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TOTAL SYNTHESIS OF NSTX-3, SPIDER TOXIN OF *NEPHILA MACULATA*

Tadashi Teshima, Takahiro Matsumoto, Tateaki Wakamiya, Tetsuo Shiba†*,
Yoshio Aramaki††, 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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A spider toxin NSTX-3 obtained from the venom of Papua New Guinean spider, *Nephila maculata* was synthesized in order to confirm its proposed structure and to supply the sample for the biological tests. The structure of NSTX-3 is now established as 2,4-dihydroxyphenylacetyl-L-Asn→Cad←Pua←L-Arg, where Cad stands for cadaverine and Pua for putreanine.

From the venom of a Papua New Guinean spider *Nephila maculata*, a new neurotoxin NSTX-3 was isolated which showed an irreversible suppression of the excitatory postsynaptic potential (EPSP) as well as the glutamate potential in the lobster neuromuscular junction. The structure of NSTX-3 was proposed as shown in Fig. 1[1], and established by our total synthesis[2]. The structure of JSTX-3,[1] a similar toxin obtained from the venom of Joro spider *Nephila clavata*, was also proposed and confirmed by synthesis.[3] After those synthetic studies, recently another synthesis of NSTX-3 as well as JSTX-3 were presented.[4] Moreover, a variety of other spider toxins such as argiopin[5], argiotoxin[6], and nephilatoxin[7] were reported to have similar blocking action on the glutamate receptor. We wish to present now the details of our synthetic study of NSTX-3 which was undertaken to confirm the proposed structure and to supply the

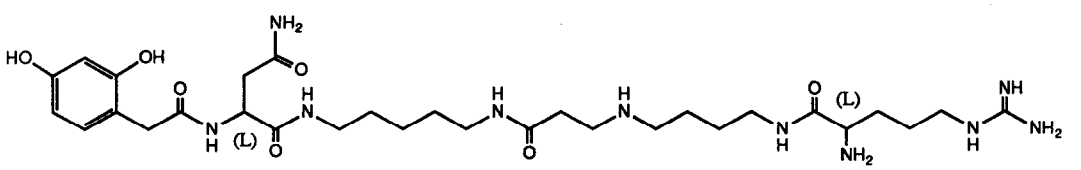

Fig. 1. Structure of NSTX-3

sample for the investigation of its biological activity.

A suitable derivative of 2,4-dihydroxyphenylacetic acid required for the successive coupling was prepared from 2,4-dihydroxybenzoic acid. Its di-*O*-benzyl derivative **3** was prepared by benzylation followed by saponification. Insertion of a methylene group between phenyl ring and carboxyl group in **3** was carried out by Arndt-Eistert method (Fig 2). Carboxyl group of **3** was converted to the acid chloride with oxalyl chloride, and allowed to react with diazomethane to give α-diazoketone which could be directly converted to the succinimide active ester by Wolff-rearrangement in the presence of *N*-hydroxy-succinimide.[8]

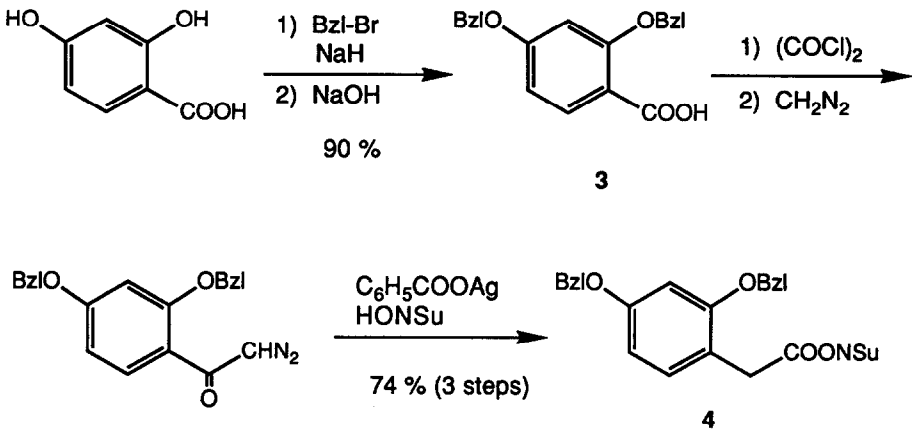

Fig. 2

On the other hand, N^8-phthaloylputreanine (**6**) was synthesized by reductive amination of 4-phthal-imidobutanal obtained from 4,4-diethoxybutylamine with β-alanine. Its preparation is shown in Fig. 3 but not described here. After protection of the secondary amino group of **6** with benzyloxycarbonyl group, it was coupled with mono-Boc-cadaverine hydrochloride (**5**). The phthaloyl group of **7** thus obtained was replaced with trichloroethoxycarbonyl (Troc) group before introduction of asparagine residue, in order to avoid a possible formation of the succinimide ring at asparagine residue under basic condition which is required for the removal of phthaloyl group by hydrazinolysis. After replacement of the protecting group at N^8 of the putreanine moiety, Boc group on another amino group in cadaverine part was removed and then coupled with Boc-L-Asn-ONp to give **8**. Dibenzyloxyphenylacetic acid was then introduced to the free amino group of asparagine obtained by deprotection of Boc group of **8** affording us the compound **9**.

Finally, compound **9** was treated with zinc powder in acetic acid to remove the Troc group. Z-L-Arg(Z_2)-OH was then coupled to N^8-free amino group of putreanine moiety. The removal of all protecting groups from the final coupling product was carried out by catalytic hydrogenation. After purification by HPLC, the synthetic compound was completely identical with the natural sample in all respects such as retention time of HPLC, ^{1}H-NMR, and biological activity. However, the hydrogenation procedure seemed not to be suitable in a small scale experiment such as for the preparation of the radio active compound,

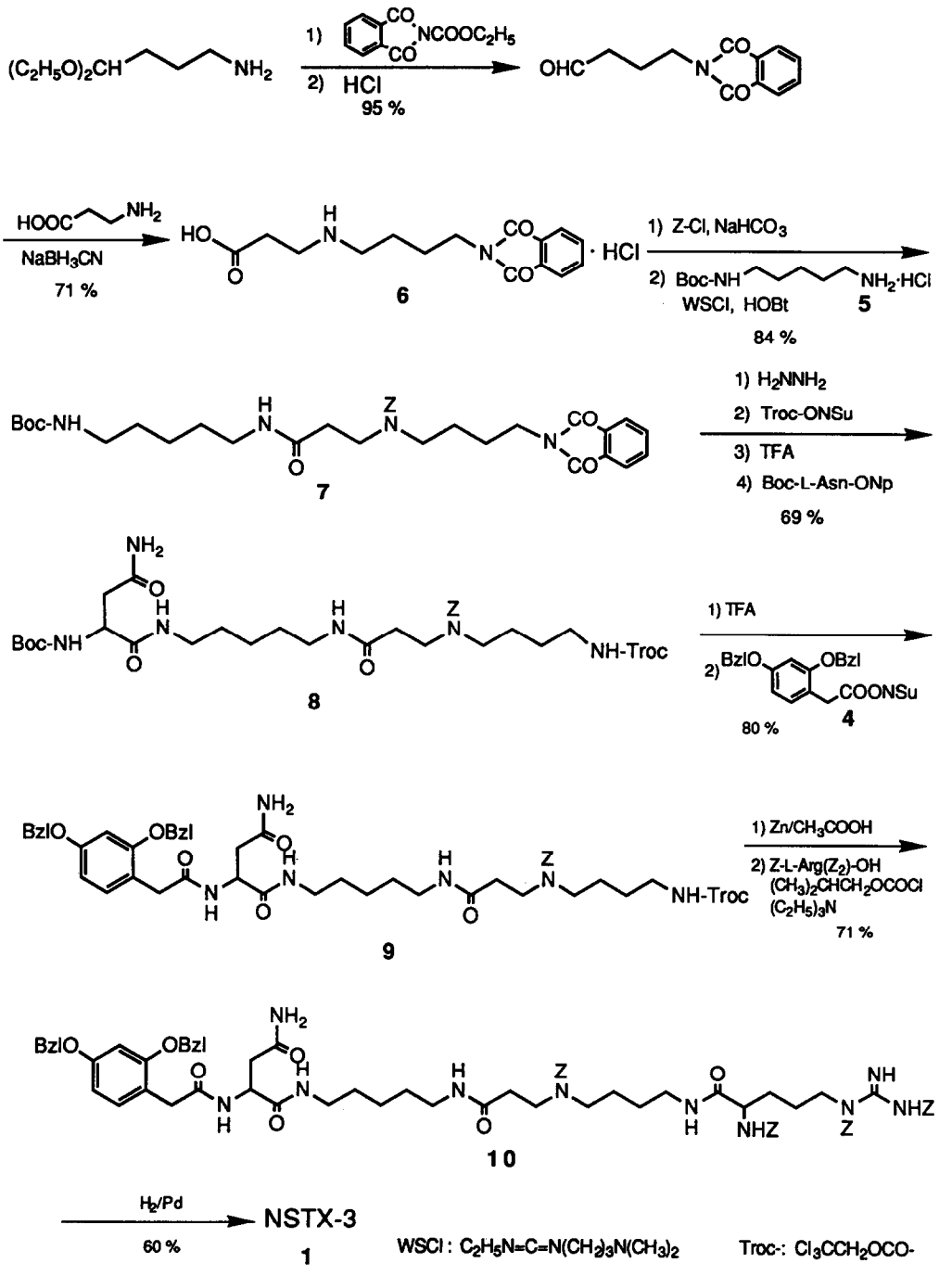
1) 2) HCl 95 %
HOOC NaBH3CN 71 %
6
1) Z-Cl, NaHCO3
2) Boc-NH WSCl, HOBt
5
84 %
7
1) H2NNH2
2) Troc-ONSu
3) TFA
4) Boc-L-Asn-ONp
69 %
8
1) TFA
2)
4
80 %
9
1) Zn/CH3COOH
2) Z-L-Arg(Z2)-OH
(CH3)2CHCH2OCOCl
(C2H5)3N
71 %
10
H2/Pd
60 %
NSTX-3
1
WSCl : C2H5N=C=N(CH2)3N(CH3)2
Troc-: Cl3CCH2OCO-

Fig. 3

because of an adsorption of the compound to the catalyst. In order to avoid this disadvantage, the deprotection was tried by trifluoromethanesulfonic acid - trifluoroacetic acid - *m*-cresol - thioanisole (5:20:6:6)[9,10]. The final product obtained by the acidolysis was identical with NSTX-3 by the hydrogenation method in view of retention time of HPLC as well as ^{1}H-NMR.

This study not only led the confirmation of the proposed structure of NSTX-3 synthetically, but also established its synthetic route for easy supply of NSTX-3 in enough amount for the investigation in neurosciences.

Experimental

Benzyl 2,4-dibenzyloxybenzoate (2)

To a solution of 2,4-dihydroxybenzoic acid (5.00 g, 32.4 mmol) in DMF (33 ml) was added NaH (60 % oil dispersion) (2.33 g, 97.2 mmol) portionwise at 0°C under stirring. Benzyl bromide (16.6 g, 97.2 mmol) was added dropwise at 0°C, and was allowed to stand overnight at room temperature after stirring at 0°C for 1 h. After addition of water to the reaction mixture, the product was extracted with ethyl acetate. The organic layer was washed with water, and dried over $MgSO_4$. The solution was concentrated *in vacuo* to give oily residue which crystallized with trituration with hexane. The crude crystal was recrystallized from ethyl acetate - hexane. Yield 8.75 g (63.4 %). mp 100-101°C. Found: C, 78.94; H, 5.76 %. Calcd for $C_{28}H_{24}O_4$: C, 79.22; H, 5.70 %.

2,4-dibenzyloxybenzoic acid (3)

To a solution of benzyl 2,4-dibenzyloxybenzoate (**2**) (12.0 g, 28.2 mmol) in dioxane (120 ml) was added 1 M NaOH (120 ml), and heated under reflux for 1.5 h. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in water. The solution was washed with ether, and extracted with ethyl acetate after acidifying the solution with 3M HCl. The organic layer was washed with water, and dried over $MgSO_4$. The solution was concentrated *in vacuo* to give crystalline product which was recrystallized from ethyl acetate - hexane. Yield 8.78 g (93.1 %). mp 122-123°C. Found: C,75.35; H, 5.42%. Calcd for $C_{21}H_{18}O_4$: C, 75.43; H, 5.43%.

2,4-dibenzyloxyphenylacetic acid succinimide ester (4)

To a solution of 2,4-dibenzyloxybenzoic acid (**3**) (3.00 g 8.97 mmol) and pyridine (783 mg, 9.89 mmol) in anhydrous benzene (30 ml) was added dropwise a solution of oxalyl chloride (2.29 g, 18.0 mmol) in anhydrous benzene (30 ml). After stirring 30 min, insoluble material was filtered off, and the filtrate was concentrated *in vacuo*. The residue was dissolved in anhydrous THF (60 ml), and excess amount of an ethereal solution of diazomethane was added to the solution at 0°C. The reaction mixture was stirred at 0°C for 30 min, and then at room temperature for 1.5 h. The solution was concentrated *in vacuo* to give yellow solid.

To a solution of diazoketone thus obtained and *N*-hydroxysuccinimide (11.3 g, 98.1 mmol) in DMF (90 ml) was added silver benzoate (414 mg, 1.79 mmol) in triethylamine (3 ml) in dark. Insoluble material was filtered off, and the filtrate was concentrated *in vacuo*. Ethyl acetate solution of the residue was washed with water, 10% citric acid solution, water, sat. $NaHCO_3$ solution, and water successively, and dried over $MgSO_4$. The organic layer was concentrated *in vacuo* to give crystalline product which was recrystallized from ethyl acetate - hexane. Yield 2.94 g (73.5%). mp 143.0-143.5°C. Found: C, 70.12; H, 4.99; N, 3.16 %. Calcd for $C_{26}H_{23}NO_6$: C, 70.10; H, 5.20; N, 3.14 %.

N-*t*-Butoxycarbonyl-1,5-diaminopentane hydrochloride (Boc-Cad-H·HCl) (5)

To a solution of 1,5-diaminopentane (5.45 g, 53.4 mmol) and triethylamine (2.70 g, 26.7 mmol) in methanol (100 ml) was added the solution of di-*t*-butyldicarbonate (5.82 g, 26.7 mmol) in THF (30 ml) dropwise for 1 h. After stirring for 1 h, the solution was concentrated *in vacuo*. A solution of the residue in water (50 ml) was neutrallized with 2M HCl, and purified by the column chromatography (Diaion HP-20, 3 x 42 cm, eluate: 50% aqueous methanol after washing with water (600 ml)). The product was recrystallized from methanol - ether. Yield 3.99 g (62.7 %). mp 115.5-117°C. Found: C, 50.22; H, 9.70; N, 11.74; Cl, 14.92 %. Calcd for $C_{10}H_{23}N_2O_2Cl$: C, 50.31; H, 9.71; N, 11.73; Cl, 14.85.

N^8-Phthaloylputreanine hydrochloride (Pht=Pua-OH·HCl) (6)

N-Ethoxycarbonylphthalimide (8.04 g, 36.7 mmol), 4-aminobutylaldehyde diethylacetal (5.63 g, 34.9 mmol), and triethylamine (3.89 g, 34.4 mmol) were dissolved in THF (40 ml), and allowed to react for 2 h. The reaction mixture was concentrated *in vacuo*, and the residue was extracted with hexane. The hexane solution was concentrated *in vacuo* and heated at 100°C under reduced pressure to remove ethylcarbamate by sublimation . Yield 9.71 g (95.2 %).

The mixed solution of *N*-phthaloyl-4-aminobutylaldehyde diethylacetal (5.06 g,17.4 mmol) thus obtained and 1M HCl (34 ml) in acetone (50 ml) was heated under reflux for 15 min. Acetone was evaporated from the reaction mixture *in vacuo*, and the residue was extracted with ether. The organic layer was washed with water, and dried over $MgSO_4$. The ethereal solution was concentrated *in vacuo*, and the oily residue was dissolved in methanol (155 ml). This solution was mixed with a solution of β-alanine (1.85 g, 20.8 mmol) in water (14 ml). After addition of acetic acid (3 ml), a solution of $NaBH_3CN$ (1.31 g, 20.9 mmol) in methanol (44 ml) was added dropwise in 20 min. After stirring at room temperature for 3 h, 6M HCl (11 ml) was added. The reaction mixture was concentrated *in vacuo*, the residue was dissolved in ethyl acetate and water. Insoluble material was filtered off, and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (Diaion HP-20, 3 x 55 cm, eluate: methanol-0.01M HCl (1:1) after washing with water (450 ml)). The product was recrystallized from methanol - ether. Yield 2.63 g (46.4 %, from *N*-phthaloyl-4-aminobutylaldehyde diethylacetal). mp 203°C (dec). Found: C, 54.83; H, 5.90; N, 8.49; Cl, 10.72%. Calcd for $C_{15}H_{19}N_2O_4Cl$: C, 55.13; H, 5.86; N, 8.57; Cl, 10.85%.

Boc→Cad←Pua(Z)=Pht (7)

To a solution of Pht=Pua-OH·HCl (**6**) (2.40 g, 7.34 mmol) and $NaHCO_3$ (1.23 g, 14.6 mmol) in water (148 ml) were added benzyl chloroformate (2.51 g, 14.7 mmol) and $NaHCO_3$ (1.24 g, 14.8 mmol) under stirring at 0°C. After stirring overnight at room temperature, the reaction mixture was acidified with 6M HCl, and extracted with ethyl acetate. The organic layer was washed with brine, and dried over $MgSO_4$. The solution was concentrated *in vacuo* to give oily product. To a mixed solution of Pht=Pua(Z)-OH thus obtained, Boc-Cad-H·HCl (**5**) (2.21 g, 9.26 mmol), and HOBt (991 mg, 7.34 mmol) in DMF (24.0 ml), WSCI (1.14 g,7.34 mmol) was added at 0°C under stirring. After stirring at room temperature overnight, the reaction mixture was concentrated *in vacuo*, and the solution of the residue in ethyl acetate was washed with 10% citric acid, brine, sat. $NaHCO_3$, and then brine again. The organic layer was dried over $MgSO_4$, and concentrated *in vacuo*. The oily residue was purified by silica-gel column chromatography (eluate: ethyl acetate - toluene (2:1)). Yield 3.57 g (79.9 %).

Boc-L-Asn→Cad←Pua(Z)-Troc (8)

A solution of Boc-Cad←Pua(Z)=Pht (**7**) (3.47 g, 5.70 mmol) and hydrazine hydrate (856 mg, 17.1 mmol) in ethanol was heated under reflux for 2 h. Insoluble material was filtered off, and the filtrate was concentrated *in vacuo*. To a solution of oily residue in ethyl acetate were added Troc-ONSu (3.31 g, 11.4 mmol) and triethylamine (576 mg, 5.70 mmol). The mixture was allowed to react for 2.5 h. The reaction mixture was washed with 10% citric acid, brine, sat. $NaHCO_3$, and brine successively, and dried over $MgSO_4$. The organic layer was concentrated *in vacuo*. The oily residue was dissolved in TFA (20 ml), and allowed to react at room temperature for 45 min. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in DMF (20 ml) to which Boc-L-Asn-ONp (2.82 g, 8.07 mmol) and triethylamine (2.02 g, 19.9 mmol) were added, and stirred at room temperature for 36 h. The reaction mixture was concentrated *in vacuo* to give the residue which was dissolved in ethyl acetate. The solution was washed with 10% citric acid, brine, sat. $NaHCO_3$, and brine successively, and dried over $MgSO_4$. After the organic layer was concentrated *in vacuo*, the residue was triturated with ether to give solid which was reprecipitated from methanol - ether. Yield 3.11 g (69.1%). mp 93-100°C. $[\alpha]_D^{19}$ -2.0°(c 1.07, DMF). Found: C, 48.89; H, 6.45; N, 10.97; Cl, 13.37 %. Calcd for $C_{32}H_{49}N_6O_9Cl_3 \cdot H_2O$: C, 48.89; H, 6.54; N, 10.69; Cl, 13.53 %.

2,4-Dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)-Troc (9)

Boc-L-Asn→Cad←Pua(Z)-Troc (**8**) (1.37 g, 1.75 mmol) was dissolved in TFA (34 ml), and stirred at room temperature for 50 min. The reaction mixture was concentrated *in vacuo*, to an oily residue which was dissolved in DMF (45 ml). To the solution were added 2,4-dibenzyloxyphenylacetic acid succinimide ester (861 mg, 1.93 mmol) and triethylamine (445 mg, 4.39 mmol), and allowed to react at room temperature for 48 h. The reaction mixture was concentrated *in vacuo*. The residue was triturated with ethyl acetate and water to give solid which was reprecipitated from DMF - ether. Yield, 1.55 g (88.6 g). mp 146-147°C. $[\alpha]_D^{19}$ -0.8°(c 1.12, DMF). Found: C, 58.27; H, 6.05; N, 8.70; Cl, 9.90 %. Calcd for $C_{49}H_{59}N_6O_{10}Cl_3 \cdot 0.5DMF \cdot 0.5H_2O$: C, 58.10; H, 6.13; N, 8.72; Cl, 10.19 %.

2,4-Dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)←L-Arg(Z_2)-Z (10)

To a solution of 2,4-dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)–Troc (**9**) (830 mg, 0.831 mmol) in acetic acid (17 ml) was added zinc powder (1.21 g), and allowed to react under sonication for 1.5 h. After filtration, hydrogen sulfide was bubbled to the filtrate, the precipitate separated out was filtered off with membrane filter (0.45 μm). The filtrate was concentrated *in vacuo* to the residue which was triturated with ether to give solid. Yield 659 mg (95.2 %).

2,4-Dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)-H·CH_3COOH thus obtained (200 mg, 0.227 mmol) was dissolved in DMF (8 ml) (solution A). To a solution of Z-L-Arg(Z_2)-OH (261 mg, 0.454 mmol) in THF (4 ml) were added triethylamine (46 mg, 0.46 mmol) and isobutylchloroformate (61 mg, 0.45 mmol) at -20°C and stirred for 10 min. This reaction mixture was added to the solution A at -20°C, and stirred for 1 h at -20°C. After stirring at room temperature for 1.5 h, the reaction mixture was concentrated *in vacuo*. The residue was triturated with ethyl acetate and water to give solid which was recrystallized from DMF - ether. Yield, 262 mg (83.3 % from 2,4-dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)-H·CH_3COOH). mp 158-159°C. $[\alpha]_D^{15}$ +2.0°(c 1.03, DMF). Found: C, 64.87; H, 6.48; N, 10.03 %. Calcd for $C_{76}H_{88}N_{10}O_{15}\cdot 1.5H_2O$: C,64.80; H,6.51; N, 9.94%.

NSTX-3 (1) by hydrogenation

2,4-Dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)←L-Arg(Z_2)-Z (**10**) (10 mg, 7.2 μmol) was dissolved in acetic acid (2 ml) and methanol (1 ml). Hydrogen was bubbled into the solution in the presence of Pd-black. Catalyst was filtered off, and the filtrate was concentrated *in vacuo*. The residue obtained was then purified by HPLC. (Cosmosil $5C_{18}$, 8 x 250 mm, eluate: acetonitrile - 0.1 % TFA (linear gradient: 0 - 60 %(30 min), flow rate: 2.0 ml/min), retention time: 15.5 min). Yield, 4.4 mg (60 %). $[\alpha]_D^{15}$ +4.3°(c 0.51, H_2O). Amino acid analysis (Hitachi amino acid analysis system 655A; 6M HCl, 48 h): Put(1), 1.00; Asp(1), 0.91; Arg(1), 1.05. NMR (in D_2O; auto-reference condition (DHO=4.65 ppm); JEOL JMN GX-400, δ (ppm)): 1.03 (*quin.*, 2H), 1.28 (*m*, 2H), 1.30 (*m*, 2H), 1.46 (*m*, 2H x 2), 1.56 (*m*, 2H), 1.75 (*m*, 2H), 2.49 (*t*, 2H), 2.60 (*m*, 2H), 2.92 (*m*, 2H x 2), 3.07 (*t*, 2H), 3.12 (*m*, 2H x 3), 3.38 (*dd*, 2H), 3.80 (*t*, 1H), 4.45 (*dd*, 1H), 6.30 (*m*, 2H), 6.94 (*d*, 1H). Retention time of HPLC: 13.1 min (TSK gel ODS-120T, 4.6 x 250 mm, eluate: acetonitrile - 0.02 % HCl (4 : 96) (isocratic), flow rate 0.5 ml/min) (natural NSTX-3: 13.1 min). Biological activity: Irreversible suppression of EPSP tested on lobster (*Palinurus japonicus*) neuromuscular synapse[11] was observed in synthetic as well as natural NSTX-3 at the sample concentration of 5 x 10^{-8} M. The same neuromuscular preparation could not be used for both compounds because the activity of this toxin was irreversible. Therefore, direct comparison of synthetic and natural compounds was not be done.

NSTX-3 (1) by acidolysis

2,4-Dibenzyloxyphenylacetyl-L-Asn→Cad←Pua(Z)←L-Arg(Z_2)-Z (**10**) (19.5 mg, 14.1 μmol) was dissolved in trifluoromethanesulfonic acid - trifluoroacetic acid - *m*-cresol - thioanisole (5:20:6:6) (370 μl) at 0°C and allowed to react for 90 min. Addition of ether to the reaction mixture to give precipitate which was washed with ether. The product was purified by HPLC in the similar manner as describe above. Yield

7.3 mg (50.3 %). Its ^{1}H-NMR spectrum (in D_2O; JEOL JMN GX-400) was superimposable to that of NSTX-3 obtained by hydrogenolysis. Retention time of HPLC: 15.5 min (Cosmosil 5C_{18}, 8 x 250 mm, eluate: acetonitrile - 0.1 % TFA (linear gradient: 0 - 60 % (30 min), flow rate: 2.0 ml/min) (NSTX-3 obtained by hydrogenolysis: 15.5 min).

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