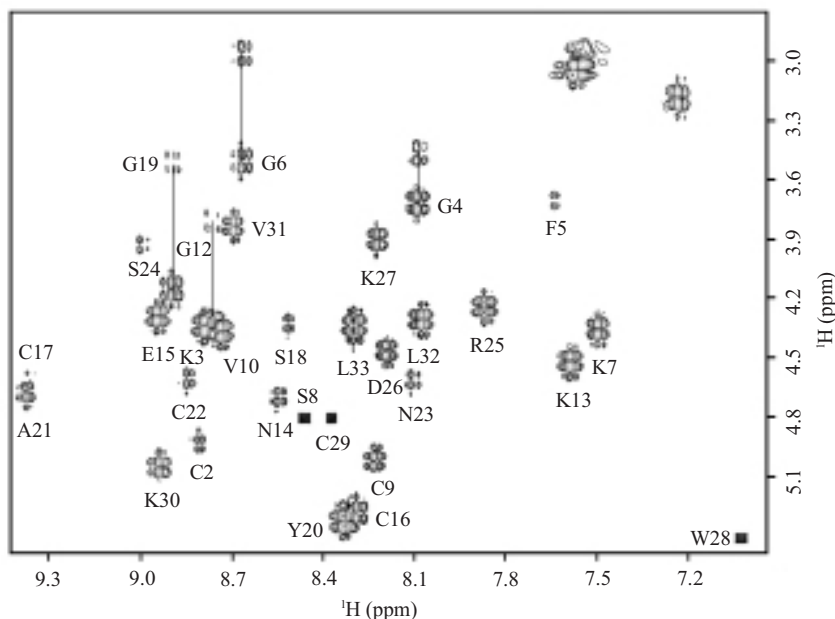

Fig.1 Fingerprint region of DQF-COSY spectrum of HNTX-I in H$_2$O. The NH$_i$-C$_\alpha$H$_i$ cross-peaks are shown. The rectangles (■) indicate the too weak or overlapped weaks
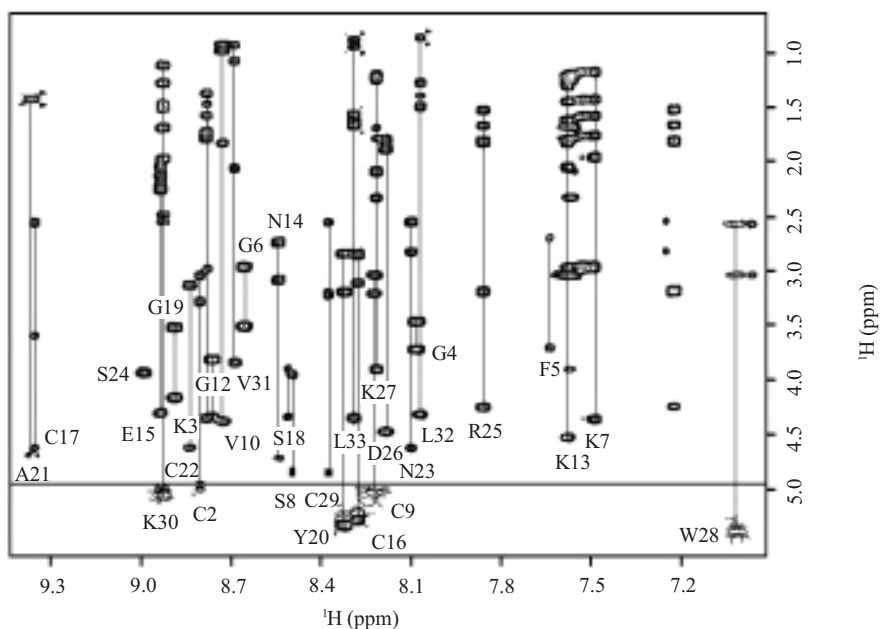

Fig.2 Fingerprint region of TOCSY spectrum of HNTX-I with a mixing time of 85 ms in H$_2$O. All the spin systems of HNTX-I expect for Glu1 and Pro11 are shown

3.2 Sequence-specific assignments

Sequence-specific assignments were undertaken in NOESY spectrum with a mixing time of 200 ms. The identified spin systems were then aligned along the primary structure of this molecule through interresidue sequential NOEs observed in the NOESY spectra. Residue types that were previously assigned were used as starting points for the sequential assignment process. When a $d_{\alpha N}$-type NOE was observed, a sequential connectivity was established only if an additional $d_{\beta N}$ or d_{NN} NOE was also observed. The spin system of residue Pro11 was achieved by the observation of strong sequential NOE cross peaks between the α proton of Val10 and the δ protons of Pro11, which also indicates that residue Pro11 in HNTX-I takes the *trans* configuration. Cys9 and Lys30 were confirmed by the observed $d_{\beta N}$ connectivities, though no sequential $d_{\alpha N(i,i+1)}$ connectivities were found. At the end of the sequential assignment procedure, almost all protons were assigned. Figure 3 shows the sequential $d_{\alpha N\ (i,i+1)}$ connectivities in the CαH-NH fingerprint region of the NOESY spectrum with a mixing time of 200 ms. Table 1 shows the summary of the chemical shifts of proton resonances of HNTX-I.
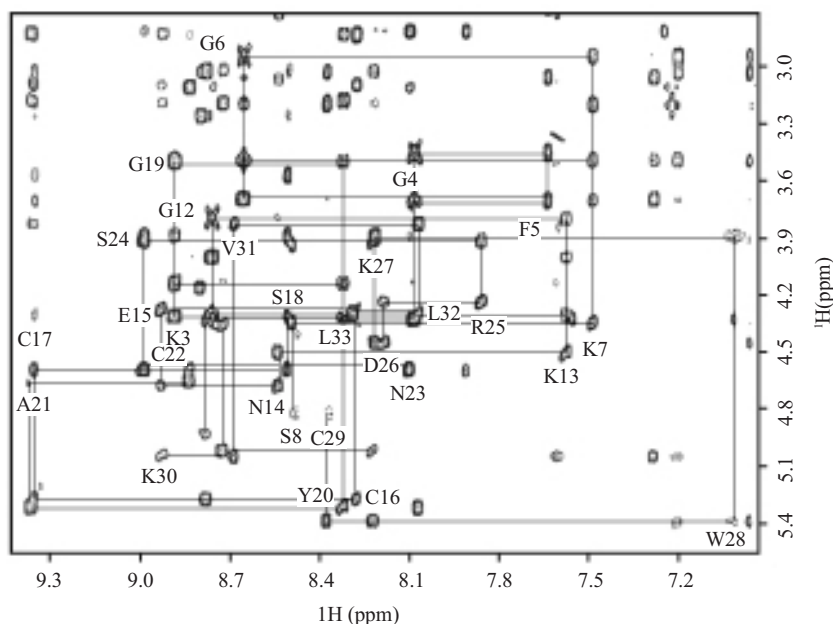

Fig.3 Sequential $d_{\alpha N(i,i+1)}$ connectivities in the CαH-NH fingerprint region of the NOESY spectrum with a mixing time of 200 ms. Sequential $d_{\alpha N}$ connectivities are shown for residues 1~8, 11~29 and 30~33 respectively

3.3 Secondary structure

The regular secondary structure elements of the HNTX-I molecule were identified according to the standard criteria described by Wüthrich[7]. The extent and relative orientation of β-strands were based on strong sequential $d_{\alpha N}$, interstrand NH-NH and NH-CαH connectivities, large $^3J_{NH-C\alpha H}$ coupling constants, and slowly exchanging amide protons, which give a discrimination of the peripheral and central strands in the β-sheet.

The NMR data summarized in Figure 4 and 5 shows a schematic diagram including the observed NOEs and hydrogen bonds. From these analyses, it was found that the main element of the secondary structure of HNTX-I is a short triple-stranded β-sheet with residues Lys7 to Cys9, Tyr20 to Asn23, and Trp28 to Val31, respectively. They are arranged in an antiparallel fashion. Analysis of the CαH chemical shifts was compatible with the three-stranded antiparallel β-sheet, in which most of the residues showed downfield shifts[8]. Figure 5 shows the β-sheet region, in good accordance with the standard criteria.

Table 1　Chemical Shifts of the assigned ¹H-NMR resonances of HNTX-Ⅰ

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	Others
E1		4.18	2.17	$C^{\gamma}H_2$ 2.49
C2	8.81	4.94	3.03, 3.27	
K3	8.78	4.34	1.70, 1.75	$C^{\gamma}H_2$ 1.35, 1.56; $C^{\delta}H_2$ 1.46; $C^{\epsilon}H_2$ 2.97
G4	8.07	3.46, 3.72		
F5	7.64	3.71	2.69, 3.07	C2, 6H 7.29; C3, 5H 7.61; C4H 7.52
G6	8.66	2.96, 3.50		
K7	7.49	4.35	1.95	$C^{\gamma}H_2$ 1.16, 1.42; $C^{\delta}H_2$ 1.57, 1.75; $C^{\epsilon}H_2$ 2.97; $N^{\epsilon}H_3^+$ 7.53
S8	8.49	4.86	3.95	
C9	8.23	5.03	3.03, 3.20	
V10	8.72	4.37	1.81	$C^{\gamma}H_3$ 0.90, 0.96
P11		4.01	1.95, 2.22	$C^{\gamma}H_2$ 1.72, 2.13; $C^{\delta}H_2$ 3.79, 4.15
G12	8.76	3.81, 4.30		
K13	7.58	4.52	1.82, 2.04	$C^{\gamma}H_2$ 1.29, 1.44; $C^{\delta}H_2$ 1.61, 1.75; $C^{\epsilon}H_2$ 2.96
N14	8.54	4.70	2.73, 3.07	$N^{\gamma}H_2$ 6.88, 7.61
E15	8.94	4.29	2.03, 2.12	$C^{\gamma}H_2$ 2.24
C16	8.28	5.27	2.84, 3.11	
C17	9.36	4.61	2.55, 3.58	
S18	8.51	4.33	3.90	
G19	8.89	3.52, 4.15		
Y20	8.33	5.32	2.83, 3.19	
A21	9.37	4.67	1.41	
C22	8.84	4.61	3.12	
N23	8.10	4.61	2.54, 2.82	$N^{\gamma}H_2$ 7.5, 7.91
S24	8.99	3.92		
R25	7.86	4.24	1.81	$C^{\gamma}H_2$ 1.52, 1.66; $C^{\delta}H_2$ 3.19; $N^{\epsilon}H_2$ 7.22
D26	8.18	4.47	1.79, 1.88	
K27	8.21	3.90	2.08, 2.32	$C^{\gamma}H_2$ 1.21; $C^{\delta}H_2$ 1.67, 1.77; $C^{\epsilon}H_2$ 3.05; $N^{\epsilon}H_3^+$ 7.57
W28	7.02	5.40	2.57, 3.04	C2H 6.97; C4H 7.22; C5H 6.88 C6H 7.24; C7H 7.45; N1H 10.18
C29	8.38	4.86	2.55, 3.20	
K30	8.93	5.04	1.67, 1.95	$C^{\gamma}H_2$ 1.10, 1.27; $C^{\delta}H_2$ 1.44, 1.49; $C^{\epsilon}H_2$ 2.47, 2.54
V31	8.68	3.83	2.04	$C^{\gamma}H_3$ 0.91, 1.06
L32	8.07	4.30	1.27, 1.48	$C^{\gamma}H$ 1.38; $C^{\delta}H_3$ 0.85
L33	8.29	4.34	1.55	$C^{\gamma}H$ 1.65; $C^{\delta}H_3$ 0.86, 0.93
CONH₂	7.04, 7.56			

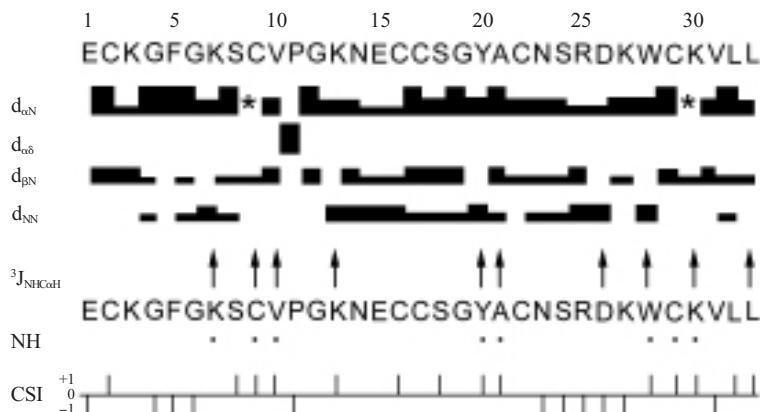Data acquired at pH4.0, 298 K, TSP as the 0 ppm internal standard. Chemical shifts are measured to ± 0.01 ppm

Fig.4 Summary of the sequential NOE connectivities, $^3J_{NH-C\alpha H}$ coupling constants and slowly exchanging backbone NH protons observed in HNTX-I. Difference in NOE intensities of the sequential $d_{\alpha N}$, $d_{\alpha\delta}$, $d_{\beta N}$, and d_{NN} connectivities are represented by block height; Covered NOEs by H_2O are indicated by an asterisk (∗); Filled circles (•) indicate slowly exchanging amide protons; Values of $^3J_{NH-C\alpha H} \geq 8.0$ Hz are indicated by ↑ ; In the chemical shift index (CSI), plus and minus signs represent +1 and −1, respectively
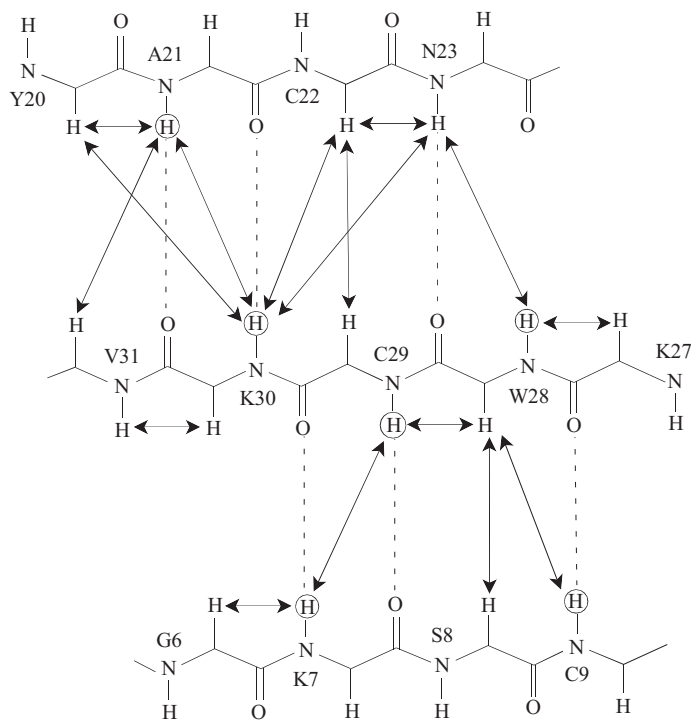

Fig.5 The triple-stranded antiparallel β-sheet (residues 7~9, 20~23, and 28~31) in HNTX-I. Arrows indicate observed NOE, dashed lines represent hydrogen bonds, and the circled protons are slowly exchanging amide protons

3.4 Structural motif

According to up-to-date record, the three-dimensional solution structures of some of spider peptide toxins have been determined by using ^{1}H-NMR spectroscopy. For example, P-type calcium channel inhibitors ω-agatoxin IVA[9] and IVB[10], and insect sodium channel antagonist μ-agatoxins[11] from the venom of the funnel web spider; insect calcium channel inhibitor ω-atracotoxin-HV1[12] from the venom of an Australian funnel web spider; N-type calcium channel inhibitor HWTX-I[13], and SHL-I[14] from the venom of the Chinese bird spider *Selenocosmia huwena*. Although these peptide toxins show low sequence homology and different biological activity, they all share the same structural scaffold known as the inhibitory cystine knot(ICK) motif[15,16]. The main

feature of ICK motif is that it consists of an embedded ring formed by two disulfide bonds and intervening polypeptide backbone that is threaded by a third disulfide bond. Furthermore, the predominant secondary structure element is a small triple-stranded antiparallel β-sheet that is intimately associated with the cystine knot. The locally structural differences determine the different bioactivities.

The characteristic structural feature of HNTX-Ⅰ is a cystine knot and a small triple-stranded antiparallel β-sheet composed of residues Lys7 to Cys9, Tyr20-Asn23 and Trp28 to Val31. The cystine knot in HNTX-Ⅰ is formed by three disulfide bonds linked as Cys2-Cys17, Cys9-Cys22 and Cys16-Cys29. Hence, it is very clear now from our analysis of the secondary structure that HNTX-Ⅰ shares the same cystine knot motif as the above neurotoxins and inhibitory polypeptides.

In conclusion, the complete sequence-specific assignments of proton resonances in the ¹H-NMR spectra of HNTX-Ⅰ were obtained. Moreover, the secondary structure element of HNTX-Ⅰ was determined based on the NMR data. HNTX-Ⅰ adopts the ICK motif. These studies provide a basis for the determination of the solution structure of HNTX-Ⅰ.

Acknowledgment

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海南捕鸟蛛毒素 - Ⅰ（HNTX-Ⅰ）的 ¹H-NMR 信号归属 和二级结构分析

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摘要：海南捕鸟蛛毒素 - Ⅰ（HNTX-Ⅰ）是从海南捕鸟蛛(Ornithoctonus hainana)的粗毒中纯化的一种新型神经毒素。应用二维 ¹H-NMR 技术研究 HNTX-Ⅰ的溶液结构特点，通过分析水和重水中的 DQF-COSY、TOCSY 和 NOESY 谱，识别出 HNTX-Ⅰ全部 33 个氨基酸残基自旋体系；通过 NOESY 谱中的 $d_{\alpha N}$、$d_{\beta N}$、d_{NN} 和 $d_{\alpha\delta}$ 联系完成了序列专一的谱峰归属，从而确认了 HNTX-Ⅰ所有的主链质子和大于 96％的侧链质子的化学位移。并通过分析 $^3J_{NH-C\alpha H}$ 耦合常数、序列间的 NOE 联系以及慢氢交换质子等，确定 HNTX-Ⅰ的二级结构主要是由三股反平行的 β- 折迭组成（Lys7-Cys9, Tyr20-Asn23 和 Trp28-Val31），这些结构特点与已经探明结构的其它蜘蛛毒素的基本相同。这些结果为完全解析 HNTX-I 的溶液三维结构奠定了基础。

关键词：HNTX-Ⅰ；　二维核磁共振；序列专一归属；二级结构

中图分类号：Q617