Characterization of a New Insecticide, Clavamine, from the Venom of a Spider, *Nephila clavata* by Use of a Synthetic Compound

Masanori YOSHIOKA,*[,a] Noriko NARAI,[a] Tadashi TESHIMA,[b] Takahiro MATSUMOTO,[b] Tateaki WAKAMIYA,[b] Tetsuo SHIBA,[c] Naomi TOKORO,[d] Tetsuo OKAUCHI[d] and Yoshiaki KONO[d]

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Setsunan University,[a] 45–1 Nagaotogecho, Hirakata, Osaka 573–01, Japan, Department of Chemistry, Faculty of Science, Osaka University,[b] 1–1 Machikaneyama, Toyonaka, Osaka 560, Japan, Peptide Institute, Protein Research Foundation,[c] 4–1–2, Ina Minoh, Osaka 562, Japan and Research Laboratories, Agricultural Chemicals Division, Takeda Chemical Industries, Ltd.,[d] Ichijoji-Takenouchi-cho, Sakyo-ku, Kyoto 606, Japan. *Received April 22, 1992*

In this paper, we compared the chemical and biological activities of natural and synthetic clavamines, *N*-[2,4-dihydroxyphenylacetyl-4-L-asparaginyl)-*N'*-[*N*-(L-arginyl-glycyl-L-alanyl)-8-amino-4-azaoctanoyl]-1,5-pentanediamine (DPA→Asn→Cad←Ptr←Ala←Gly←Arg) [DPA, 2,4-dihydroxyphenylacetic acid; Cad, 1.5-pentanediamine (cadaverine); Ptr, 8-amino-4-azaoctanoic acid (putreanine, or carboxyethylputrescine)], and found that both were identical. Both compounds showed the same *Rf* value in thin-layer chromatography and the same retention time in high performance liquid chromatography (HPLC). Both hydrolyzates consisted of the same components, such as Gly, DPA, Ala, Asp, Arg and Ptr in gas chromatography. The Edman degradation of both compounds also gave exactly the same sequence. The ^{1}H-NMR spectrum and fast atom bombardment (FAB) mass spectrum of the synthetic compound coincided with those of the natural one. Both were active on the insects used. Clavamine was the main component of the insecticidal activity in the crude venom. By this synthetic study, the structure of a new venom insecticide was thus established. The synthetic compound in abundant quantity will be advantageous for practical use as an insecticide over natural ones, because the latter is available only in small amounts. Also, it will be useful for the exploitation of a new field of biochemistry for polyamines.

Keywords spider toxin; clavamine; *Nephila clavata*; insecticide; polyamine; putreanine; 2,4-dihydroxyphenylacetic acid; peptide

Introduction

Recently, a new group of interesting polyamines has been identified in the venom of spiders.[1–3] Even though these polyamine toxins are weakly toxic to humans, they are remarkably paralytic to insects. The biological activities of these toxins are being studied in neurochemistry. Some toxins block the L-glutamate receptor in the nervous system of not only insects but also mammals. Other toxins are active in the release of histamine from mast cells. In the venoms, there are still a variety of other polyamines which have not yet been purified and their biological actives remain to be determined.[4] Although the structures of these toxins are complicated and they are difficult to synthesize, some toxins have been prepared chemically such as JSTX-3,[5] NSTX-3,[6] and argiopine.[7] These toxins contain 2,4-dihydroxyphenylacetylasparaginyl-1,5-pentanediamine as the common structural moiety as shown in Fig. 1. They are useful ligands for studying the glutamate receptor.[8]

In the previous paper, we purified and estimated a new polyamine derivative which was named clavamine, and its proposed structure is shown in Fig. 1. Clavamine has strong insecticidal activity against intact insects,[9] whose motor neurons are regulated by L-glutamate. However, it

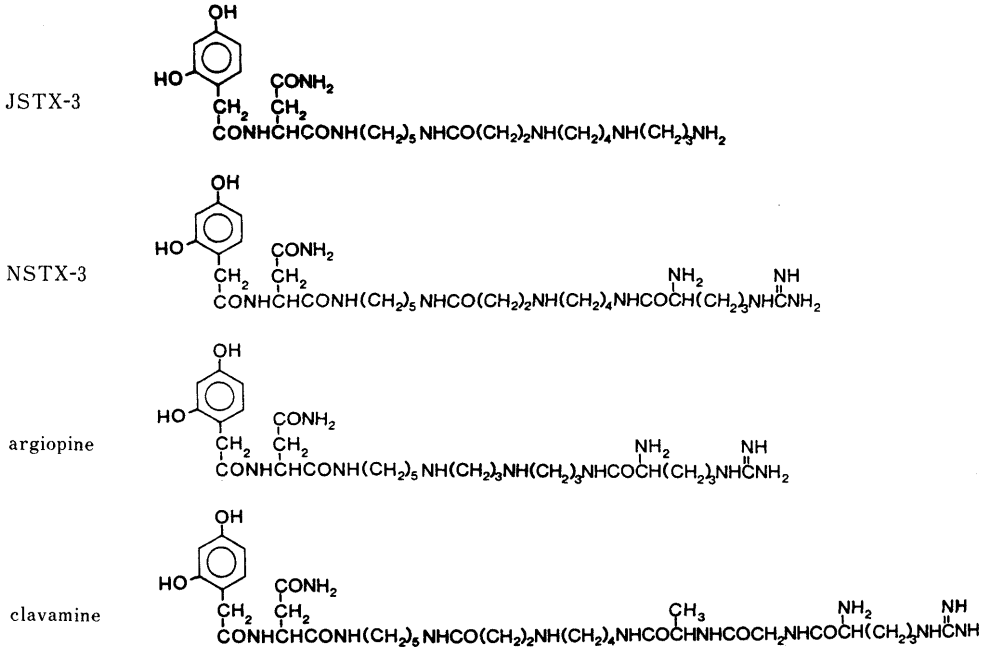
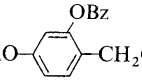
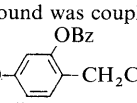


Fig. 1. Structures of Spider Toxins Identified

is not enough to identify the complicated structure of clavamine by chemical analyses and spectrometries. Thus, we chemically synthesized clavamine for further study.[10] In this paper, we compared the chemical properties and biological activities of both natural and synthetic clavamines.

Materials and Methods

Materials The natural clavamine was purified as described in the previous paper.[9] The synthesis of clavamine was carried out starting from a synthetic intermediate of NSTX-3, *i.e.*, BzlO-  -$CH_2CO\rightarrow$ Asn$\rightarrow$Cad$\leftarrow$Ptr(Z), in which Bzl and Z are benzyl and benzyloxycarbonyl groups, respectively. The compound was coupled with Ala, Gly and Arg, successively to build up to BzlO-  -$CH_2CO\rightarrow$Asn$\rightarrow$Cad$\leftarrow$Ptr(Z)$\leftarrow$Ala$\leftarrow$Gly$\leftarrow$Arg(Z_2)-Z. The coupling product was deprotected with CF_3SO_3H-CF_3COOH-thioanisole-*m*-cresol. The final product was further purified by preparative HPLC. All amino acids used for the synthesis were of L-form. Venom glands collected from joro spiders were homogenized in equivalent volumes of water (1 gland/1 ml) by ultrasonification and the supernatant was lyophilized to give a crude extract. The activity GU (gland unit) was expressed as the gland equivalent in the number of glands. The details were described in our previous paper.[9,10] Putreanine sulfate was obtained from Sigma, St. Louis. All other reagents were commercially available.

Chemical Analyses Chemical characteristics of the natural and synthetic clavamines were compared to each other by HPLC, thin-layer chromatography (TLC), Edman degradation and dansylation, gas chromatography (GC), ^{1}H-NMR spectrometry, and fast atom bombardment-mass spectrometry (FAB-MS) under the same conditions described in the previous paper.[9]

Bioassay Using Mosquito Larvae The original method for bioassay was described in the previous paper.[11] First or second instar larvae of the mosquito, *Culex pipiens molestus*, were grown in our laboratory. Five larvae per tube were released into tubes (7 mm in diameter, 60 mm in height) containing mixtures of 100 μl of water and 100 μl of the test solutions. For preparation of the test solutions, to 100 μl of 3×10^{-6} M clavamine or the venom extract from *Nephila clavata* was added 100 μl of 1.6 M citrate buffer at pH 7.0. The solution was further diluted 10-fold with the buffer. Dead and heavily intoxicated individuals were counted 3 h after the release. Each test was repeated three times.

Bioassay Using German Cockroach Adult German cockroaches, *Blattella germanica*, were maintained in the laboratory. Each cockroach was anesthetized under CO_2. Into the thoracic body cavity was injected 1 μl of the test solution in water with a microsyringe. After the injection, the cockroach was confined in a polyethylene cup (6 cm in diameter, 4.5 cm in height), so that poisoning symptoms were easily observed. For each test solution, 10 individuals were used.

Results

As shown in Fig. 2, the synthetic as well as the purified natural clavamine showed a single spot of the same *Rf* value in TLC. In HPLC, both compounds were eluted at the same retention time as shown in Fig. 3. In GC, both hydrolyzates showed exactly the same composition of Gly, Cad, DPA, Ala, Asp, Arg and Ptr as shown in Fig. 4.

The Edman degradation gave the same products such as Arg, Gly and Ala in this order. The ^{1}H-NMR spectrum of the synthetic compound corresponded to that of the natural one as shown in Fig. 5. The same was true in a FAB-MS as shown in Fig. 6. The parent mass of 793(M+1) is apparent in the use of a large amount of the synthetic sample.

The biological activity is a crucial problem. Both synthetic and natural compounds showed almost the same extent of insecticidal activity on the wrigglers after 3 h.

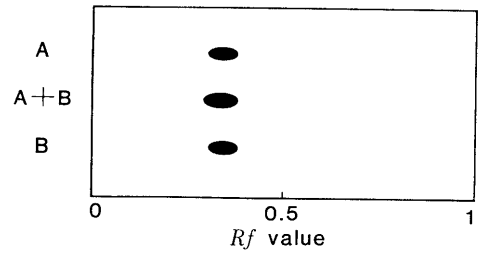


Fig. 2. TLC of Synthetic (A) and Natural (B) Toxins, and the Spot of A was Overlapped with the Spot of B(A+B)

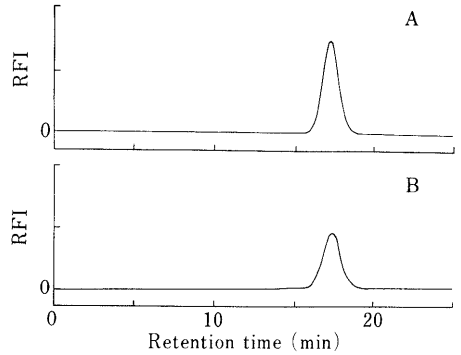


Fig. 3. HPLC Profiles of Synthetic (A) and Natural (B) Toxins

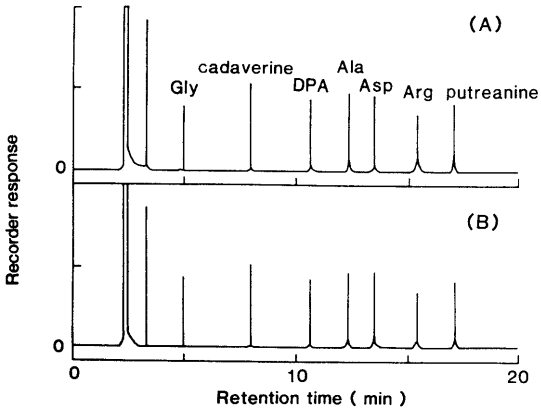


Fig. 4. Gas Chromatographic Analyses of the Hydrolyzates of Synthetic (A) and Natural (B) Toxins

The mortalities were 90 and 70% at 6.7×10^{-8} M solutions of the natural and synthetic clavamine, respectively, the concentrations of which corresponded to several GU. In the previous paper,[11] 5 GU of the crude extract showed 100% mortality.

We also examined the insecticidal activity of synthetic clavamine on the cockroach to determine whether it is a representative constituent in the activity of the crude venom extract. In order to first check the assay procedure, we used kainate, an agonist to the glutamate receptor, as a standard insecticide. When 1 μl of 4×10^{-4} M kainate was injected, the cockroach recovered from the anesthesia in 20 min but remained in an upside down posture and started to move slowly in a normal posture in 60 min. For 60 min, the legs and antennae were tight. At 2×10^{-3} and 1×10^{-2} M kainate, the cockroach returned to normal in 90 min and one day, respectively. When 0.1 GU of the crude extract of the venom was injected, the cockroach

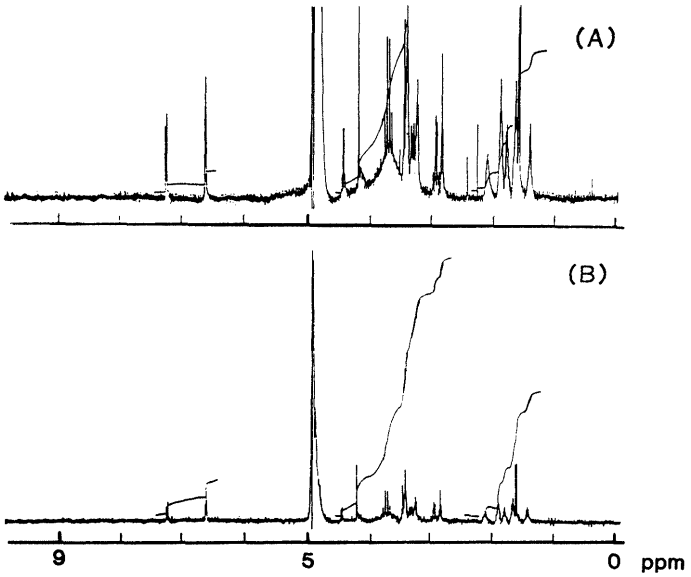


Fig. 5. ^{1}H-NMR Spectra of the Synthetic (A) and Natural (B) Toxins

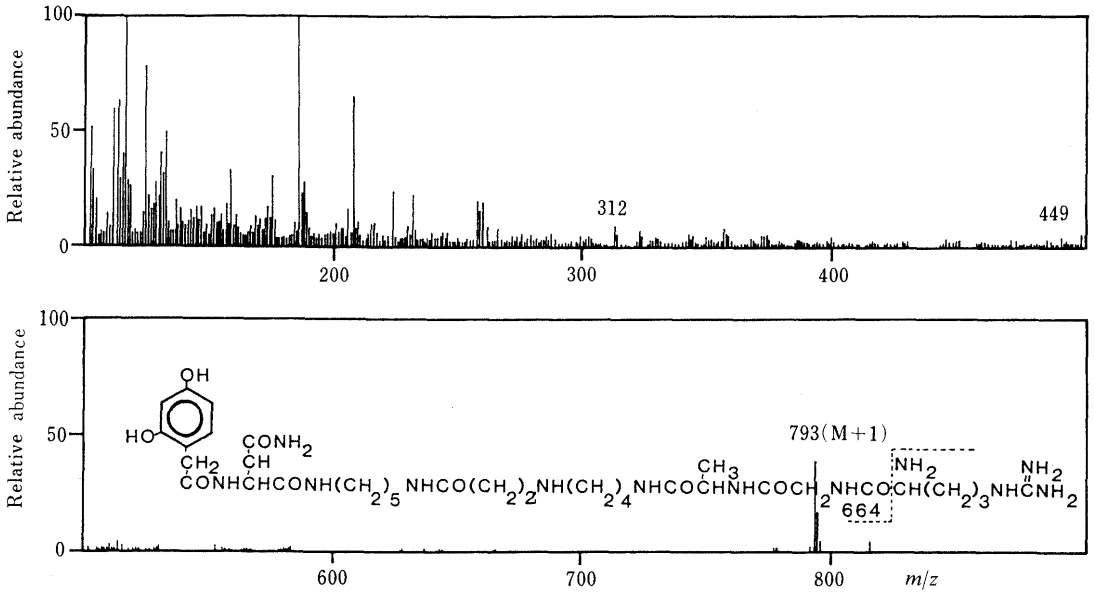


Fig. 6. FAB-MS of Synthetic Clavamine

became normal in 1—3 h. During 3 h, the legs and antennae were relaxed and weakly responsive to stimulation. When 1 GU of the crude extract was injected, it took a day for the cockroach to return to normal. No rigidity was observed in the legs or antennae, when the crude extract was injected with the kainate. When 1 μl of 1.3×10^{-2} M synthetic clavamine corresponding to 0.05—0.1 GU in the assay of the larvae was injected, only 1 to 3 h were necessary for the insect to recover. At 5×10^{-2} M clavamine, the insect became normal in one day. Thus, it was semi-quantitatively and qualitatively proven that clavamine was the main active component in the venom.

Discussion

It was proven that synthetic clavamine was chemically and biologically identical with the natural one. The analytical methods such as TLC, HPLC, GC, ^{1}H-NMR and FAB-MS adopted in this study are useful not only for analyzing the polyamines in the venoms but also for exploring new polyamines. The main difficulties of purification and identification were chelate formations with metal ions, as shown in the previous paper.[12] The chemical characteristics of these chelates will be described elsewhere.

It seems very interesting to study the mechanism of clavamine as related to bioorganisms. Friedel and Nentwig examined the immobilizing and lethal effects of crude venoms on six kinds of spiders, cockroaches and common mealbeetles. According to their consideration, the immobilizing effect of locomotion of the insects was more crucial to the prey capture than the lethal effect.[13] In our previous report, the crude venom of *N. clavata* was shown to be effective for immobilizing cockroaches, larvae of the tobacco cutworm, and adults of the green rice leafhopper, whose movements were supposedly driven by glutamate transmission.[11] Clavamine is lethal to wrigglers at a

concentration as low as 10^{-8} M solution. Toki *et al.* described that 4-hydroxyindole-3-acetylpolyamines identified in the same venom of *N. clavata* also were toxic to wrigglers.[14] The concentrations of their derivatives used, however, were 10000 times higher than what we used in testing. JSTX-3 and NSTX-3 identified in the venoms of *N. clavata* and *N. maculata* respectively, have the common structural feature of 2,4-dihydroxyphenylacetylpolyamines as a clavamine, but they were not lethal at all to the wrigglers. Thus, clavamine might be the main active component involved in the insecticidal effect of the venom, considering its comparable activity with that of the crude extract to the wriggler and the cockroach. The posture of clavamine intoxication is opposite to the rigid posture of the kainate. The antennae and legs of the cockroach intoxicated by the clavamine are soft and relaxed. This phenomenon is interesting regarding the mechanism of the insecticidal effect.

In conclusion, the structure and biological activity of clavamine were established. Together with JSTX-3 and NSTX-3, the synthetic clavamine may be used to investigate neurochemical effects on insects and thereby to develop new insecticides and drugs for neurological disorders of the brain.

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