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STRUCTURE-ACTIVITY RELATIONSHIP OF NSTX-3, SPIDER TOXIN OF *NEPHILA MACULATA*

Tadashi Teshima, Takahiro Matsumoto, Tateaki Wakamiya, Tetsuo Shiba†,
Terumi Nakajima††, and Nobufumi Kawai†††

Department of Chemistry, Faculty of Science, Osaka University,
Toyonaka, Osaka 560, Japan.
†Peptide Institute, Protein Research Foundation,
Minoh, Osaka 562, Japan.
†† Department of Analytical Chemistry, Faculty of Pharmaceutical Science,
The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.
††† Department of Neurobiology, Tokyo Metropolitan Institute for
Neurosciences, Fuchu, Tokyo 183, Japan.

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In a study on structure-activity relationship of spider toxin NSTX-3 obtained from venom of *Nephila maculata*, nine analogs or fragments of natural toxin were synthesized. Among them, des-Arg-NSTX-3 was found to be the minimum structure for blocking the excitatory transmission in the crustacean neuromuscular synapse. A positive charge of the arginine part was concluded to play the most important role for exhibition of the activity.

A new neurotoxin NSTX-3 isolated from the venom of a Papua New Guinean Spider (*Nephila maculata*) showed the irreversible suppression of the excitatory postsynaptic potential (EPSP) as well as glutamate potential in the lobster neuromuscular junction. The whole structure of NSTX-3 was proposed as shown in Fig. 1[1] that was established by total synthesis by us.[2] At the same time, the proposed structure of JSTX-3 from the venom of Joro Spider (*Nephila clavata*)[1] was also determined synthetically.[3] Moreover, a variety of other spider toxins such as argiopin[4], argiotoxin[5], were reported to have similar blocking action on the glutamate receptor. These toxins are characterized to consist of a variety of related compounds

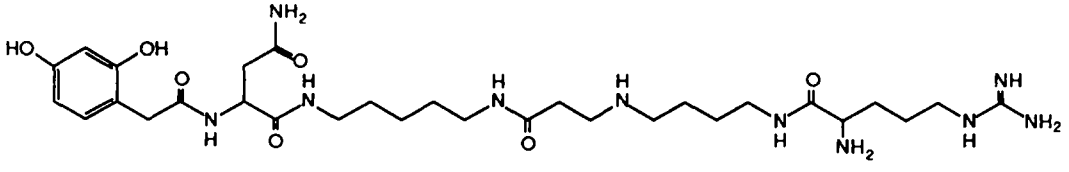


Fig. 1. The Structure of NSTX-3

with a novel structure containing phenolic group in addition to polyamine moiety.[6] It is, therefore, of interest to know how the unique toxin structure act on the glutamate receptor with high affinity.

In this study, we synthesized several analogs of NSTX-3 and examined biological activity to elucidate the structure-activity relationship.

All analogs were prepared from **1**, **3**, and **6** of the synthetic intermediates in the total synthesis of NSTX-3 as shown in Fig. 2. The shorter analogs, **2**, **4**, and **11**, were obtained by deblocking of **1**, **3**, and **6**, respectively. Substituted derivatives **12**, **13**, **14**, **15**, and **16**, were prepared by couplings of suitable protected amino acids (or acetic acid) active ester with **6** followed by deprotection. Data for amino acid analysis, retention time of HPLC, and specific optical rotation of all these compounds were listed in Table 2. Their NMR spectra (data are not shown) were

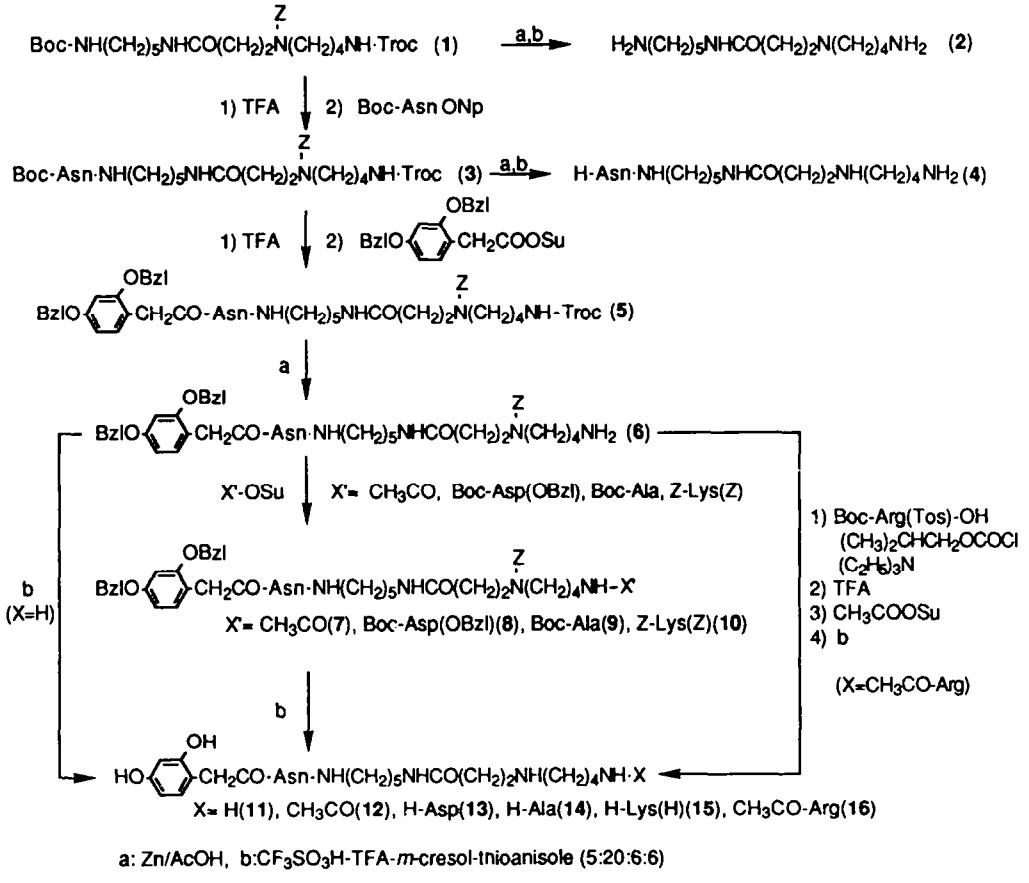


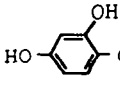
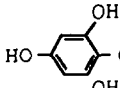
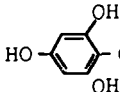
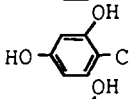
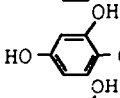
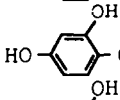
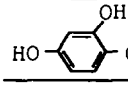
Fig.2 Synthetic Scheme of NSTX-3 Analogs.

consistent with their structures.

The suppressions of the excitatory postsynaptic potential in the lobster neuromuscular junction of all synthetic analogs were measured, results being shown in Table 1. Both of Cad←Pua (**2**) and Asn→Cad←Pua (**4**) were found to be inactive. On the other hand, 2,4-dihydroxyphenylacetyl-Asn→Cad←Pua (**11**) showed the irreversible suppression of EPSP, whereas the activity of acetyl derivative (**12**) of des-Arg-NSTX-3 was reversible and extremely weak.

Substituted derivatives of **11**, *i.e.*, **13**, **14**, and **15** in which Arg of NSTX-3 was replaced with acidic, neutral, and basic amino acids respectively were next synthesized. Ala derivative (**14**) and Lys derivative (**15**) showed the irreversible activity in strength of 0.05 and 0.2 respectively, while Asp derivative (**13**) was very weak and reversible. From the results of activities of these compounds, it may be concluded that a positive charge in ω-position in Arg part was very important in order to show minimum irrevers-

Table 1. Relative activities of suppression of the EPSP in the lobster neuromuscular junction and their mode of NSTX-3 Analogs

Compound	Relative Activity	mode
 CH$_2$CO-Asn→Cad←Pua←Arg-H (NSTX-3)	1	I
Cad←Pua (**2**)	no	-
Asn→Cad←Pua (**4**)	no	-
 CH$_2$CO-Asn→Cad←Pua-H (**11**)	0.1	I
 CH$_2$CO-Asn→Cad←Pua-Ac (**12**)	<0.001	R
 CH$_2$CO-Asn→Cad←Pua←Asp-H (**13**)	<0.001	R
 CH$_2$CO-Asn→Cad←Pua←Ala-H (**14**)	0.05	I
 CH$_2$CO-Asn→Cad←Pua←Lys-H (**15**)	0.2	I
 CH$_2$CO-Asn→Cad←Pua←Arg-Ac (**16**)	<0.001	R

I: irreversible, R: reversible
Cad:1,5-pentanediamine (Cadaverine),
Pua:8-amino-4-azaoctanoic acid (Putreanine)

ible activity. Ac-Arg derivative (**16**) with only one positive charge in guanidino group of Arg was expected to show the irreversible suppression. However, unexpectedly it manifested only weak reversible activity, indicating that α-amino group of Arg was also requisite to show the irreversible activity.

Hashimoto *et al.* synthesized 2,4-dihydroxyphenylacetyl-Asn→Cad-H known as reversible activity against the suppression of EPSP. Taking account of this fact with the results obtained in this study, des-Arg-NSTX-3 was now assumed as the minimum structure responsible for the irreversible activity. Therefore the arginine part should be recognized as an important component to influence the mode of suppression (reversible or irreversible) as well as the strength. In addition, a number of plus charge and presence of α-amino group seemed to be much concerned with the mechanism to show the irreversible activity.

Experimental

Biological assay of synthesized toxins

Blocking activity of the compounds on the glutamate receptor were tested on lobster (*Palinurus japonicus*) neuromuscular synapse as described previously.[7] Percent inhibition of the excitatory postsynaptic potential (EPSP) was measured at 10 min after applying the compounds to the neuromuscular synapse.

Preparations of **4**

The compounds **3** (100 mg, 130 μmol) as intermediate of total synthesis of NSTX-3[2] was dissolved in acetic acid (2.8 ml). To the solution, zinc powder (200 mg, 3.06 mmol) was added and allowed to stand for 35 min under sonication. After filtration of excess zinc powder, the filtrate was concentrated *in vacuo*. The residue was treated with CF_3SO_3H-TFA-*m*-cresol-thioanisole (5:20:6:6)[8] at 0°C for 1.5 h. The crude product was purified by HPLC (column: Cosmosil $5C_{18}$, 8 mm x 250 mm, CH_3CN - 0.1 % TFA, linear gradient 0 - 30 % (15 min)). Yield 37 mg (40 %).

Compound **2** was obtained in the same manner. Yield 78 %.

Preparation of **11**

Compound **6** (30.3 mg, 34.3 μmol) was treated with CF_3SO_3H-TFA-*m*-cresol-thioanisole (5:20:6:6) at 0°C for 1.5 h. Ether was added to the reaction mixture to give a precipitate. A solution of the precipitate in water was

washed with ether, and purified by HPLC (column: Cosmosil $5C_{18}$, 8 mm x 250 mm, CH_3CN - 0.1 % TFA, linear gradient 0 - 30 % (15 min)). Yield 16.3 mg (64.4 %).

General method for preparation of compound **12-15**

A solution of compound **6** (50.0 mg, 56.6 μmol), Boc-Asp(OBzl)-OSu (47.6 mg, 113 μmol) and triethylamine (5.7 mg, 56.6 μmol) was stirred at room temperature for 20 h. The reaction mixture was concentrated *in vacuo*, and ethyl acetate and water were added to the residue to give solid. The solid thus obtained was dissolved in CF_3SO_3H-TFA-*m*-cresol-thioanisole (5:20:6:6) (1.48 ml) at 0°C and stirred for 1 h. To the reaction mixture, ether was added to give powder. A solution of the powder in water was washed with ether, and purified by HPLC (Cosmosil $5C_{18}$, 8 x 250 mm, 10% CH_3CN - 0.1 % TFA, retention time 15.7 min). Yield 19.5 mg (40.4 %).

Compound **12**, **14**, and **15** were prepared in the same manner as described above. Yield **12**: 48 %, **14**: 37 %, **15**: 23 %.

Acetyl NSTX-3 (**16**)

To a solution of Boc-Arg(Tos)-OH (96.9 mg, 226 μmol) in THF (2 ml), triethylamine (22.9 mg, 226 μmol) and isobutylchloroformate (30.9 mg, 226 μmol) were added at -20°C and stirred for 10 min. at -20°C. The mixed anhydride thus obtained was added to the solution of **6** (100 mg, 113 μmol)

Table 2. Amino acid analyses, retention times on HPLC, and specific optical rotations of NSTX derivatives

Synthetic Analogs	molar ratio					HPLC*	$[\alpha]_D^{15}$ **
	Asp	Ala	Lys	Pua	Arg		
2						10.2	-
4	1.05			1.00		11.1	3.0(0.35)
11	0.94			1.00		14.6	3.6(0.42)
12	0.96			1.00		15.7	4.6(0.50)
13	1.98			1.00		14.8	-4.3(0.47)
14	0.91	1.11		1.00		14.9	-5.0(0.30)
15	1.02		1.11	1.00		14.7	-4.3(0.49)
16	0.91			1.00	0.95	15.4	3.0(0.53)

Cad was not determined.

* Retention time of HPLC. Conditions of HPLC: Cosmosil $5C_{18}$, 8 mm x 250 mm, CH_3CN - 0.1 % TFA, linear gradient 0 - 30 % (15 min), detection: UV at 220 nm.

** Specific optical rotation in water. Concentrations were shown in parentheses.

and triethylamine (11.4 mg, 113 μmol) in DMF (4 ml) at -20°C. The reaction mixture was stirred at -20°C for 1 h and then at room temperature for 20 h. The reaction mixture was concentrated *in vacuo*, and the residue was solidified by the addition of ethyl acetate and water. Yield 113 mg (82 %).

This solid (92 mg, 75 μmol) was dissolved in TFA (2 ml) and stirred for 1 h at room temperature. The solution was concentrated in vacuo and the residue was washed with ether to give the solid of debutoxycarbonyl peptide. Yield 85 mg (91 %).

The product (10 mg, 8.0 μmol) was coupled with CH_3COOSu and deblocked in the similar manner as the preparation of **13**. Yield 2.6 mg (40 %)

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