Finding of Primitive Polyamine Toxins in the Venom of a Joro Spider, *Nephila clavata*

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A series of joro spider toxins, novel polyamines sharing a common moiety of 2,4-dihydroxyphenylacetyl cadaverine, have been identified using various bioassays, such as inhibition of a glutamatergic transmission and insecticidal activity. In this paper, we tried to chemically find still unknown polyamine toxins in the venom of a joro spider, *N. clavata*, by several analytical methods based on the characteristics of the common moiety. An aqueous extract from 3000 venoms was separated by preparative high performance liquid chromatography (HPLC). The polyamine toxins were detected by monitoring the fluorescence produced in an on-line reaction of *o*-phthalaldehyde with amino groups and UV absorption of the phenol group. Two compounds in minute quantity were purified and analyzed by gas-liquid chromatography (GC) and HPLC, which we specifically developed for the simultaneous determination of amino acids and polyamines of the toxins. Judging from the constituents of the hydrolysate by GC and HPLC and the molecular weights determined by fast atom bombardment mass spectrometry, the two compounds were estimated to be *N*-(2,4-dihydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-*β*-alanyl) cadaverine and *N*-(4-hydroxyphenylacetyl-L-asparaginyl)-*N'*-(3-aminopropyl-*β*-alanyl) cadaverine. These compounds were small in content and molecular weight compared with hitherto known toxins. Both were presumed to be biochemically primitive toxins and were named spidamine and joramine, respectively.

Keywords *Nephila clavata*; spidamine; joramine; 2,4-dihydroxyphenylacetic acid; 4-hydroxyphenylacetic acid; *N*-(3-aminopropyl)-*β*-alanine

Spider venoms have been known to contain a variety of substances affecting important neurotransmission.[1-3] Among orb-web spider venoms, toxins derived from a joro spider, *Nephila clavata*, were first found to irreversibly block glutamatergic transmission in a lobster neuromuscular synapse.[4] The toxins called Joro spider toxins (JSTX) specifically blocked the transmission of a squid giant synapse,[5] a mammalian hippocampal synapse,[6,7] as well as the lobster synapse.

The structures of JSTX-1, -2, -3 and -4 were characterized by Aramaki *et al.*[8] with NSTX-3, which was derived from the venom of a New Guinean spider, *Nephila maculata*.[9,10] Both JSTX-3 and NSTX-3 have 2,4-dihydroxyphenylacetyl-L-asparaginyl-1,5-pentanediamine derivatives as a common moiety. JSTX-3 was a good ligand for studying glutamate receptors. Two subtypes of the receptors to quisqualate (Q) and kainate (Ka) were efficiently blocked with JSTX-3, but not the other subtype to *N*-methyl-D-aspartic acid (NMDA) in the extrasynaptic. Neither were the presynaptic receptors to Q and Ka blocked.[11] Similar spider toxins have also been reported by Grishin and colleagues[12] and by Adams *et al.*[13] from *Argiope lobata*. The physiological properties of these toxins in blocking glutamate receptors have been studied by Usherwood and colleagues.[2,14]

Yoshioka *et al.* reported the isolation and identification of another polyamine toxin which was stronger than JSTX-3 in an *in vivo* insecticidal activity assay from the same venom of *N. clavata*.[15,16] These polyamine toxins consist of 2,4-dihydroxyphenyl-acetic acid, amino acids and polyamines, as shown in Fig. 1. In addition, *N*-phenyl-acetyl and *N'*-amino acid residues in both ends of the polyamine were essential to the insecticical activity, although JSTX-3 was also active in excitatory postsynaptic potential (EPSP) as described in the previous paper.[15]

During the purification of the polyamine toxins by high performance liquid chromatography (HPLC), there appeared a number of peaks assumed to contain unknown polyamines. These polyamines were previously missed, because their peak heights and activities were low.

In this paper, we tried to chemically find these unknown polyamines in the venom using several analytical methods which we specifically designed for the polyamine toxins. In HPLC for each step of the purification, the toxins were detected by monitoring the fluorescence produced in the reaction of *o*-phthalaldehyde (OPA) with the amino groups and UV absorption of the phenol group. The hydrolysates of the purified compounds were analyzed by HPLC and gas-liquid chromatography (GC), designed to simultaneously detect amino acids and polyamines.

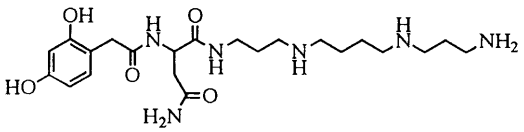
Materials and Methods

Materials Synthetic JSTX-3[17,18] was kindly donated by Takeda Chemical Industries, Ltd., Osaka. *p*-Hydroxyphenylacetic acid (4-HPA) and heptafluorobutyric anhydride (HFBA) were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Hydrogen chloride–*n*-butanol was obtained from Tokyo Kasei Chemical Industries, Ltd., Tokyo. 4-Dimethylaminoazobenzene-4'-sulfonyl chloride (Dabsyl-Cl) was obtained from Pierce, Ltd., Rockford, IL, U.S.A. All the other chemicals of reagent grade were commercially available.

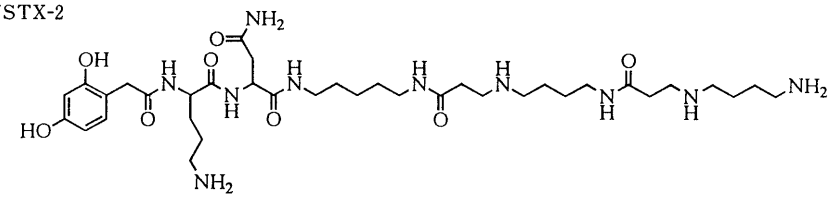
Extraction of Toxins from Venoms Three thousand joro spiders, *N. clavata*, were collected in the Kinki district, a central area of Japan. Approximately 500 pairs of venom glands were taken out and placed into 60 ml of boiled water in a test tube for each extraction for 30 min, and then homogenized with a glass rod. This extraction was repeated 6 times. All the homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was lyophilized to give 0.75 g in dry weight and was stored at −80 °C before use. One-third (0.25 g) of the lyophilizate was used for analytical HPLC and preparative HPLC in a small scale. The remainder (0.5 g) was used for preparative HPLC in a large scale.

Analytical HPLC with a Multi-Channel Detector (HPLC-M) The

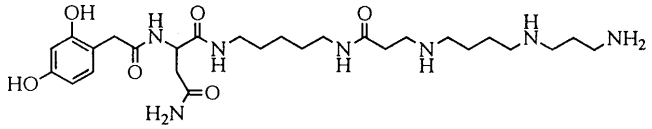
JSTX-1



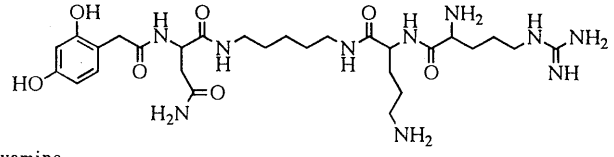
JSTX-2



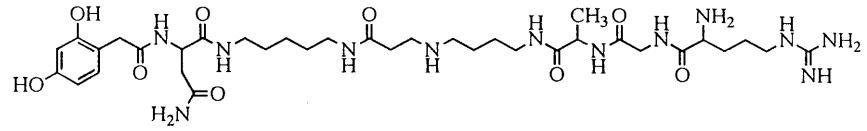
JSTX-3



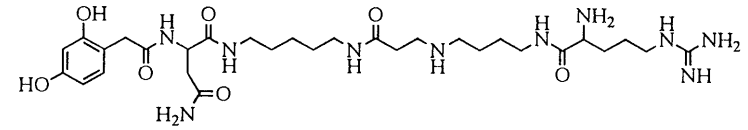
JSTX-4



clavamine



NSTX-3



argiopine

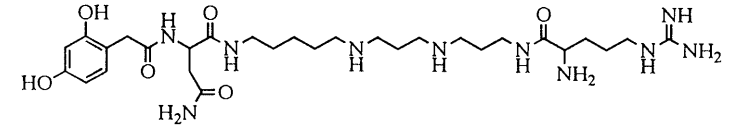


Fig. 1. Structures of Polyamine Toxins Identified

chromatographic systems consisted of an intelligent HPLC pump (880-PU) for solvent delivery, a 2-line degasser (880-51), a solvent mixing module (880-31), a column oven (860-CO) and a multi-channel detector (Multi-340) from Japan Spectroscopic Co., Ltd. (JASCO), Tokyo, and a 7125 Rheodyne sample injector. The data of the chromatograms were analyzed by a computer, PC-9801-DX, and were printed out by a printer PC-pr201/80A (Nippon Electronics Co., Ltd., Tokyo). A reversed phase column (MCI Gel ODS-1MU, 4.6 mm i.d. × 250 mm, donated by Mitsubishi Kasei Corporation, Yokohama) was used for separation under the condition of a linear gradient elution from water containing 0.1% trifluoroacetic acid (TFA) to 70% acetonitrile containing 0.1% TFA. The flow rate was set at 1.0 ml/min. The eluate was monitored by the multi-channel detector.

Tandem HPLC with an On-Line UV/Fluorescence Detector (HPLC-UF) The chromatographic system consisted of another intelligent HPLC pump, a Familic-300S pump for delivery of a 0.16% OPA solution, a 3-line degasser (880-50), a ternary gradient unit (880-02), a column oven (TU-300), an intelligent UV/VIS detector (875-UV), a fluorescence spectrophotometer (FP-110) and a circulating water bath (TC-100) for a column from JASCO, and another sample injector. The length of a reaction coil of 0.25 mm in diameter (i.d.) was 1.2 m, and the temperature was kept at 50 °C. The chromatogram was recorded by a Unicorder U-228 from Nippon Denki Kagaku Co., Ltd., Tokyo. A reversed phase column (MCI Gel ODS-1MU, 4.6 mm i.d. × 250 mm) was used for separation under the condition of a linear gradient elution from water containing 0.1% TFA to 20% acetonitrile containing 0.1% TFA. The flow rate was set at 1.0 ml/min. The eluate was monitored by a UV absorption at 280 nm, and a fluorescence of 455 nm excited at 340 nm. Relative fluorescence intensity (RFI) was measured.

1st Preparative HPLC Two-thirds of the lyophilized extract (0.5 g) was dissolved in 2 ml of water. Each 0.5 ml aliquot was applied to the following preparative HPLC. Preparative HPLC was performed using a reversed phase column (MCI Gel ODS-1MU, 20 mm i.d. × 250 mm) coupled with a UV monitor at 214 nm. The chromatographic system consisted of another intelligent HPLC pump, another 2-line degasser, a solvent mixing module (880-30), the other column oven , an intelligent UV/VIS detector (UV-970) and an integrator (807-IT) from JASCO. Another sample injector, 7125 Rheodyne, was used. The reversed phase column was under the condition of a linear gradient elution system from water containing 0.1% TFA to 20% acetonitrile containing 0.1% TFA.

The flow rate was set at 10.0 ml/min. The eluate was monitored by the UV absorption at 214 nm and collected every 1 min into 30 fractions. Small aliquots of the fractions were analyzed by HPLC-UF and GC. Fractions from No. 12 to No. 20 had a UV absorption maximum at 280 nm by the multi-channel detector, and their amino groups were reactive with OPA. These fractions were pooled and lyophilized for the 2nd preparative HPLC.

2nd Preparative HPLC The fractions from No. 12 to No. 20 collected by 1st preparative HPLC were dissolved in 1 ml of water. Each 50 μl aliquot was applied to the following preparative HPLC. Preparative HPLC was performed using a reversed phase column (MCI Gel ODS-1MU, 4.6 mm i.d. × 250 mm) coupled with another UV monitor at 280 nm. The flow rate was set at 1.0 ml/min. Other chromatographic conditions were the same as in the 1st preparative HPLC system. A chromatogram showed 15 peaks. All the fractions containing each peak were analyzed by HPLC-UF. The 8th peak showed both UV absorbance and a fluorescence reaction. All the 8th peaks were repeatedly collected and lyophilized for the 3rd preparative HPLC.

3rd Preparative HPLC The residue in the 8th peaks was dissolved in 500 μl of water. Each 50 μl aliquot was applied to the following preparative HPLC. Preparative HPLC was performed using a reversed phase column (Superiorex-ODS, 4.6 mm i.d. × 150 mm, was donated by Shiseido Corporation, Tokyo) coupled with the UV monitor at 280 nm. The flow rate was set at 1.0 ml/min. The other chromatographic conditions were the same as in the 1st preparative HPLC system. The chromatogram showed 4 peaks. All the peaks were analyzed by HPLC-UF. In the 2nd peak, polyamine toxins were present and applied to a 4th preparative HPLC.

4th Preparative HPLC The lyophilized residue in the 2nd peak was dissolved in 500 μl of water. Each 100 μl aliquot was applied to the following preparative HPLC. Preparative HPLC was performed using a reversed phase column (MCI Gel ODS-1MU, 4.6 mm i.d. × 250 mm) coupled with a UV monitor at 280 nm. The flow rate of the eluate was set at 1.0 ml/min. The other chromatographic conditions were the same as in the 1st preparative HPLC system. The chromatogram showed 5 peaks. All the peaks were analyzed by HPLC-UF. The 1st (83 μg) and the 2nd peak (10 μg) were collected and analyzed by HPLC of Dabsyl derivatives (HPLC-D), GC and fast atom bombardment mass spectrometry (FAB-MS).

Acid Hydrolysis of a Purified Sample A portion of a purified compound 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. The hydrolysate was dried with a centrifugal evaporator, Speed Vac Concentrator (Savant, Inc., N.Y.) *in vacuo* at room temperature and the residue was redissolved with 100 μl of water. A portion of this solution was analyzed by HPLC-UF.

HPLC-D Another portion of the purified sample in preparative HPLCs was also analyzed by a dabsylation method.[19-22] Derivatization with Dabsyl-Cl was usually carried out in a glass-stoppered tube (0.5 × 4.5 cm).

The portion in the tube was dissolved in 15 μl of 50 mM $NaHCO_3$ buffer (pH 9.0). After being mixed with 20 μl of 4 mM Dabsyl-Cl in 50% acetonitrile, the sample solution in the tube was heated at 70 °C for 10 min. A 5 μl-aliquot of this reaction mixture was injected to a HPLC analyzer, in which a reversed phase column (LiChrosper 100RP-18, 4.0 mm i.d. × 250 mm, Merck, Darmstadt) at 55 °C was used for separation under the condition of a programmed gradient from 20 mM acetate buffer (pH 6.6) to acetonitrile. The flow rate was set at 1.0 ml/min. The eluate was monitored by UV absorption at 436 nm.

GC A small amount of the purified compound in the preparative HPLCs was dissolved in 100 μl of 6 N HCl in a vial. The air phase in the vial was displaced with N_2 gas. This sample was hydrolyzed at 115 °C for 24 h. The hydrolysate was dried with the centrifugal evaporator *in vacuo* at room temperature. Into this vial, 50 μl of *n*-butanol containing 10% HCl was added. The air phase in the vial was displaced with N_2 gas. The vial was heated at 55 °C for 3 h and dried. To make a heptafluorobutyryl (HFB) derivative, 50 μl of acetonitrile and 10 μl of HFBA was added into the vial and the air phase in the vial was displaced with N_2 gas. After the reaction mixture was heated at 80 °C for 90 min, the solvent in the vial was removed with the centrifugal evaporator. The residue was dissolved in 25 μl of ethyl acetate.[23] A half microliter of the solution was injected to a gas chromatograph GC-14A (Shimadzu, Kyoto) equipped with a capillary column (0.32 mm i.d. × 50 m, donated by Gasukuro Kogyo Inc., Tokyo) coated with 100% silicone Neutrabond-1. The splitless column was heated under the condition of a programmed gradient from 50 °C to 255 °C at 90 min. The pressure of the carrier gas, N_2, was 0.5 kg/cm^2. The flow rate was 55 ml/min. The eluate was detected with a flame ionization detector (FID).

FAB-MS Positive ion atom bombardment mass spectra were measured by a mass spectrometer JMS-DX300 (JEOL, Tokyo) with a glycerin matrix at 3 kV.

Results

The sum of the crude extracts was 0.75 g in dry weight after lyophilization. A part of the crude extracts was analyzed by HPLC-M. JSTX-3 and JSTX-like compounds, which were later designated as compounds-Y and -Z, were eluted in the earlier retention time as shown in Fig. 2.

In the earlier fractions, the existence of JSTX-like compounds was estimated by HPLC-UF under the same separation condition. These fractions, with a UV absorption at 280 nm, were positive for the reaction of amino groups with the OPA reagent. To purify these fractions in the large scale, preparative HPLC was performed with a reversed phase column by monitoring UV absorption at 214 nm, selected for monitoring not only the target toxins but also other ingredients. All fractions were collected and analyzed by GC. It was observed that fractions No. 1 through No. 11 contained free amino acids and polyamines. Especially, fraction No. 5 through No. 8 contained polyamines. Fraction No. 12 through No. 20 contained conjugated polyamines like cadaverine derivative, and amino acids such as aspartic acid (Asp) and glutamic acid (Glu), which appeared after the acid hydrolysis. Fractions after No. 21 had no amino acids or

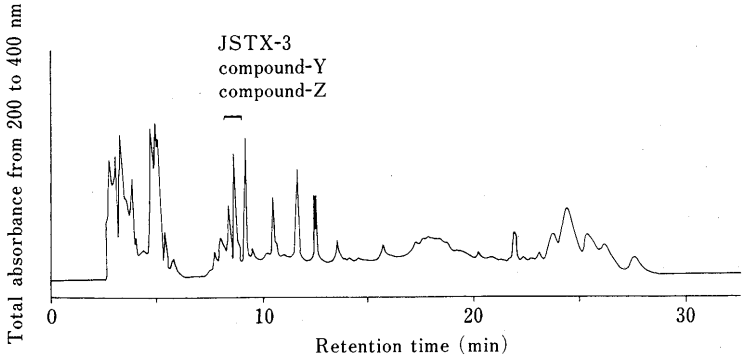


Fig. 2. HPLC Profile of Crude Extract of Venoms

In a range from 8 to 9 min in retention time, JSTX-3, compound-Y and compound-Z appeared and are explained in the text.

polyamines. Thus, the fractions No. 12 through No. 20 were pooled and lyophilized for the 2nd preparative HPLC. In a chromatogram of the 2nd preparative HPLC, there were 15 peaks which were detected by HPLC-UF. In the peaks of No. 7 and No. 8, there were compounds strong in both the UV absorption at 280 nm and the fluorescent reaction. The two fractions were lyophilized, and the residue of peak No. 8 showed characteristic signals due to a 2,4-dihydroxyphenyl group in NMR, but residue of peak No. 9 did not. The 8th peak was purified in following the 3rd preparative HPLC.

In the 3rd preparative HPLC, there appeared mainly two peaks with two front and tailed shoulders which did not have characteristic UV absorption. The first main peak was analyzed by HPLC-UF, HPLC-M, HPLC-D and NMR. In this peak, a compound was identified as known JSTX-3. The 2nd peak also had characteristic absorption, but was presumed to be unknown polyamine toxins from NMR. The peak was further purified by the 4th preparative HPLC.

In a chromatogram of the 4th preparative HPLC, there appeared five peaks. The 1st and 2nd peaks showed a characteristic UV absorption maximum at 280 nm and a fluorescent reaction by HPLC-UF, which indicated the presence of separated polyamine toxins. The 3rd peak had tryptophan, judging from HPLC-D and NMR. The 4th and 5th peaks did not have characteristic UV absorption, nor the reaction of an amino group by HPLC-D.

The 1st and 2nd peaks showed single peaks in HPLC-UF, as shown in Fig. 3. These peaks were collected and analyzed with HPLC-UF. In the chromatogram, as shown in Fig. 3, both peaks were proved pure in the UV absorption, and the fluorescence reaction in which the early peaks appeared at the void volume was neglectable as blank. The compounds in both peaks were designated as compound-Y and compound-Z, respectively. In the UV absorption spectra, both showed the same spectra, with a maximum at 280 nm as in JSTX-3. Based on the absorbance at 280 nm, we obtained 83 μg of compound-Y, 10 μg of compound-Z and 1.6 mg of JSTX-3 from 2000

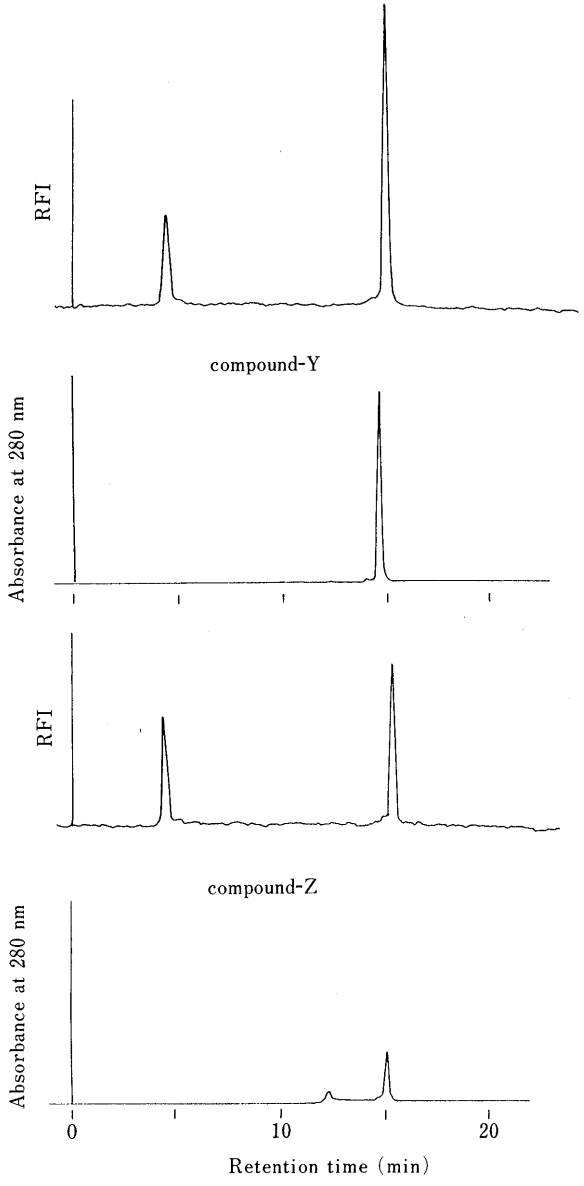


Fig. 3. Tandem HPLC System On-Line Detected by UV Absorption and Fluorescence

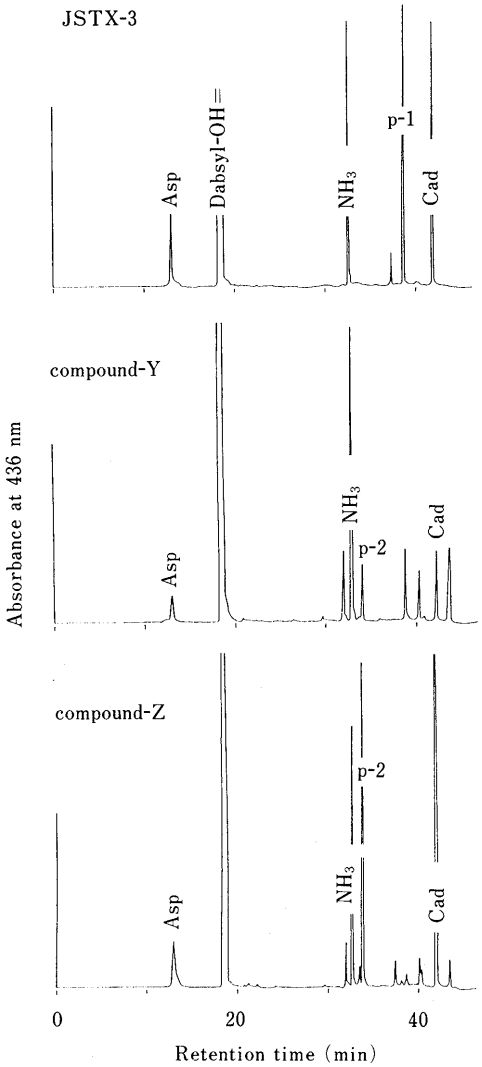


Fig. 4. Chromatograms of Dabsyl Derivatives of Hydrolysates of JSTX-3 and Toxins Purified

Dabsyl-OH; hydrolysate of Dabsyl-Cl, NH_3; ammonia, Cad; cadaverine, Asp; aspartic acid.

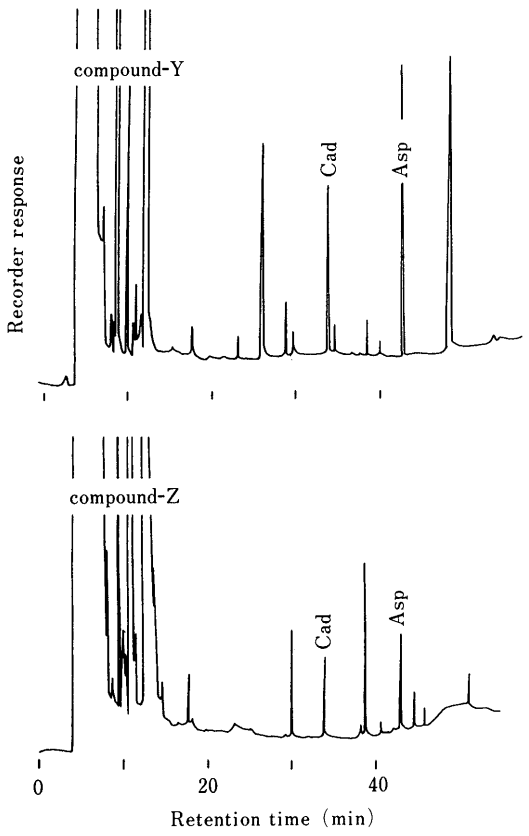


Fig. 5. Gas-Liquid Chromatograms of HFB Derivatives of Hydrolysates of Toxins Purified

See the lengend of Fig. 4.

pairs of the venoms. In order to identify the two unknown compounds, portions of the purified materials were hydrolyzed and the hydrolysates analyzed by HPLC-D. In the chromatograms shown in Fig. 4, both the compounds produced Asp and cadaverine (Cad), just like JSTX-3. An unknown peak (p-1) in JSTX-3 was assumed to be *N*-(8-amino-5-azaoctyl)-*β*-alanine. The other peaks not designated were assumed to be by-products of the incomplete hydrolysis, because the peak height of Asp and Cad were low compared with those peaks in compound-Z, when the same amount was hydrolyzed. In the same sense, another unknown peak (p-2) in the chromatograms of compound-Y and compound-Z were assumed to be *N*-(3-aminopropyl)-*β*-alanine, which were later estimated from the mass spectrum of compound-Y or compound-Z by FAB-MS.

The hydrolysates were further analyzed by GC after derivatization with HFBA. In the chromatograms shown in Fig. 5, Asp and Cad, were found again. To confirm these estimations, the hydrolysates were separated by HPLC-M. In chromatograms (data not shown), the retention time corresponding to 2,4-DHPA from compound-Y overlapped with the one from JSTX-3. The retention time of authentic 4-HPA also overlapped with the one from compound-Z. Each peak was further collected and lyophilized. The lyophilized samples were directly analyzed by FAB-MS. A peak at 150 *m/z* was assigned to a dehydrated parent ion of $[M-H_2O]^+$ from the 2,4-DHPA fractions of both JSTX-3 and compound-Y. It was difficult to detect a parent ion of 4-HPA from compound-Z.

Other portions of both purified materials were also applied to FAB-MS without the hydrolyses. As shown in Fig. 6, parent ions were identified by taking into consideration the known structures shown in Fig. 1 and the constituents found in the above experiments. A common fragment ion at 241 was present in all three compounds.

Discussion

It was possible to find two new polyamine toxins in the venoms using the above analytical methods, HPLC, GC and FAB-MS. These methods will be useful to detect other polyamine toxins under investigation. Of course, it is difficult to estimate both structures by only the present methods using fragment analyses by the HPLC and FAB-MS without taking the previously identified structures, especially the common moiety, into consideration. Both structures were also confirmed by a high resolution NMR at 600 MHz. 2,4-Dihydroxyphenylacetyl, or 4-hydroxyphenylacetyl, α and *β*-protons of asparagine (Asn), methylene protons of Cad and all the protons of *N*-(4-aminobutyl)-*β*-alanyl were assigned by 2-dimensional correlated spectroscopy (COSY). This spectral analysis of the larger preparation will be described in the following paper. These analytical methods were sensitive enough to use minute quantities of the purified sample in every step, as well as to detect minor compounds which had been overlooked in previous experiments. The concentrations of both compounds were extremely low compared with those of JSTX-3 and clavamine.

Both compounds we found were very small in molecular weight compared with the previously found toxins, except JSTX-1, as shown in Fig. 1. It is, however, supposed that both have the minimum structure for the biological activity to the glutamatergic transmission from a study on the structure–activity relationship of a JSTX-3 derivative.[17] In fact, both compounds were found to participate in the EPSP in the lobster synapse.[6] The details will be described in the following paper. It is biosynthetically obvious that compound-Z is produced by one less step than compound-Y, which has one more hydroxyl group in the *o*-position of the acetyl group. The presence of compound-Y, *p*-hydroxyphenylacetyl polyamine, suggests that the dihydroxyphenylacetyl group is produced from tyrosine, but not from 6-hydroxy-tryptophan. Another interesting point is that the *N*-(4-aminobutyl)-*β*-alanyl residue in some JSTX's or clavamine is replaced by *N*-(3-aminopropyl)-*β*-alanyl lacking one methylene. In the polyamine toxins, the *N'*-terminal of the common moiety is extended by conjugation of linker polyamines and/or amino acids, except in JSTX-1. Thus, both of the new polyamine toxins we found are more primitive than the polyamine toxins identified so far, as shown in Fig.1. Both compounds will be key compounds to begin investigative biosynthesis of the polyamine toxins. Thus, we shall name compound-Y and compound-Z spidamine and joramine, as shown in Fig. 7, respectively. Further studies on their biochemistry are under investigation.

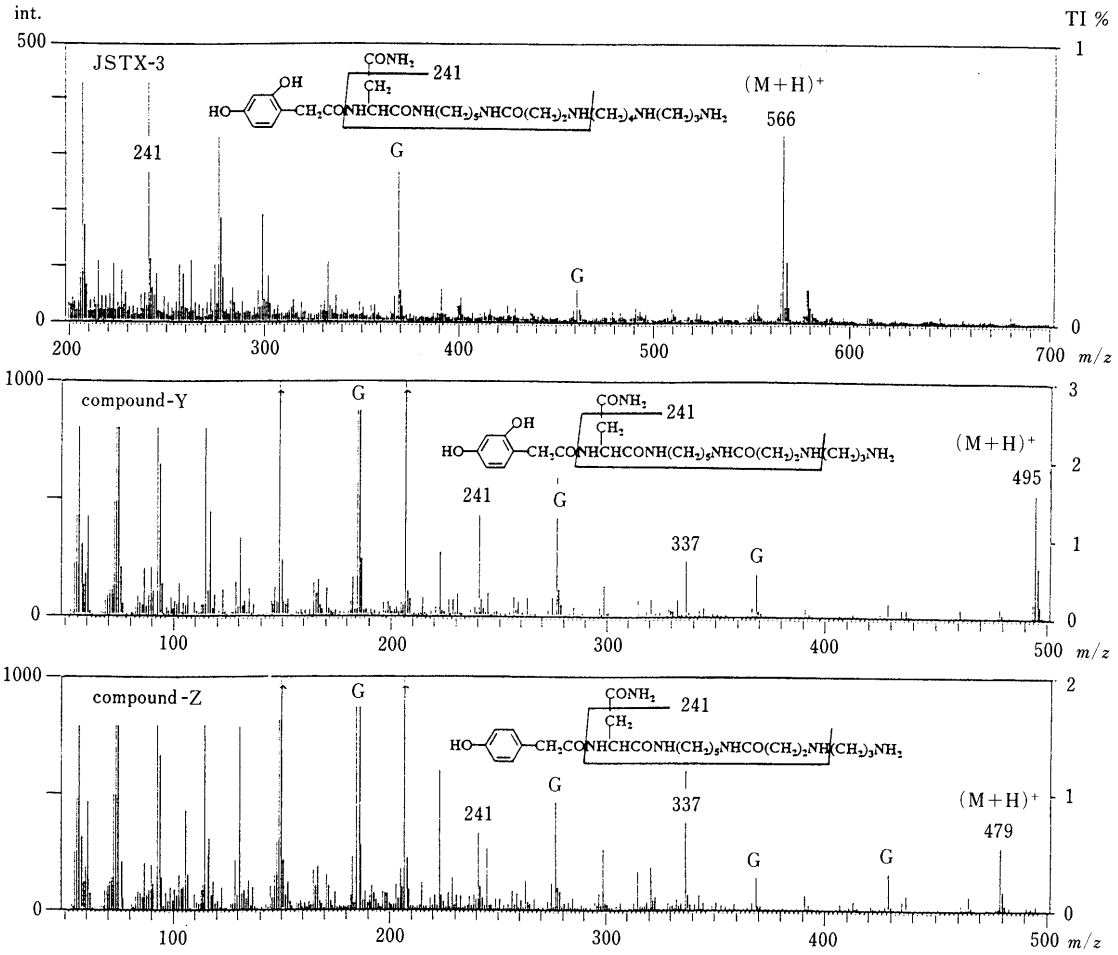


Fig. 6. Positive Ion Spectra in FAB-MS of JSTX-3 and Toxins Purified
G means fragment ions due to glycerin.

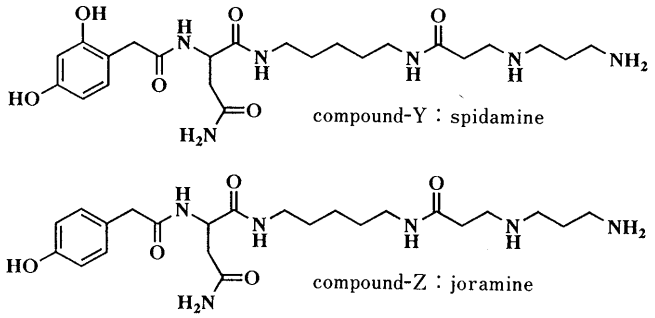


Fig. 7. Structures of Polyamine Toxins Purified

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