

Total Synthesis of the Novel Spider Toxin NPTX-594 from *Nephila madagascariencis*[1]

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A novel spider toxin, NPTX-594, comprised of four constituents: i.e., 2,4-dihydroxyphenylacetic acid (Dhpa), asparagine, 4,8-diaza-1,12-dodecanediamine (Dada), and lysine, was chemically synthesized. The synthetic compound was completely identical with the natural product as regards ^{1}H NMR and mass spectra. The structure of NPTX-594 was thus determined synthetically to be N^{12}-(Dhpa-Asn)-N^1-Lys-Dada, as proposed by spectrometric elucidation.

Spider toxins are known to be potent and specific blockers of glutamate receptor,[2] and seem to be usable as unique tools for elucidating the mechanism of glutamate neurotransmission in the brain. A novel acylpolyamine toxin (NPTX-594) was isolated from the venom of a Madagascar spider (*Nephila madagascariencis*) and/or a Brazilian spider (*Nephila clavipes*).[3] On the basis of the mass spectrometric analysis of natural product, the plausible structure **1** was proposed to NPTX-594; this compound is comprised of four constituents, i.e., 2,4-dihydroxyphenylacetic acid (Dhpa), Asn, 4,8-diaza-1,12-dodecanediamine (Dada), and Lys (Fig. 1).[4] However, because of shortage of natural sample, synthetic study was required to confirm the structure of this novel spider toxin, and to elucidate the biological activity.

Results and Discussion

Many acylpolyamines, such as JSTX.[5] NSTX.[6] argiopine,[7] NPTX,[8,9] and others[10] have been found in the venom glands of several kinds of spiders, such as *Nephila clavata*, *Nephila maculata*, *Argiope lobata*, and *Nephilengys borbonica*. These acylpolyamines are generally classified into six types based on their structural features, as shown in Fig. 2.[9c] NPTX-594 is a novel compound belonging to type B, i. e., the Lys residue instead of the putreanine (8-amino-4-azaoctanoic acid) unit or the Arg residue in known nephilatoxins binds to the 1-amino group of Dada.

In the synthesis of acylpolyamine-type spider toxins, we must find an effective strategy to introduce the acyl groups into both the terminal amino groups of the polyamine residue.[11,12] In the present study, the Lys-Dada bond was first formed, and then the Dhpa-Asn part was introduced into the other amino terminus of the Dada residue.

On the basis of the synthetic plan mentioned above, the polyamine part was first prepared by application of the reductive *N*-alkylation method. 3-(Troc-amino)propionaldehyde (**4**) prepared from 3-aminopropionaldehyde diethyl acetal (**2**) was connected with **2** in the presence of $NaBH_3CN$. The secondary amino group newly formed was protected with the Z group to give 4-Z-7-(Troc-amino)-4-azaheptanal diethyl acetal (**5**), which was then converted into the corresponding aldehyde **6** by acid hydrolysis (Scheme 1).

In order to construct a C4-chain unit in the Dada residue, we next required *N*-Boc-1,4-butanediamine (**8**). Although **8** can be prepared from commercially available 1,4-butanediamine, selective monoacylation of α,ω-diamines using general acylating reagents is often difficult and not reproducible.[11c,13] Therefore, we employed an alternate method reported by Mattingly[14] on the basis of the reduction of *N*-Boc-4-azidobutylamine (**7**) prepared from 4-aminobutanol. Although Mattingly hydrogenated the azide compounds by use of 10% Pd–C as a catalyst under pressure, we carried out the conversion of **7** into **8** with PPh_3 in THF and H_2O. When the hydrogenation was carried out using 10% Pd–C at atmospheric pressure, we observed a very interesting result that a major product was not the primary amine **8** but the secondary amine, i. e., $(BocNHCH_2CH_2CH_2CH_2)_2NH$.[15]

The aldehyde derivative **6** and the amine component **8** were connected in a manner similar to that mentioned in the preparation of **5**, followed by benzyloxycarbonylation to obtain fully protected Dada derivative **9** (Scheme 2). The other three acyl components in the molecule of **1** were used as their active ester derivatives for coupling with the amino groups. For this purpose, Z-Lys(Z)-OSu (**11**)[16] and Dhpa(Bzl)$_2$-OSu (**17**)[11c] were prepared according to known methods, and Boc-Asn-ONp (**14**) was of commercial origin.

The connection of four components was carried out as shown in Scheme 3. At first, the Troc group in the Dada derivative **9** was removed with Zn/AcOH, and the freed 1-amino group was coupled with **11**. Next, the Boc group of the product **12** was removed in the usual way, and **14** was coupled with the 12-amino group of the Dada residue in the compound **13** to

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give **15**. The Boc group of **15** was removed again, and **17** was coupled with the α-amino group of the Asn residue to obtain the fully protected NPTX-594 (**18**). Finally, all protecting groups of **18** were removed by catalytic hydrogenation, and the crude product was purified by preparative RPHPLC. The thus-obtained synthetic compound **1** was completely identical with natural product in respects of ^{1}H NMR (Fig. 3) and FAB-MS/MS[17] spectra. As a result of the present work, we determined

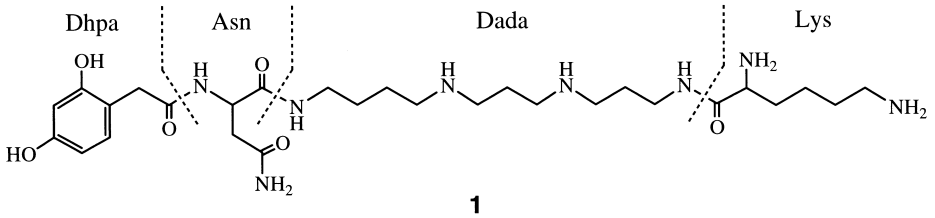


Fig. 1. Proposed structure of NPTX-594.

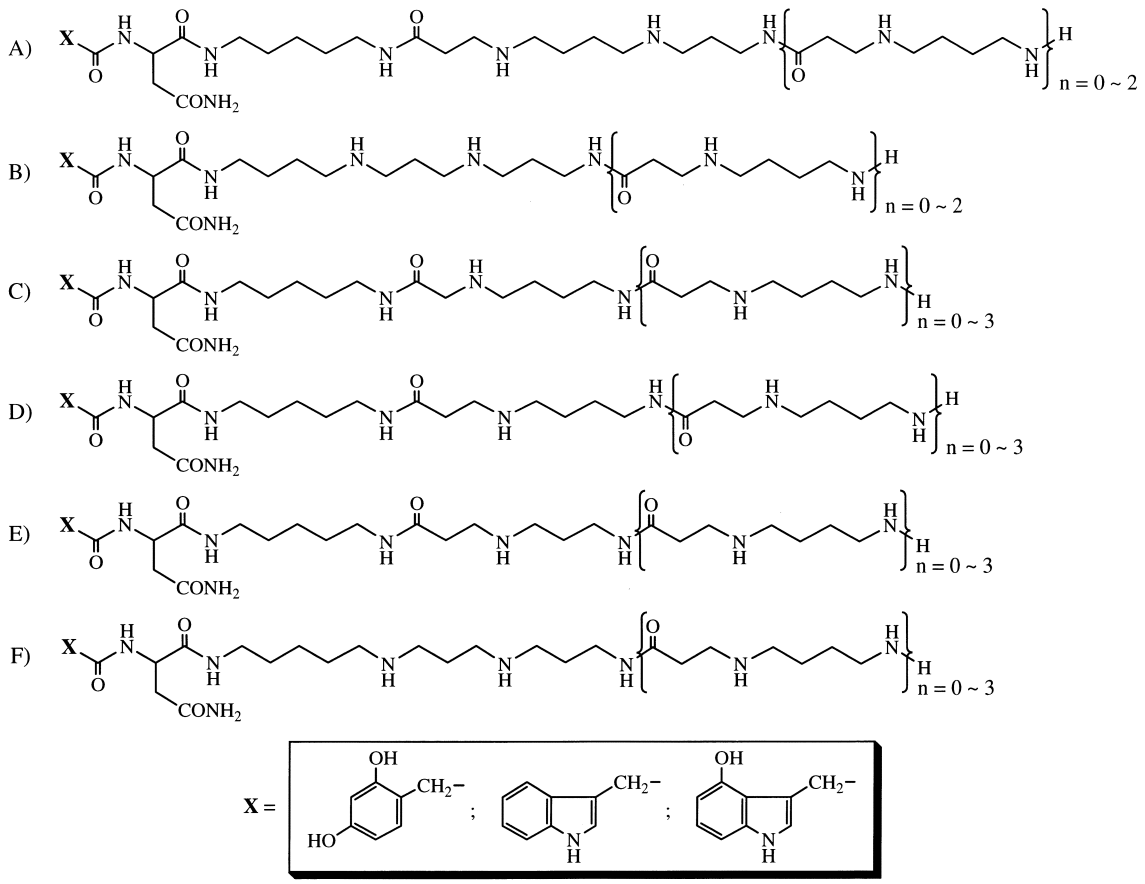
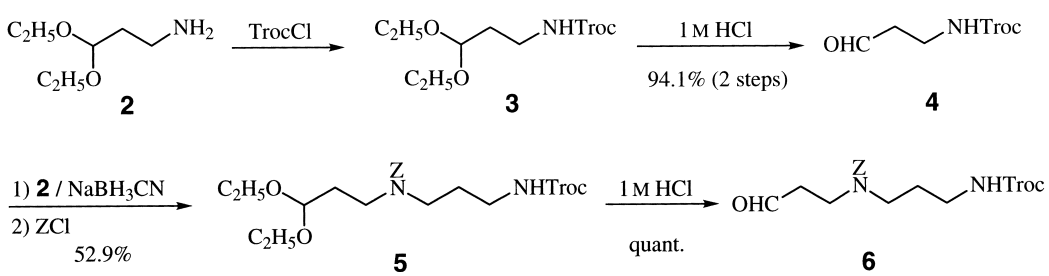
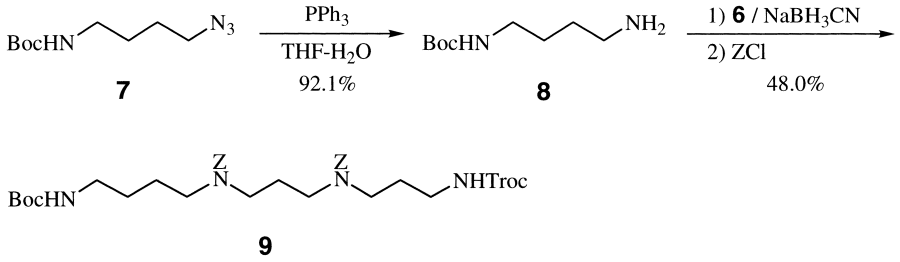


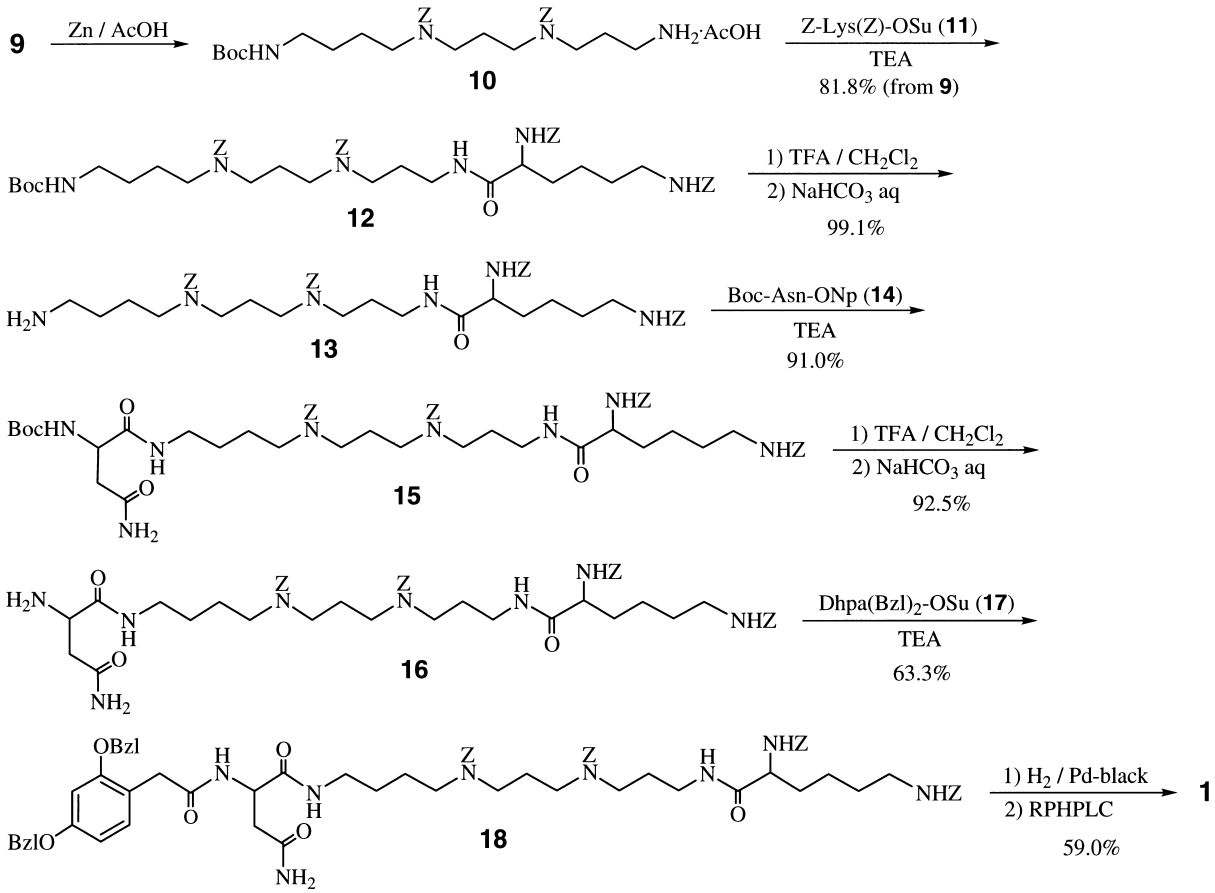
Fig. 2. Classification of acylpolyamine-type spider toxins. In some cases, putreanine unit, $-\{COCH_2CH_2NH(CH_2)_4NH\}_nH$, is replaced by the arginine residue.



Scheme 1.



Scheme 2.



Scheme 3.

the structure of NPTX-594 synthetically.

Although we are not able to compare the biological activity of the synthetic compound with that of natural one due to shortage of natural sample, NPTX-594 shows sufficient neurotoxicity, i. e., the ED_{50} value to paralyze crickets (*Grillus bimaculatus*) was of 0.38–0.30 μg/g insect.[18] The syntheses of several analogs of NPTX-594 are being currently undertaken from the standpoint of the structure-activity relationship study of type B nephilatoxins.

Experimental

The melting point is uncorrected and was measured by Yanaco MP-J3 (Yanaco Co., Ltd., Kyoto, Japan). Specific rotations were measured by DIP-140 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). ^{1}H NMR spectra were recorded on JEOL GX-270 (270 MHz, JEOL Co., Ltd., Tokyo, Japan), Varian Mercury 300 (300 MHz, Varian Co., Ltd., USA), or Bruker DMX-500 (500 MHz, Bruker Co., Ltd., Germany) spectrometer. The chemical shifts are given in δ values from TMS used as the internal standard. HRFAB-MS was carried out on a JEOL JMS-700 TKM mass spectrometer (JEOL Co., Ltd., Tokyo, Japan). Silica-gel column chromatography was carried out with Merck silica gel 60 (Art. 7734, 70–230 mesh). RPHPLC was performed on Cosmosil 5C_{18}-AR (4.6 $\times$ 150 mm, Nacalai Tesque, Kyoto, Japan) for analysis and YMC-Pack ODS-AM (20 $\times$ 250 mm, YMC Co., Ltd., Kyoto, Japan) for preparative purification. 3-Aminopropionaldehyde diethyl acetal was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Boc-Asn-ONp was purchased from Peptide Institute, Inc., Osaka, Japan.

3-(2,2,2-Trichloroethoxycarbonylamino)propionaldehyde (4). To a solution of 3-aminopropionaldehyde diethyl

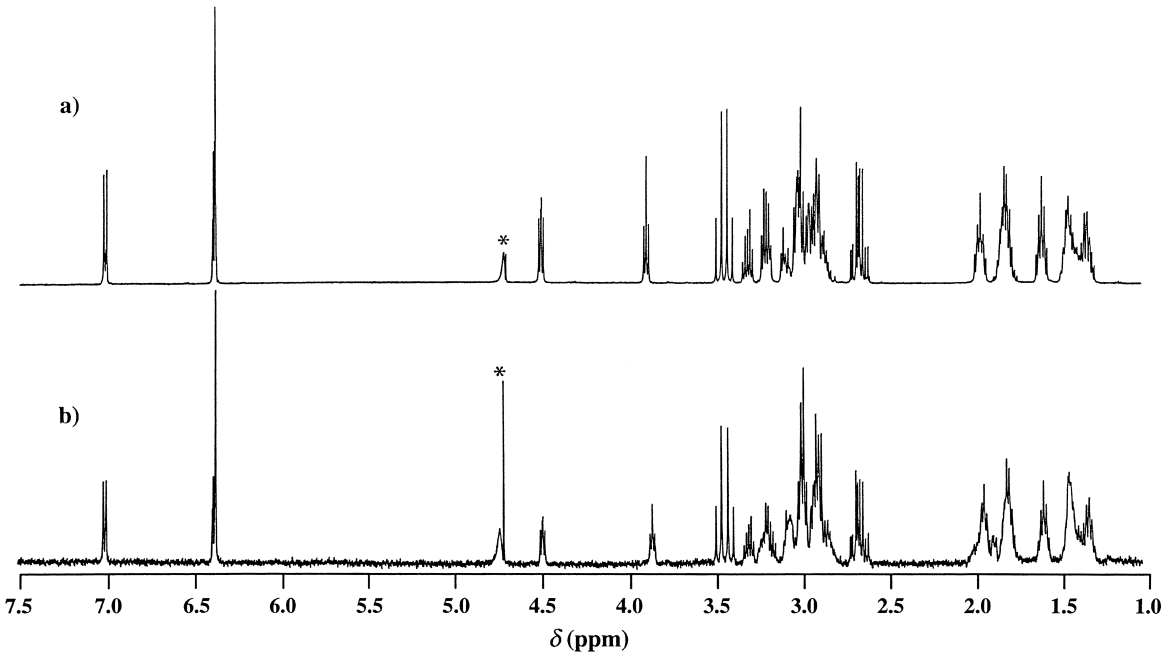


Fig. 3. 500 MHz ^{1}H NMR spectra of NPTX-594 in D_2O: (**a**) synthetic compound and (**b**) natural product. Peaks asterisked are of HDO.

acetal (**2**) (4.55 g, 30.9 mmol) in THF (100 mL) were added TEA (3.44 g, 34.0 mmol) and 2,2,2-trichloroethoxycarbonyl chloride (7.20 g, 34.0 mmol). The reaction mixture was stirred for 10 min at 0 °C and additionally for 2 h at r. t. The precipitated insoluble material was filtered off. The filtrate was concentrated in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (50 mL × 3), saturated aqueous $NaHCO_3$ (50 mL × 3), and brine (50 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo to give 3-(2,2,2-trichloroethoxycarbonylamino)propionaldehyde diethyl acetal (**3**) as a colorless oil (9.96 g, quant.). To a solution of the thus-obtained compound **3** in acetone (200 mL) was added 1 M HCl (10 mL). The reaction mixture was heated under reflux for 30 min. The solvent was removed in vacuo, and the residue was dissolved in diethyl ether (200 mL). The solution was washed with water (50 mL × 3), saturated aqueous $NaHCO_3$ (50 mL × 3), and brine (50 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo to give **4** as a colorless oil (7.23 g, 94.1%). The thus-obtained crude product was used for the next reaction without further purification.

4-Benzyloxycarbonyl-7-(2,2,2-trichloroethoxycarbonylamino)-4-azaheptanal Diethyl Acetal (5). To a solution of compound **4** (4.23 g, 17.0 mmol) in MeOH (200 mL) were added **2** (3.00 g, 20.4 mmol) and AcOH (2.93 mL, 51.0 mmol), and the reaction mixture was stirred for 1 h at r. t. To the solution was added dropwise $NaBH_3CN$ (2.14 g, 34.0 mmol) in MeOH (20 mL) at r. t. over a 30-minute period, and the reaction mixture was additionally stirred overnight. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (200 mL). The solution was washed with 10% citric acid (50 mL × 3), saturated aqueous $NaHCO_3$ (50 mL × 3), and brine (50 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo. To a solution of the residue in THF (100 mL) was added TEA (1.89 g, 18.7 mmol) under cooling at 0 °C. To this solution was added dropwise ZCl (3.19 g, 18.7 mmol) in THF (40 mL) at 0 °C over a 15-minute period, and the reaction mixture was additionally stirred for 3 h at r. t. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (30 mL × 3), saturated aqueous $NaHCO_3$ (30 mL × 3), and brine (30 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo. The crude product was purified by silica-gel column chromatography (180 g, 2.0 × 70 cm, benzene:AcOEt = 5:1). The fractions containing the desired product were combined, and concentrated in vacuo to give **5** as a colorless oil (4.62 g, 52.9%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (6H, t, CH_3 × 2), 1.88 (4H, m, CH_2 × 2), 3.18–3.75 (12H, m, CON–CH_2 × 3 and OCH_2 × 2), 4.50 (1H, t, O–CH–O), 4.73 (2H, s, CH_2/Troc), 5.17 (2H, dd, PhCH_2) and 7.35 (5H, s, Ph); HRFAB-MS: found *m/z* 513.1309 $(M+H)^+$ (calcd for $C_{21}H_{31}Cl_3N_2O_6$ + H: 513.1326).

4-Benzyloxycarbonyl-7-(2,2,2-trichloroethoxycarbonylamino)-4-azaheptanal (6). To a solution of compound **5** (3.35 g, 6.52 mmol) in acetone (100 mL) was added 1 M HCl (6 mL), and the reaction mixture was heated under reflux for 30 min. The solvent was removed in vacuo, and the residue was dissolved in diethyl ether (100 mL). The solution was washed with water (20 mL × 3), saturated aqueous $NaHCO_3$ (20 mL × 3), and brine (20 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was concentrated in vacuo to give **6** as a colorless oil (2.87 g, quant.). The thus-obtained crude product was used for the next reaction without further purification.

***N*-*t*-Butoxycarbonyl-1,4-butanediamine (8).** To a solution of *N*-Boc-4-azidobutylamine (**7**)[14] (22.6 g, 106 mmol) in THF (50 mL) were added PPh_3 (30.4 g, 116 mmol) and water (5 mL) under cooling at 0 °C. The mixture was stirred for 2 h at 0 °C and for 21 h at r. t. The solvent was removed in vacuo, and the residue was treated with 10% citric acid (100 mL) and AcOEt (30 mL). The aqueous layer separated was basified with 2 M NaOH, and the alkaline solution was extracted with $CHCl_3$ (50 mL × 3). The extract was dried over anhydrous $MgSO_4$, and the solvent was then removed in vacuo to give **8** as a colorless oil (18.3 g, 92.1%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.44 (9H, s, $(CH_3)_3C$), 1.51–1.71 (4H, m, CH_2 × 2), 2.76 (2H, t, NH_2CH_2) and 3.14 (2H, m, $BocNHCH_2$); HRFAB-MS: found *m*/*z* 189.1604 $(M + H)^+$ (calcd for $C_9H_{20}N_2O_2$ + H: 189.1594).

4,8-Bis(benzyloxycarbonyl)-N^{12}-*t*-butoxycarbonyl-N^1-(2,2,2-trichloroethoxycarbonyl)-4,8-diaza-1,12-dodecanediamine (9). To a solution of compound **6** (1.50 g, 3.41 mmol) in MeOH (100 mL) were added **8** (0.770 g, 4.09 mmol) in MeOH (10 mL) and AcOH (0.584 mL, 10.2 mmol). The reaction mixture was stirred for 1 h at r. t., and then $NaBH_3CN$ (0.430 g, 6.82 mmol) in MeOH (10 mL) was added dropwise at r. t. over a 30-minute period; the reaction mixture was next stirred overnight. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (30 mL × 3), saturated aqueous $NaHCO_3$ (30 mL × 3), and brine (30 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo. To a solution of the oily residue in THF (100 mL) was added TEA (0.387 g, 3.75 mmol) at 0 °C. To the solution was added dropwise ZCl (0.640 g, 3.75 mmol) in THF (10 mL) at 0 °C over a 15-minute period, and the reaction mixture was additionally stirred for 3 h at r. t. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (30 mL × 3), saturated aqueous $NaHCO_3$ (30 mL × 3), and brine (30 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent removed in vacuo. The crude product was purified by silica-gel column chromatography (180 g, 2.0 × 70 cm, benzene:AcOEt = 6:1). The fractions containing the desired product were combined and evaporated in vacuo to give **9** as a colorless oil (1.22 g, 48.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (9H, s, $(CH)_3C$), 1.29–1.88 (8H, m, CH_2/Dada × 4), 2.80–3.40 (12H, m, CON–CH_2 × 6), 4.72 (2H, s, CH_2/Troc), 5.11 (4H, dd, $PhCH_2$ × 2), 7.37 (10H, s, Ph × 2); HRFAB-MS: found *m*/*z* 745.2586 $(M+H)^+$ (calcd for $C_{34}H_{47}Cl_3N_4O_8$ + H: 745.2538).

4,8-Bis(benzyloxycarbonyl)-N^1-[N^α,N^ε-bis(benzyloxycarbonyl)lysyl]-N^{12}-*t*-butoxycarbonyl-4,8-diaza-1,12-dodecanediamine (12). To a solution of compound **9** (1.23 g,1.65 mmol) in AcOH (36 mL) and water (4 mL) was added zinc dust (3.24 g, 49.5 mmol). The suspension was stirred for 4 h at r. t., and then insoluble materials were filtered off. The filtrate was concentrated in vacuo, and the residue was dissolved in diethyl ether (50 mL). The solution was extracted with water (10 mL × 5), and the extract was lyophilized to obtain 4,8-bis(benzyloxycarbonyl)-N^{12}-*t*-butoxycarbonyl-4,8-diaza-1,12-dodecanediamine acetate (**10**) as a colorless powdery substance. To a solution of the thus-obtained **10** in DMF (20 mL) were added TEA (0.184 g, 1.82 mmol) and Z-Lys(Z)-OSu (**11**)[16] (0.930 g, 1.82 mmol). The reaction mixture was stirred for 24 h at r. t. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (30 mL × 3), saturated aqueous $NaHCO_3$ (30 mL × 3), and brine (30 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo. The crude product was purified by silica-gel column chromatography (40g, 3.0 × 40 cm, $CHCl_3$:MeOH = 100:1). The fractions containing the desired product were combined and concentrated in vacuo to give **12** as a colorless oil (1.30 g, 81.8%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (9H, s, $(CH_3)_3C$), 1.19–1.90 (6H, m, β-, γ-, and δ-CH_2/Lys; 8H, m, CH_2/Dada × 4), 2.88–3.49 (14H, m, CON–CH_2 × 6 and ε-CH_2/Lys), 4.13 (1H, m, α-CH/Lys), 5.07 (8H, dd, $PhCH_2$ × 4), 7.32 (20H, s, Ph × 4); HRFAB-MS: found *m*/*z* 967.5144 $(M+H)^+$ (calcd for $C_{53}H_{70}N_6O_{11}$ + H: 967.5151); $[\alpha]_D^{19}$ −2.7° (*c* 1.0, MeOH).

4,8-Bis(benzyloxycarbonyl)-N^1-[N^α,N^ε-bis(benzyloxycarbonyl)lysyl]-N^{12}-[N^α-(*t*-butoxycarbonyl)asparaginyl]-4,8-diaza-1,12-dodecanediamine (15). Compound **12** (1.30 g, 1.35 mmol) was dissolved in CH_2Cl_2 (24 mL) and TFA (6 mL). The solution was stirred for 30 min at r. t. and then concentrated in vacuo. The residue was dissolved in AcOEt (100 mL), and the solution was washed with saturated aqueous $NaHCO_3$ (30 mL × 3) and brine (30 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo to give 4,8-bis(benzyloxycarbonyl)-N^1-[N^α,N^ε-bis (benzyloxycarbonyl) lysyl] - 4,8 - diaza -1,12-dodecanediamine (**13**) as a colorless oil (1.16 g, 99.1%). To a solution of the thus-obtained compound **13** in DMF (30 mL) were added N^α-(*t*-butoxycarbonyl)asparagine *p*-nitrophenyl ester (**14**) (0.522 g, 1.47 mmol) and TEA (0.148 g, 1.47 mmol), and the reaction mixture was stirred for 3 h at r. t. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (20 mL × 3), saturated aqueous $NaHCO_3$ (20 mL × 3), and brine (20 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was concentrated in vacuo. The crude product was purified by silica-gel column chromatography (50 g, 3.0 × 20 cm, $CHCl_3$:MeOH = 20:1). The fractions containing the desired product were combined and concentrated in vacuo to give **15** as a colorless oil (1.32 g, 91.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (9H, s, $(CH_3)_3C$), 1.22–1.90 (6H, m, β-, γ-, and δ-CH_2/Lys; 8H, m, CH_2/Dada × 4), 2.46–2.91 (2H, m, β-CH_2/Asn), 2.92–3.40 (14H, m, CO–N–CH_2 × 6 and ε-CH_2/Lys), 4.10 (1H, m, α-CH/Lys), 4.40 (1H, m, α-CH/Asn), 5.07 (8H, dd, $PhCH_2$ × 4), 7.32 (20H, s, Ph × 4); HRFAB-MS: found *m*/*z* 1081.5625 $(M+H)^+$ (calcd for $C_{57}H_{76}N_8O_{13}$ +H: 1081.5610); $[\alpha]_D^{19}$ −3.3° (*c* 1.0, MeOH).

4,8-Bis(benzyloxycarbonyl)-N^1-[N^α,N^ε-bis(benzyloxycarbonyl)lysyl]-N^{12}-{N^α-[2,4-bis(benzyloxy)phenylacetyl]asparaginyl}-4,8-diaza-1,12-dodecanediamine (18). A solution of compound **15** (1.32 g, 1.22 mmol) in 20% TFA/CH_2Cl_2 (20 mL) was stirred for 30 min at r. t. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with saturated aqueous $NaHCO_3$ (30 mL × 3) and brine (30 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent

was concentrated in vacuo to give 4,8-bis(benzyloxycarbonyl)-N^1-[N^α,N^ε-bis(benzyloxycarbonyl)lysyl]-N^{12}-asparaginyl-4,8-diaza-1,12-dodecanediamine (**16**) as a colorless oil (1.11 g, 92.5%).

To a solution of a part of compound **16** (0.214 g, 0.219 mmol) in DMF (10 mL) were added 2,4-bis(benzyloxy)phenylacetic acid succinimidyl ester[11c] (**17**) (0.107 g, 0.241 mmol) and TEA (24.4 mg, 0.241 mmol), and the reaction mixture was stirred for 3 h at r. t. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (50 mL). The solution was washed with 10% citric acid (10 mL × 3), saturated aqueous $NaHCO_3$ (10 mL × 3), and brine (10 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, and then the solvent was removed in vacuo. The crude product was purified by silica-gel column chromatography (10 g, 1.0 × 20 cm, $CHCl_3$:MeOH = 100:1). The fractions containing desired product were combined and concentrated in vacuo. The residue was triturated with hexane to obtain **18** as colorless crystals, and the thus-obtained crude product was recrystallized from diethyl ether and hexane (0.181 g, 63.3%). Mp 130–134 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.20–1.72 (6H, m, β-, γ-, and δ-CH_2/Lys; 8H, m, CH_2/Dada × 4), 2.39 and 2.44 (each 1H, dd, β-CH_2/Asn), 2.90–3.21 (14H, m, CO–N–CH_2/Dada × 6 and ε-CH_2/Lys), 3.42 (2H, s, PhCH_2/Dhpa), 3.89 (1H, m, α-CH/Lys), 4.51 (1H, m, α-CH/Asn), 4.96–5.06 (8H, PhCH_2/Z × 4), 5.04 and 5.08 (each 2H, s, PhCH_2/Bzl), 6.53 (1H, dd, C^3-Ph /Dhpa), 6.68 (1H, d, C^5-Ph /Dhpa), 7.08 (1H, d, C^6-Ph /Dhpa), and 7.24–7.38 (30H, Ph/Z and Bzl × 6); HRFAB-MS: found *m*/*z* 1311.6325 $(M+H)^+$ (calcd for $C_{74}H_{86}N_8O_{14}$ + H: 1311.6342); $[\alpha]_D^{19}$ −3.5° (*c* 1.0, MeOH).

***N*^{12}-[*N*$^\alpha$-(2,4-Dihydroxyphenylacetyl)asparaginyl]-*N*^{1}-lysyl-4,8-diaza-1,12-dodecanediamine (NPTX-594) (1).** To a solution of compound **18** (50.0 mg, 0.0382 mmol) in MeOH (10 mL) and AcOH (20 mL) was added Pd black (50 mg). The reaction mixture was stirred under an atmosphere of hydrogen for 2 h at r. t. The catalyst was filtered off, and the filtrate was concentrated in vacuo. The crude product was dissolved in water and filtered through Millipore® filter (Millipore Corp., Type: HA, pore size: 0.45 μm); the filtrate was then lyophilized. The crude product was finally purified by preparative RPHPLC (10–40% CH_3CN–0.1% TFA/8.0 mL min^{-1}). The fractions containing the desired compound were combined and lyophilized. The thus-obtained powdery substance was dissolved in 0.1 M HCl (10 mL), and the solution was lyophilized again to obtain **1** as hydrochloride[19] (16.7 mg, 59.0%). ^{1}H NMR (500 MHz, D_2O; auto-reference condition: HDO = 4.75 ppm) δ 1.37 (2H, m, γ-CH_2/Lys), 1.40–1 52 (4H, m, $C^{10}H_2$ and $C^{11}H_2$/Dada), 1.62 (2H, m, δ-CH_2 /Lys), 1.83 (2H, m, β-CH_2/Lys), 1.85 (2H, m, C^2H_2/Dada), 1.97 (2H, m, C^6H_2/Dada), 2.64 and 2.70 (each 1H, dd, β-CH_2 /Asn), 2.88 (2H, m, C^9H_2/Dada), 2.92 (2H, m, ε-CH_2/Lys), 2.96 and 3.03 (each 2H, m, C^5H_2, C^7H_2/Dada), 3.01 (2H, m, C^3H_2/Dada), 3.09 and 3.21 (each 1H, m, $C^{12}H_2$/Dada), 3.21 and 3.31 (each 1H, m, C^1H_2/Dada), 3.45 (2H, m, CH_2/Dhpa), 3.89 (1H, m, α-CH/Lys), 4.49 (1H, m, α-CH/Asn), 6.37 (1H, s, C^3-Ph/Dhpa), 6.38 (1H, d, C^5-Ph/Dhpa), and 7.02 (1H, d, C^6-Ph/Dhpa); FAB-MS: found *m*/*z* 595.3946 $(M+H)^+$ (calcd for $C_{28}H_{50}N_8O_6$ + H: 595.3935); $[\alpha]_D^{19}$ −7.9° (*c* 1.0, H_2O).

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3 NPTX-594 indicates a nephilatoxin whose molecular weight is 594. Details about the isolation and structure determination will be reported soon in Natural Toxins.

4 Abbreviations according to IUPAC-IUB commission, Eur. J. Biochem., 138, 9 (1984), are used. Asn: L-asparagine; Boc: *t*-butoxycarbonyl; Boc_2O: di-*t*-butyl dicarbonate; Bzl: benzyl; DCHA: dicyclohexylamine; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HRFAB-MS: high resolution fast atom bombardment mass spectrometry; HOBt: 1-hydroxybenzotriazole; HOSu: *N*-hydroxysuccinimide; Lys: L-lysine; Ms: methanesulfonyl; Np: *p*-nitrophenyl (or 4-nitrophenyl); RPHPLC: reversed-phase high-performance liquid chromatography; TEA: triethylamine; TFA: trifluoroacetic acid; THF: tetrahydrofuran; Troc: 2,2,2-trichloroethoxycarbonyl; TrocCl: 2,2,2-trichloroethoxycarbonyl chloride; Z: benzyloxycarbonyl; ZCl: benzyloxycarbonyl chloride.

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19 Although elemental analysis was not carried out, we assume that the synthetic compound is obtained as tetrahydrochloride.