Large-Scale Purification and Further Characterization of Spidamine and Joramine from Venom of Spider, *Nephila clavata*

Tadashige CHIBA,[a] Toshifumi AKIZAWA,[a] Motomi MASTUKAWA,[a] Hidemitsu PAN-HOU,[a] Nobufumi KAWAI,[b] and Masanori YOSHIOKA*,[a]

Faculty of Pharmaceutical Sciences, Setsunan University,[a] 45–1, Nagaotoge-cho, Hirakata, Osaka 573–01, Japan and Department of Physiology, Jichi Ika University,[b] Kawachi-gun, Tochigi 329–04, Japan.
Received June 7, 1995; accepted July 31, 1995

We purified a new type of polyamine neurotoxins called spidamine and joramine in the venom of a spider, *Nephila clavata*, on a large-scale and confirmed their structures by chemical analysis and NMR spectra. Spidamine was *N*-(2,4-dihydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-β-alanyl)-1,5-pentanediamine, whereas joramine was *N*-(4-hydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-β-alanyl)-1,5-pentanediamine.

We also studied the toxins biological activity on glutamatergic transmission using lobster neuromuscular synapses. The blocking activity of both toxins on the excitatory post synaptic potentials (EPSPs) in the lobster muscle was about one tenth time less than JSTX-3, the most potent toxin in the venom of *Nephila clavata*. Interestingly, spidamine which has 2,4-dihydroxyphenyl group showed an irreversible block of EPSPs, whereas joramine possessing 4-hydroxyphenyl group had a reversible block.

Key words spidamine; joramine; excitatory post synaptic potential; NMR; large-scale purification; spider toxin

Since the discovery of a novel blocker of glutamate receptors in the venom of a spider,[1] numerous reports have been made on the isolation of toxic substances, biological activities and chemical characteristics of spider toxins; so far more than 20 species of the Araneidae family are known to contain the blocker of the glutamate receptor.[2] Spider toxin was the first blocker found from natural sources and it has attracted great interest since the glutamate receptors play the most important role in brain functions.[3,4] It is reasonable to assume that spiders use their venom to paralyze prey whose muscles are innervated by glutamatergic nerves. A major component of the toxic substance in the venom of *Nephila clavata* (joro spider), called JSTX-3 was found to block the glutamate receptors in nervous systems of crustacean neuromuscular synapses, squid giant synapses, and mammalian brain synapses.[5] Recently recombinant study of glutamate receptor subunit genes expressed in Xenopus oocytes reportedly showed that JSTX-3 has a subunit specific blocking action.[6] Several JSTX's inducing JSTX-3 were purified and identified by Aramaki *et al.*[7] Yoshioka *et al.*[8] identified the main insecticide in the same venom as clavamine which had no activity forward the excitatory post synaptic potential (EPSP) of the lobster.

We earlier[9] screened other novel spider toxins by HPLC with attention to the chemical specificity of the common moiety of JSTX-3 and clavamine such as 2,4-dihydroxyphenylacetyl-L-asparaginyl-1,5-pentanediamine from two thousand venom glands. We proposed the chemical structures of two novel polyamine toxins to *N*-(2,4-dihydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-β-alanyl)-1,5-pentanediamine and *N*-(4-hydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-β-alanyl)-1,5-pentanediamine on the basis of constituents of amino acid–polyamine analyzed by HPLC and FAB-MS. The two compounds were called spidamine and joramine, respectively (Fig. 1). It was difficult to measure the NMR spectra of these compounds, however, because the amounts obtained were minimal. These spider toxins seemed to have the blocking action to EPSP, although the intensity and mechanism could not be defined because of the minimal quantity. These compounds were small in content and molecular weight compared with hitherto known toxins. Knowledge of the chemistry of these polyamine toxins is still incomplete, but several toxins of this kind have been identified and synthesized to date. Complete understanding of the components is necessary to make leading compounds for therapeutic drugs and

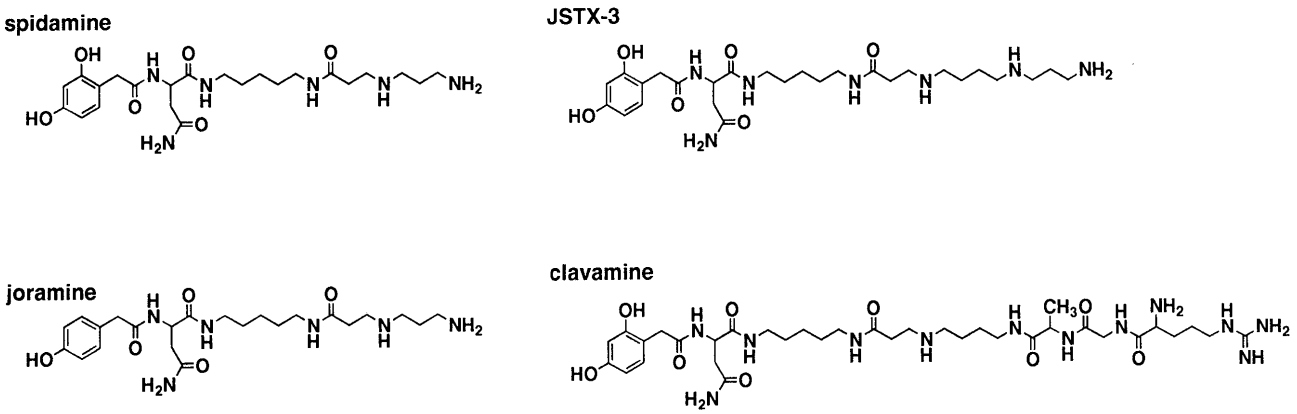

Fig. 1. Structures of Phenolic Polyamine Toxins Found in Joro Spiders

* To whom correspondence should be addressed.

insecticides.

We repurified the two compounds from venom glands on a large-scale to confirm their structures and blocking activity for this paper.

Experimental

Materials A natural JSTX-3 was purified from 2000 venom glands of the joro spider as described.[9] Deuterium oxide for NMR spectroscopy was obtained from Merck (Darmstadt, Germany). All the other chemicals of reagent grade were commercially available.

Blocking Activity on the Glutamate Receptor The blocking activity of the toxins was assayed on the lobster neuromuscular synapse as described.[9] We estimated reduction of the EPSP amplitude at 15 min after bath application of the solution containing the toxin with appropriate concentrations following HPLCs. Gland unit (GU) was defined as the activity of one gland to EPSP.

Component Analyses of Purified and Hydrolyzed Spidamine and Joramine Amino group and phenol group of both toxins were detected by monitoring the fluorescence produced in the reaction of *o*-phthalaldehyde (OPA) with the amino groups and the UV absorption, respectively. A portion of a purified sample was dried *in vacuo* and hydrolyzed at 115 °C for 24 h with 100 μl of 6 N HCl in an evacuated, sealed glass tube under reduced pressure.

Another portion of the hydrolysate was analyzed by dabsylation method[10,11] and heptafluorobutyryl (HFB) derivative method[12] as described.[9]

Aqueous Extraction of Toxins from Venom Joro spiders were collected in the Kinki district, a central area of Japan. The wet weight of 75.53 g was removed from 52342 glands. Twenty grams of venom glands was homogenized with 400 ml of 0.3% NaCl, the remaining glands were treated in the same manner. The supernatant of the extract was separated by centrifugation at $1000 \times g$ for 10 min. It was boiled in a water-bath for 2 min and filtered through a membrane filter (Millipore, Tokyo). The boiled filtrate was concentrated *in vacuo* to approximately 60 ml, and stored at −80 °C until used.

Purification of Active Fractions by Gel Filtration Total volume of the concentrate was adjusted to 75.53 ml with water. A 5 ml portion of this solution was stored as the standard sample for the assay and the remainder of 70.53 ml was passed through a column of Sephadex G-10 (6 × 30 cm, Pharmacia Fine Chemicals, Uppsala) equilibrated and eluted with 0.5% acetic acid. The chromatogram was recorded by monitoring UV absorbance with a Mini UV Monitor II (ATTO, Tokyo) at 254 nm, and the eluent was fractionated into 5 ml fractions. These fractions were pooled into four (Fig. 2) on the basis of the chromatogram. Molecular weight of the constituents in each fraction was estimated by gel filtration on a column (2 × 30 cm, Pharmacia Fine Chemicals) of Biogel P-2 using synthesized peptide standards (Ala–Lys–Asn–Phe–Phe; 625, Lys–Gly–Pro–Gly–Tyr; 521, Gly–Leu–Tyr; 351, Tyr; 181). Fraction No. 2 was inhibitory to EPSP. The fraction was concentrated *in vacuo* and stored at −80 °C until used.

Ion Exchange Chromatography of Fraction No. 2 from Gel Filtration The volume of fraction No. 2 was diluted with 35.3 ml of water. Thirty two ml of this solution was subjected onto a column (5 × 37 cm) of CM Sephadex C-25, eluted with a linear gradient solvent system from 0 to 1 M NaCl and then continuously with an isocratic mode with 2 M NaCl in 100 mM acetate buffer (pH 5.0). The chromatogram was recorded by monitoring UV absorption at 254 nm. The eluent was fractionated by 5 ml each and divided into 9 fractions on the basis of the chromatographic pattern (Fig. 3). Fraction No. 6 contained the activity inhibitory to EPSP. All fractions were concentrated *in vacuo* and stored at −80 °C until used.

The First Reversed Phase HPLC of Fraction No. 6 from Ion Exchange The dried residue of fraction No. 6 was extracted three times with 150 ml of methanol and 50 ml. The supernatant was separated by centrifugation at $1000 \times g$ for 15 min and concentrated to 2 ml *in vacuo*. The residue was subjected to a reversed phase HPLC with Capcell Pak C18 (15 × 250 mm, Shiseido Corporation, Ltd., Tokyo) by monitoring UV absorption at 210 nm to detect not only the phenol group, but also amide groups. The elution was under a linear gradient from water to 30% acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (TFA) for 30 min. The flow rate was set at 10.0 ml/min. The dried residues of the three peaks appearing (Fig. 4) were measured by ^{1}H-NMR spectroscopy (data not shown). Peak No. 2 showed signals of a mixture of 2,4-dihydroxyphenylacetyl (2,4-DHPA) and 4-hydroxyphenylacetyl (4-HPA) moiety.

The Second Reversed Phase HPLC of Peak No. 2 from the First HPLC Peak No. 2 was purified again with analytical HPLC system with Biofine RPCS C18 (4.5 × 250 mm, Japan Spectroscopic Corporation: JASCO, Ltd., Tokyo). Two peaks identified as spidamine and joramine later were collected by monitoring UV spectra with a MULTI-340 multi channel detector under the conditions of linear gradient from water to 70% CH_3CN containing 0.1% TFA for 30 min. The flow rate was set at 1.0 ml/min.

^{1}H-NMR Spectroscopy Each purified sample was dissolved in 0.5 ml of deuterium oxide. ^{1}H-NMR spectra were recorded using JEOL 400 GSX (JEOL, Tokyo) at 400 MHz and GE Omega 600 (Shimadzu, Kyoto) spectrometers at 600 MHz, respectively. The probe temperature was maintained at 30 °C. Signals of HDO and H_2O at 4.721 ppm were used as an internal standard. In 1-dimensional spectroscopy and 2-dimensional correlated spectroscopy (COSY), the H_2O and HDO signals were suppressed by gated irradiation. Spectra were generally recorded with 2048 complex points and 64 or 160 for each free induction decay. Data processing was usually carried out on a SUN 4 workstation (SPARC Station 330, C. Itoh Techno Science Co., Ltd., Tokyo).

FAB-MS[9] Positive ion FAB-MS spectrum for the sample was measured by a mass spectrometer JMS-DX 300 (JEOL, Tokyo) with glycerin matrix at 3 kV.

Results

The supernatant of the venom extract was roughly separated into 4 fractions by gel filtration as shown in Fig. 2. The EPSP inhibitory activity was found in fraction No. 2 corresponding to the low molecular weight region (approximately MW 500) (data not shown). Fraction No.2 was further separated into three active fractions, Nos. 6, 7 and 8 by continuous the ion-exchange chromatography as shown in Fig. 3. Fraction No. 7 had the most activity to EPSP among the three. The yields of the three fractions according to weight, UV absorbance at 275 nm and the activity by EPSP are summarized in Table 1. After two purification steps, specific activity of three of the fractions was 30 fold higher than the one of the venom extract.

Fraction No. 7 contained JSTX-3 and other JSTXs which were active to EPSP. Fraction No. 8 contained clavamine which was inactive to EPSP. Fraction No. 6 was assumed to contain spidamine and joramine of lower

Table 1. Yields of the Purifications

Procedure	Yield (%)						Specific activity (ratio)			
	Weight (g)		Absorbance at 275 nm		Total activity (GU)		GU/g		GU/absorbance	
Crude extract	9.69	(100)	28050	(100)	89	(100)	9.2	(1)	3.2	(1)
Gel filtration (No. 2)	2.90	(29.9)	5315	(18.9)	70	(78.6)	24.1	(2.6)	13.2	(4.2)
Ion exchange (fr. 6, 7, 8)	0.19	(1.9)	3070	(10.9)	52	(58.4)	280.0	(30.5)	16.9	(5.3)
Ion exchange (fr. 7; JSTXs)	1.3×10^{-2}	(1.4×10^{-1})	1639	(5.8)	38	(42.7)	2923	(318)	23.2	(7.3)
Purified spidamine (fr. 6)	4×10^{-4}	(4×10^{-3})	18.1	(6.5×10^{-2})	0.93	(1.0)	2330	(253)	0.51	(0.16)
Purified joramine (fr. 6)	1.5×10^{-4}	(1.5×10^{-3})	7.0	(2.5×10^{-2})	0.34	(0.4)	2270	(246)	0.44	(0.14)

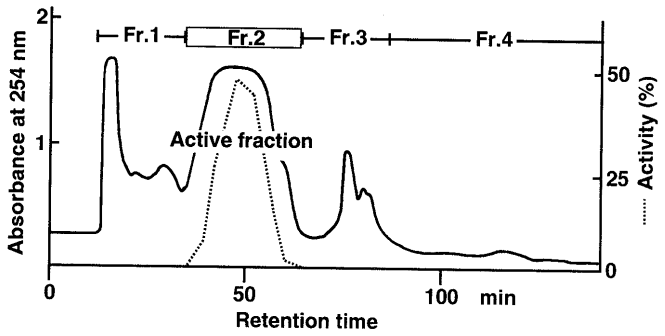

Fig. 2. Chromatogram of Gel Filtration for the Crude Extract and EPSP Inhibitory Activity

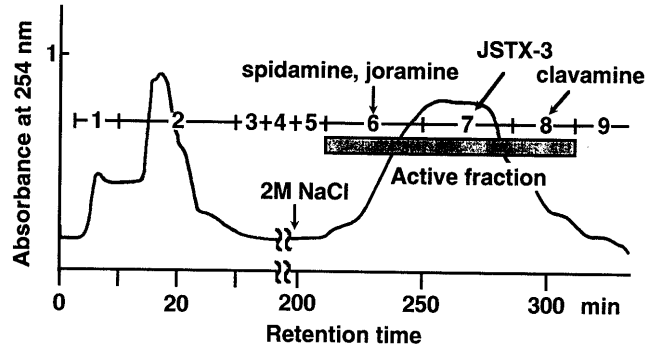

Fig. 3. Chromotogram of Ion Exchange for Active Fraction No. 2 and EPSP Inhibitory Activity

Fractions containing each polyamine toxin are shown by the arrows.

molecular weight than the JSTX-3 and clavamine and was concentrated to 2 ml and applied to the first reversed phase HPLC. Three peaks appeared as shown in Fig. 4. Spidamine and joramine were found in peak No. 2 by component analysis with the multi channel detector and by measuring the ^{1}H-NMR. This peak was continuously subjected to the 2nd reversed phase HPLC with Biofine RPC-C18. As shown in Fig. 5, the two compounds were purified. The amounts of spidamine and joramine obtained were 400 and 150 μg from the 52342 glands, respectively. The total activities by reversed phase HPLC were almost recovered, although they were 1% for spidamine and 0.4% for joramine from the crude extract (Table. 1). The specific activities for both toxins were about 250 fold higher.

The structures of the two toxins were identified in the following way.

Spidamine and joramine possessed the UV absorption maximum at 280 nm, and the spectrum of spidamine was the same as JSTX-3. Joramine showed a similar UV spectrum, but it was not exactly same as JSTX-3 as had been expected. The hydrolysates of each toxin gave asparatic acid, cadaverine and an OPA positive component by amino acid–polyamine analysis with HPLC (data not shown) as shown in the previous paper.[9] Furthermore, this hydrolysates after HFB derivatization were analyzed by GLC. Asparatic acid, cadaverine and a HFB-positive component also appeared (data not shown)[9] previous paper. Compared to the FAB-MS of JSTX-3, a characteristic fragment at m/z 241 assigned to the asparaginyl 3-aminopropyl-β-alanine moiety in JSTX-3 appeared in the purified spidamine and joramine. The molecular ions of spidamine and joramine were found

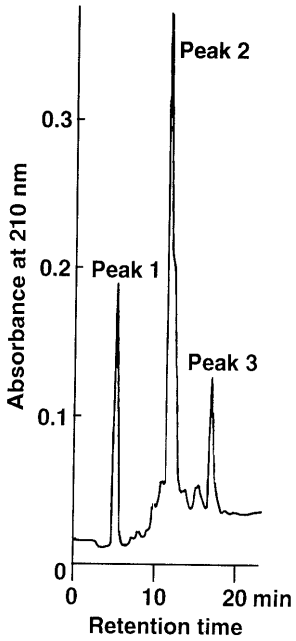

Fig. 4. Chromatogram of the 1st Reversed Phase HPLC for Active Fraction No. 6 from Ion Exchange

Peak 1, unknown; peak 2, spidamine and joramine; peak 3, tryptophan.

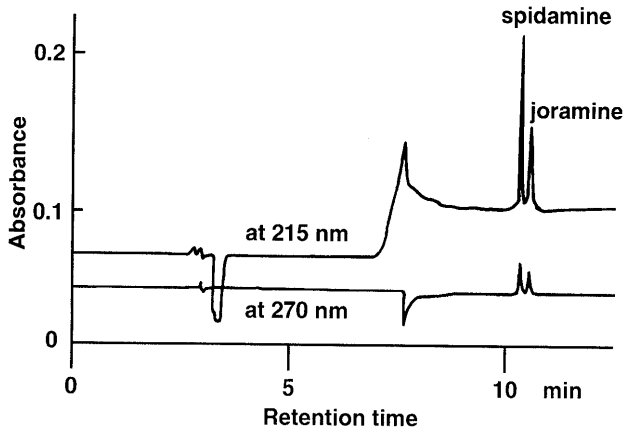

Fig. 5. Chromatogram of the 2nd Reversed Phase HPLC for Fraction No. 2 from the First HPLC

at 495 and 479 as $[M+H]^+$, respectively, as shown.[9]

In the COSY spectrum of spidamine shown in Fig. 6, cross peaks of each moiety were connected by lines. Lines a to d are each component of spidamine corresponding to 2,4-DHPA; line a, asparagine (Asn); line b, 3-aminopropyl-β-alanine (Ampa); line c, cadaverine; line d, respectively.

Three aromatic protons appearing at 6.48 ppm (m, 2H) and 7.10 ppm (d, $J=8.9$ Hz, 1H) were connected by line a, and assigned to the signals due to protons in 2,4-DHPA moiety. The methylene protons appearing at 3.52 ppm (d, $J=15.5$ Hz, 1H) and 3.55 ppm (d, $J=15.5$ Hz, 1H) were assigned to the geminal protons of methylene in 2,4-DHPA moiety. It was interesting that these protons had to be immobilized at the position 2 of the 2,4-DHPA moiety.

A signal of α-proton of asparagine residue found at 4.62 ppm (dd, $J=5.7$, 4.6 Hz, 1H) was connected to β-protons appearing at 2.72 ppm (dd, $J=13.4$, 8.0 Hz, 1H) and 2.78 ppm (dd, $J=14.6$, 5.5 Hz, 1H). This connection is shown as line b.

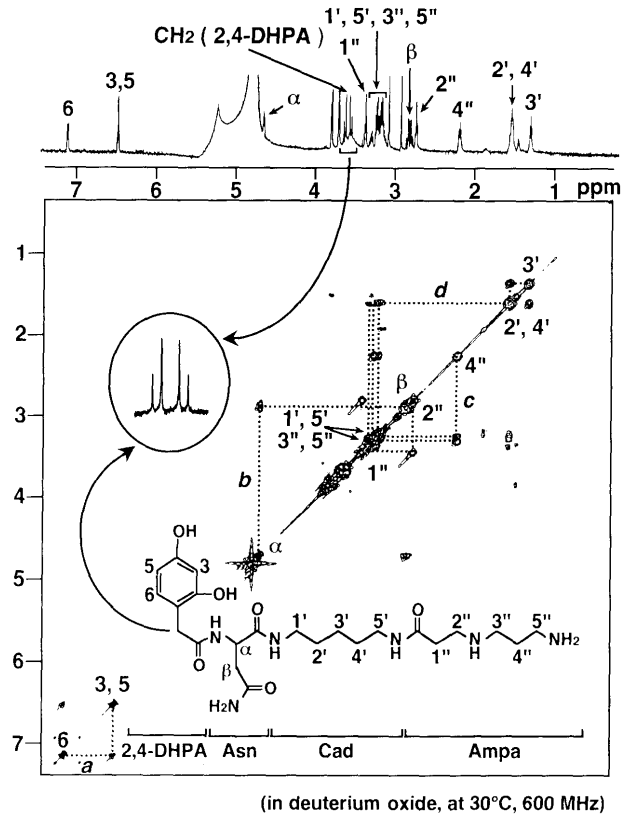

Fig. 6. COSY Spectra of the Purified Spidamine

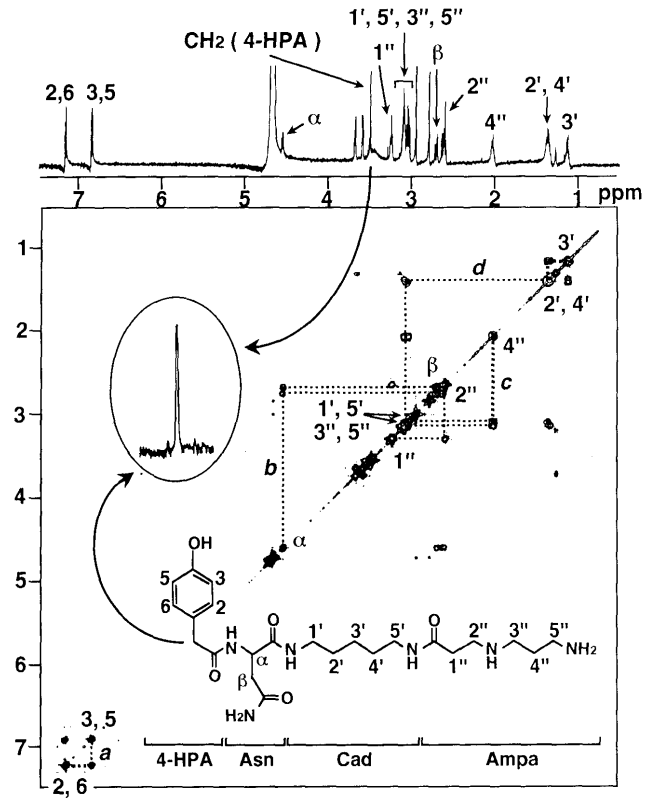

Fig. 7. COSY Spectra of the Purified Joramine

The COSY spectrum of joramine showed four aromatic protons appearing at 6.88 ppm (d, $J=8.7$ Hz, 2H) and 7.19 ppm (d, $J=8.7$ Hz, 2H) and all protons were identified as shown in Table 2.

These data suggested the existence of 4-HPA group in joramine, in place of 2,4-DHPA of spidamine. This was strongly supported by the fact that the signal of its methylene appeared at 3.55 ppm as a singlet for two protons. These respective signals are shown by expanded spectra in circles of Figs. 6 and 7. Other signals were almost the same as spidamine.

Table 2. ^{1}H-NMR Spectral Data for JSTX-3, Spidamine and Joramine

		JSTX-3	Spidamine	Joramine
2,4-DHPA	-CH_2	3.54, d (15.0)[a]	3.52, d (15.5)	
		3.68, d (15.0)	3.55, d (15.5)	
	-3 and -5	6.46, m	6.47, m	
	-6	7.10, d-like (9.1)	7.10, d-like (8.9)	
4-HPA	-CH_2			3.55, s
	-2 and -6			7.19, d (8.7)
	-3 and -5			6.88, d (8.7)
Asn	-α	4.61, dd (5.8, 4.6)	4.62, dd (5.7, 4.6)	4.61, dd (6.1, 5.8)
	-β	2.73, dd (13.6, 7.7)	2.72, dd (13.4, 8.0)	2.68, dd (13.8, 8.1)
		2.77, dd (13.6, 5.8)	2.78, dd (14.6, 5.5)	2.77, dd (14.3, 5.7)
Cad	-1′	3.25, m	3.21, m	3.14, m
	-2′	1.44, m	1.44, m	1.43, m
	-3′	1.19, t-like	1.20, q (7.7)	1.20, q (7.1)
	-4′	1.44, m	1.44, m	1.43, m
	-5′	3.13, m	3.14, m	3.14, m
Pta	-1″	2.66, td (1.0, 6.6)		
	-2″	3.30, td (1.0, 6.6)		
	-3″ and -6″	3.11, m		
	-4″ and -5″	1.78, m		
Ampa	-1‴		2.67, td (6.8, 0.9)	2.68, t (7.1)
	-2‴		3.32, td (6.8, 0.9)	3.32, t (7.1)
	-3‴		3.18, t (7.9)	3.18, t (8.0)
	-4‴		2.11, m	2.11, m
	-5‴		3.11, t (7.9)	3.11, t (8.0)
Amp	-1⁗	3.11, m		
	-2⁗	2.09, m		
	-3⁗	3.15, m		

In deuterium oxide, at 30 °C, 600 MHz. Abbreviations, 2,4-DHPA, 2,4-dihydroxyphenylacetic acid; 4-HPA, 4-hydroxyphenylacetic acid; Asn, asparagine; Cad, cadaverine; Pta, putreanine; Ampa, 3-aminopropyl-β-alanine; Amp, aminopropyl. *a*) Coupling constants (J, Hz) are shown in parenethes.

Thus, the components of spidamine and joramine were 2,4-DHPA, asparagine, 2-aminopropyl-β-alanine and cadaverine, and 4-HPA, asparagine, 2-aminopropyl-β-alanine and cadaverine, respectively. All the common moieties of both compounds to JSTX-3 were overlapped in the chemical shift as shown in Table 2.

Thus, by NMR and FAB-MS, the structures of spidamine and joramine were completely identified as *N*-(2,4-dihydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-β-alanyl)-1,5-pentanediamine and *N*-(4-hydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-β-alanyl)-1,5-pentanediamine.

The blocking activity of the two neurotoxins was compared with JSTX-3 using the lobster neuromuscular synapse. The potencies of both toxins were approximately one tenth less than that of JSTX-3. Joramine blocked EPSP, and this was completely reversed by washing the preparation with saline as shown in various analogs in the previous paper.[13,14] In contrast, spidamine blocked EPSP irreversibly, which was not recovered by washing in exactly the same manner as JSTX-3.[9]

Discussion

Adequate amounts of spidamine and joramine for NMR

were obtained in the present paper, although the yields of both compounds were lower than previously expected.[9] The yield of spidamine was one eighth of that expected, while the yield of joramine was two thirds. The difference may be due to the fact that custom resins in the gel filtration and ion exchange were used for the purifications of large amounts of the crude extract in this experiment. The total yield of active fractions containing mainly JSTX-3 in three steps was 58.3% as shown in Table 1. We previously adopted all purifications by HPLCs which should be efficient on a small scale. In the present experiment, original contents of spidamine and joramine were roughly estimated by considering the recoveries of all the steps as 100%, although the contents were dependent on the individual spiders. The content of spidamine was 7.6 ng/gland (15 pmol/gland), while that of joramine was 2.9 ng/gland (6.0 pmol/gland). Contents in a single gland were in a range measurable for both toxins by the HPLC used with the fluorescence reaction, once standards are available. Contents may actively be several tenths lower than JSTX-3. For the activity to EPSP, both were one tenth less than JSTX-3. Thus, neither compound is the main toxin to EPSP nor an intermediate to JSTX-3 judging from their structures. They might play an unknown role in the venom which will be discoverd later. We have already found an important activity of spidamine to some enzyme, which will be described elsewhere.

It is very interesting that spidamine blocked EPSP irreversibly, while joramine blocked it reversibly. In studies of structure activity correlation of synthesized analogs to JSTX-3, whole polyamine frames were essential in the irreversible reaction, while the intermediate frames ,were in the reversible. Substituting dihydroxy groups of 2,4-DHPA by other functional groups, the whole frames were also reversible.[13-16] This experiment distinctly focussed on 2,4-dihydroxyphenyl group being fundamentally essential for the irreversible reaction, whereas the polyamine frame is involved in the binding because the frames of all group are exactly same. 2,4-Dihydroxyphenyl groups such as resorcinol are possibly a reactive species to a covalent bond in the binding sites. In the rat brain synaptosome, Pan-Hou *et al.*[17] reported that other synthesized analogs bound reversibly to the glutamate binding site depending on substituents of the phenol group and methylene of 2,4-DHPA and the asparagine residue. It is likely that JSTX-3 has site specific actions to glutamatergic transmissions of various prey.

In fact, the stereo-structure of spidamine is very different from that of joramine as shown in NMR-spectra in Figs. 6 and 7. It is crucial to the irreversible reaction of JSTX-3 and spidamine that *ortho*-hydroxyl group in the phenol ring makes a rigid conformation with the asparagine residue as shown in a highly resolved nuclear Overhauser spectroscopy (NOESY) of a synthesis spidamine. The details will be described elsewhere.

Acknowledgments The authors would like to thank Dr. Masatoshi Nishi and Mr. Shoji Inoue of their faculty for skilled measurements of NMR and FAB-MS.

They also thank students of their faculty: Messrs. Nagatoshi Miyagawa, Taroh Yamashita, Kohichi Matsuda, Yasuyuki Suzuki, Toshihide Takagi and Miss Meigi Koh for technical assistance in catching the spiders and for performing the preliminary experimental analyses.

References

1) Kawai N., Miwa A., Abe T., *Brain Res.*, **247**, 169—171 (1982).
2) Jackson H., Usherwood P. N. R., *Trends Neurosci.*, **11**, 278—283 (1988).
3) Cotman C. W., Iversen L. L., *TINS.*, **10**, 263—265 (1987).
4) Collingridge G. L., Bliss T. V. P., *TINS.*, **10**, 288—293 (1987).
5) Kawai N., Miwa A., Saito M., Pan-Hou H., Yoshioka M., *J. Physiol.* (Paris), **79**, 228—231 (1984).
6) Blaschke M., Keller B. U., Rivosecchi R., Hollmann M., Heinemann S., Konnerth A., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 6528—6532 (1993).
7) Aramaki Y., Yasuhara T., Higashijima T., Yoshioka M., Miwa A., Kawai N., Nakajima T., *Proc. Jpn. Acad.*, **62B**, 359—362 (1986).
8) Yoshioka M., Narai N., Kawai N., Numata M., Nakajima T., *Biog. Amines.*, **7**, 375—384 (1990).
9) Chiba T., Akizawa T., Matsukawa M., Pan-Hou H., Yoshioka M., *Chem. Pharm. Bull.*, **42**, 1864—1869 (1994).
10) Chang J-Y., Knecht R., Braun D. G., *Biochem. J.*, **199**, 547—555 (1981).
11) Chang J-Y., Knecht R., Braun D. G., *Biochem. J.*, **203**, 803—806 (1982).
12) Pearce R. J., *J. Chromatogr.*, **136**, 113—126 (1977).
13) Asami T., Kagechika H., Hashimoto Y., Shudo K., Miwa A., Kawai N., Nakajima T., *Biomedi. Res.*, **10**, 185—189 (1989).
14) Teshima T., Matsumoto T., Wakamiya T., Shiba T., Nakajima T., Kawai N., *Tetrahedron*, **46**, 3813—3818 (1990).
15) Kawai N., Miwa A., Shimazaki K., Sahara Y., Robinson H. P. C., Nakajima T., *Comp. Biochem. Physiol.*, **98**, 87—95 (1991).
16) Kawai N., Nakajima T., *J. Toxicol.*, **9**, 203—223 (1990).
17) Pan-Hou H., Suda Y., Sumi M., Yoshioka M., Kawai N., *Brain Res.*, **476**, 354—357 (1989).