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Brief Article

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General Synthesis of β-Alanine-Containing Spider Polyamine Toxins and Discovery of *Nephila* Polyamine Toxins 1 and 8 as Highly Potent Inhibitors of Ionotropic Glutamate Receptors

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Supporting Information

ABSTRACT: Certain spiders contain large pools of polyamine toxins, which are putative pharmacological tools awaiting further discovery. Here we present a general synthesis strategy for this class of toxins and prepare five structurally varied polyamine toxins. Electrophysiological testing at three ionotropic glutamate receptor subtypes reveals that two of these, *Nephila* polyamine toxins 1 (NPTX-1) and 8 (NPTX-8), comprise intriguing pharmacological activities by having subnanomolar IC_{50} values at kainate receptors.

INTRODUCTION

The ionotropic glutamate (iGlu) receptors are ligand-gated ion channels that mediate the majority of excitatory synaptic transmission in the vertebrate brain and are crucial for normal brain function. Dysfunction of iGlu receptors is involved in a range of neurological and psychiatric diseases, and iGlu receptors are considered important drug targets for brain diseases.[1−3] In particular, inhibition of iGlu receptors is a promising strategy for the treatment of a wide range of diseases such as pain, stroke, and Alzheimer's disease.[1,4,5] Today one drug targeting iGlu receptors has been approved, memantine, an open-channel blocker of the *N*-methyl-D-aspartate (NMDA) subtype of iGlu receptors, used in the treatment of Alzheimer's disease.[6,7]

Polyamine toxins are a group of small molecule natural products found in spiders and wasps that like memantine are open-channel blockers of iGlu receptors.[8−10] Polyamine toxins have found valuable use as pharmacological tools to explore the neurophysiological role of iGlu receptors based on their high affinity and selectivity for iGlu receptors[8−10] and their ability to discriminate Ca^{2+} permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate iGlu receptor subtypes.[11] Polyamine toxins from spiders are biosynthesized in a combinatorial manner, providing a cocktail of structurally related compounds (Figure 1), most of which remain to be explored biologically.[10,12,13] Thus, spider polyamine toxins have the general structure as depicted for the prototypical polyamine toxin argiotoxin-636 (ArgTX-636, **1**, Figure 1), composed of an aromatic headgroup, an optional amino acid linkage, a polyamine backbone, and an optional amino acid tail.[10,12,13]

We have recently developed a synthetic methodology for the synthesis of **1** and analogues using solid-phase synthesis.[14] The procedure, however, does not provide direct access to the large group of polyamine toxins, such as Joro spider toxin 3 (JSTX-3, **2**), which contains a β-alanine moiety in the polyamine region. JSTX-3 is among the most studied and employed spider toxins, as it displays potent inhibitory activity particularly at AMPA receptors and in contrast to **1** displays only limited activity at NMDA receptors.[10] However, despite these intriguing biological properties, the challenges of preparing **2** and analogues have prevented systematic structure−activity relationship (SAR) studies to improve pharmacological properties. We therefore decided to explore a revised strategy for the synthesis

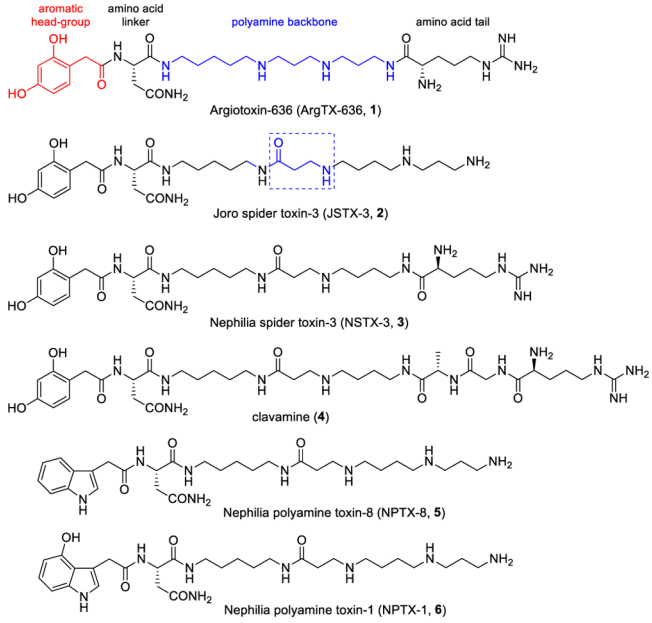

Figure 1. General structures of polyamine toxins found in spiders, illustrated with ArgTX-636 (**1**) and indicating the four variable regions. JSTX-3 (**2**), NSTX-3 (**3**), clavamine (**4**), NPTX-8 (**5**), and NPTX-1 (**6**) are structurally related toxins, having a β-alanine moiety in the polyamine backbone (indicated in JSTX-3) and variations in three of the four regions of the general structure.

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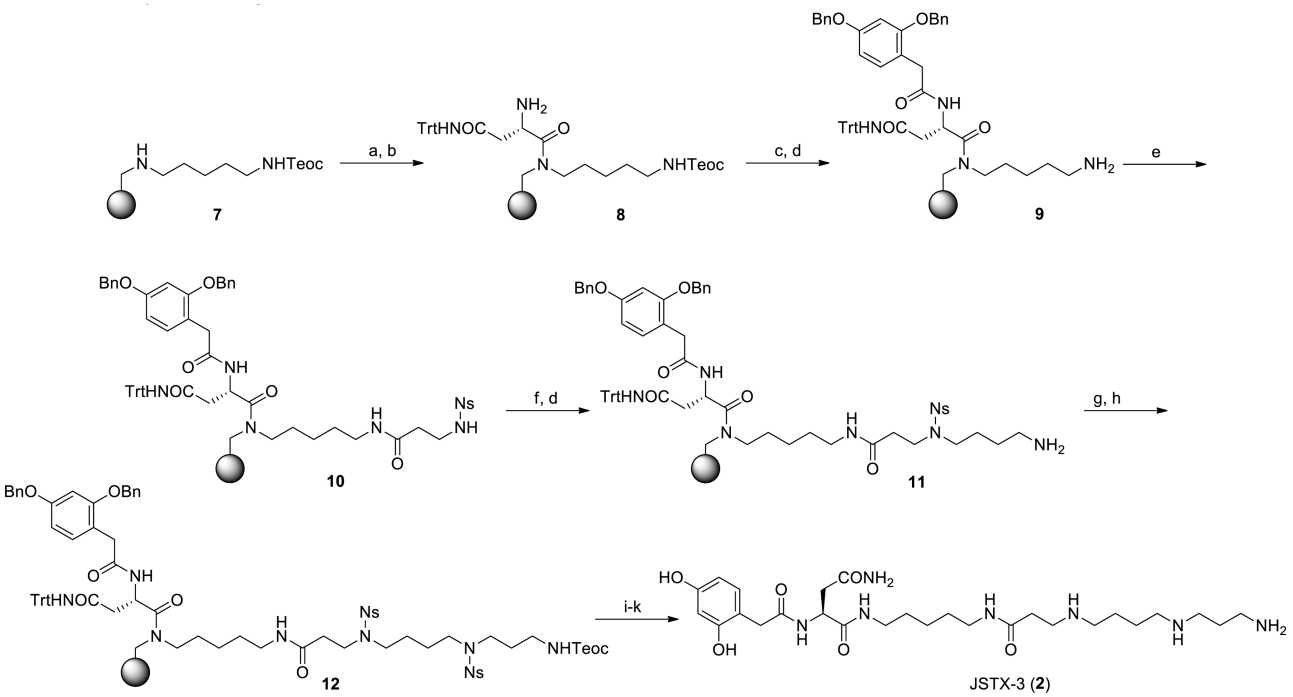
Scheme 1. Synthesis of JSTX-3 (2)[a]



[a]Reagents and conditions: (a) Fmoc-N^{γ}-Trt-L-asparagine, HATU, DIPEA; (b) 20% piperidine in DMF; (c) 2-(2,4-bis(benzyloxy)phenyl)acetic acid, HATU, DIPEA; (d) TBAF, 55 °C; (e) 3-(2-nitrophenylsulfonamido)propanoic acid, HATU, collidine; (f) 2-(trimethylsilyl)ethyl 4-hydroxybutylcarbamate, Bu_3P, ADDP; (g) 2-nitrobenzenesulfonyl chloride, collidine; (h) 2-(trimethylsilyl)ethyl 3-hydroxypropylcarbamate, Bu_3P, ADDP; (i) DBU, 2-mercaptoethanol; (j) TFA/DCM/TIPS/H_2O (75:20:2.5:2.5); (k) H_2, $Pd(OH)_2/C$.

of a range of "β-alanine-containing" polyamine spider toxins (Figure 1).

CHEMISTRY

The methodology developed for the synthesis of **1** was used as a starting point, i.e., anchoring a monoprotected diamine at a backbone amide linker (BAL) **7**. The loading of monoprotected diamine was improved using $NaBH(OAc)_3$ in DMF/AcOH (9:1) instead of $NaCNBH_3$ in DMF/AcOH (99:1). Next the linker amino acid was coupled, where HATU and DIPEA in CH_2Cl_2/DMF (9:1) were used in the coupling of resin-bound secondary amine to the linker amino acid (Asn) to provide **8**, and subsequently the aromatic acyl group and deprotection of the primary amine furnished **9**. Next, we explored the key step for introducing the 3-aminopropanoic acid moiety for the synthesis of **2** using standard coupling reagents, a seemingly straightforward operation. However, the desired product underwent fast elimination and the acrylamide side product was obtained as a major component (see Supporting Information, Scheme S1). We then systematically evaluated bases, solvents, and reaction times, which showed that the combined use of HATU and collidine in DMF for 30 min provided the desired intermediate **10** in >80% purity and good yield. In subsequent steps, the polyamine moiety of JSTX-3 was gradually built up, providing **11** and finally the fully protected and resin-bound version of JSTX-3 (**12**). This compound was cleaved from the resin, with concomitant deprotection of most protecting groups providing dibenzyl JSTX-3. The final solution-phase debenzylation step was improved by using $Pd(OH)_2/C$ (Pearlman's catalyst) and glacial acetic acid instead of Pd/C and MeOH.[14]

The synthesis of **2** was thus achieved using the revised procedures, performing the 13-step synthesis on solid-phase and deprotection of the benzyl groups in solution (Scheme 1). Purification was easily achieved by preparative HPLC, providing **2** in >97% purity and a 3% overall yield, corresponding to 77% per step. We then selected representative polyamine toxins *Nephila* spider toxin 3 (NSTX-3, **3**), clavamine (**4**), *Nephila* polyamine toxin-8 (NPTX-8, **5**), and *Nephila* polyamine toxin-1 (NPTX-1, **6**), which are all isolated from the spider *Nephila clavata* and contain a β-alanine moiety in the polyamine moiety but represent structurally different classes, with variations in three of the four regions of the general polyamine toxin structure (Figure 1). In **3** and **4** the headgroup and linker amino acid are similar to those in **2**, whereas the polyamine is modified, including addition of amino acid tails. On the other hand, **5** and **6** contain the same polyamine moiety as **2** but have different headgroups. Some of these toxins have been synthesized before by solution phase methods,[15−20] but except for **2**, none of them have previously been biologically evaluated. The synthesis of these four polyamine toxins was readily achieved by the general synthetic methodology, using appropriate building blocks, providing the target compounds **3**−**6** in high purity and good to excellent overall yield (see Supporting Information).

RESULTS AND DISCUSSION

The potency of the five toxins **2**−**6** to block representative members of the major iGlu receptor subfamilies from rat was evaluated using two-electrode voltage-clamp electrophysiology on *Xenopus oocytes* expressing the NMDA receptor subtype GluN1/2A, the AMPA receptor subtype GluA1, and the kainate receptor subtype GluK1. The concentration−inhibition rela-

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Table 1. Inhibitory Potency of 2−6 at Recombinant iGlu Receptors

compd	IC_{50} (nM)[a]		
	GluN1/2A	GluA1	GluK1
ArgTX-636 (**1**)	177 [153−205][b]	135 [121−151][b]	NT[c]
JSTX-3 (**2**)	1710 [1287−2271]	65 [63−68]	19 [19−20]
NSTX-3 (**3**)	5406 [5275−5540]	137 [129−145]	164 [157−171]
clavamine (**4**)	>10000	1078 [959−1211]	2575 [2500−2653]
NPTX-8 (**5**)	150 [144−154]	27 [23−32]	0.82 [0.67−0.99]
NPTX-1 (**6**)	1390 [1210−1610]	51 [45−57]	0.33 [0.30−0.36]

[a]Mean IC_{50} values (95% confidence interval in brackets) determined from 6 to 12 experiments at a membrane potential of −60 mV. [b]Data from Nelson et al.[14] [c]NT: not tested.

tionship was determined at these receptor subtypes at a membrane potential of −60 mV (Table 1) and −80 mV (Figure 2). Initially, we verified that **2** is a very potent AMPA

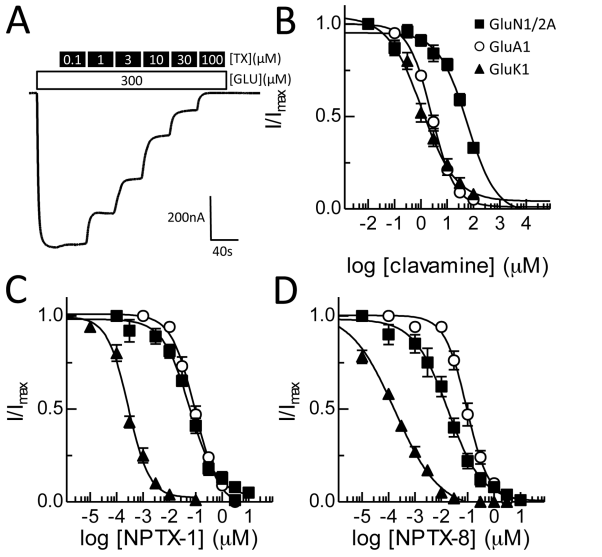

Figure 2. Inhibitory potency of toxins at recombinant iGlu receptor subtypes. (A) Representative two-electrode voltage-clamp current recording illustrating the standard testing protocol. Oocytes expressing iGlu receptor were exposed to maximally effective concentrations of agonist, followed by increasing concentrations of the test toxin plus agonist. (B−D) Composite concentration−response curves for clavamine (**4**) (B), NPTX-1 (**6**) (C), and NPTX-8 (**5**) (D) at GluN1/2A (■), GluA1 (○), and GluK1 (▲) receptors at membrane potentials of −80 mV. Error bars are SEM and are shown when larger than symbol size.

receptor antagonist with an IC_{50} of 65 nM (Table 1).[21,22] However, when tested at GluK1 containing receptors, **2** was even more potent with an IC_{50} of 19 nM, while potency at NMDA receptors was significantly less (IC_{50} = 1.7 μM, Table 1). In contrast, **4** showed drastically reduced affinity for all tested subtypes (IC_{50} > 1 μM; Table 1). **3**, on the other hand, showed reasonable potency at AMPA and kainate receptors with IC_{50} values of 137 and 164 nM, respectively, but only very modest activity at NMDA receptors (IC_{50} = 5.4 μM).

The most remarkable results came from studying the effect of **5** and **6** at AMPA and in particular at kainate receptors. Both compounds were quite potent inhibitors of AMPA receptors, with IC_{50} values of 27 and 51 nM for **5** and **6**, respectively, thus being equally or slightly more potent than **2** (Table 1). However, at kainate receptors, **5** and **6** demonstrated unprecedented potency and had IC_{50} values in the picomolar range with 0.82 and 0.33 nM for **5** and **6**, respectively. We explored the voltage dependency of inhibitory activity of **5** and **6** by determining IC_{50} at membrane potential of −80 mV along with **2** (see Supporting Information, Table S1). A hallmark of polyamine channel blockers is that they show increased potency at lower membrane potential, and according to this, we generally observed increases in potency at all receptor subtypes when lowering the membrane potential from −60 to −80 mV. **5** and **6** showed IC_{50} values of 0.28 and 0.19 nM, respectively, at GluK1 receptors, with similar relative increase in potency at NMDA and AMPA receptors. To the best of our knowledge these toxins are thereby the most potent polyamine toxins at a kainate receptor described to date and among the most potent iGlu receptor ligands known. In addition, **6** shows a remarkable selectivity for GluK1 receptors, with >40000-fold preference for kainate over the GluN1/2A type NMDA receptors and >150-fold preference over the GluA1 type AMPA receptors. The GluK1 construct used here is a double cysteine mutant (GluK1-Y506C-L768C) that prevents the receptor from desensitization and allows robust measurement of GluK1 activity in oocytes.[23] Thus, further studies on native kainate receptors are needed to fully explore their true pharmacological potential.

Structurally, **5** and **6** are characterized by having a 2-(4-hydroxy-1*H*-indol-3-yl)acetic acid or 2-(1*H*-indol-3-yl)acetic acid headgroup, whereas **2** has a 2,4-dihydroxyphenylacetic acid headgroup, while their amino acid linker and polyamine moiety are identical. Thus, it is tempting to speculate that the indole headgroup plays a key role for the striking potency at GluK1 receptors. In general, the pharmacology of open ion channel blockers of kainate receptors is much less explored than for AMPA and NMDA receptors, where numerous compounds have been identified and subsequent SAR studies performed.[1] In one case, however, derivatives of the wasp polyamine toxin philanthotoxin-433 (PhTX-433) were demonstrated to be potent inhibitors of homomeric GluK1.[24] Thus, **5** and **6** are promising starting points for studying this underexplored area of kainate receptor pharmacology and are also obvious candidates for future SAR studies.

■ CONCLUSION

We have developed a general and efficient method for the synthesis of β-alanine-containing spider polyamine toxins. The versatility was demonstrated by the synthesis of five representative toxins, and biological evaluation of these toxins led to the discovery of **5** and **6** as exceptionally potent kainate receptor antagonists, and **6** with a noticeable selectivity relative to NMDA and AMPA receptors. Considering the importance of the prototypical polyamine toxins as pharmacological tools, we consider **5** and **6** as highly promising tools for such studies and as attractive templates for future SAR studies.

EXPERIMENTAL SECTION

General Synthesis Procedure. See Supporting Information.

(*S*)-*N*^{1}-(5-(3-(4-(3-Aminopropylamino)butylamino)-propanamido)pentyl)-2-(2-(2,4-dihydroxyphenyl)acetamido)-succinamide Tris(2,2,2-trifluoroacetate) (JSTX-3, 2).[18] Yield: 3.7 mg (2.6%). ^{1}H NMR (300 MHz, CD_3OD): δ 6.95 (d, J = 8.4 Hz, 1H), 6.34 (d, J = 2.4 Hz, 1H), 6.28 (dd, J = 8.1, 2.4 Hz, 1H), 4.66 (t, J = 6.4 Hz, 1H), 3.53 (d, J = 15.0 Hz, 1H), 3.41 (d, J = 14.7 Hz, 1H), 3.29–3.17 (m, 4H), 3.17 −2.95 (m, 10H), 2.69 (d, J = 6.3 Hz, 2H), 2.66–2.50 (m, 2H), 2.17–1.99 (m, 2H), 1.88–1.67 (m, 4H), 1.58–1.34 (m, 4H), 1.34–1.16 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 175.2, 174.9, 173.2, 171.9, 159.1, 157.3, 132.9, 114.3, 108.1, 103.9, 52.1, 48.4, 48.0, 46.0, 45.1, 40.3, 40.1, 38.8, 38.0, 37.7, 31.8, 29.9, 25.6, 24.9, 24.5, 24.3. HRMS (EI) exact mass calcd for $C_{27}H_{48}N_7O_6$ [MH$^+$] 566.3666; found 566.3651. Purity (ELSD): 97%.

(*S*)-*N*^{1}-((*R*)-1,5-Diamino-1-imino-6,15-dioxo-2,7,12,16-tetraazahenicosan-21-yl)-2-(2-(2,4-dihydroxyphenyl)acetamido)-succinamide Tris(2,2,2-trifluoroacetate) (NSTX-3, 3).[16] **3** was prepared in similar manner to JSTX-3. Instead of steps g and h, acylation with Boc-L-Arg(Pbf)-OH is performed, similar to step b. Yield: 9.1 mg (5.4%). ^{1}H NMR (300 MHz, CD_3OD) δ 6.93 (d, J = 8.0 Hz, 1H), 6.33 (d, J = 2.5 Hz, 1H), 6.26 (dd, J = 8.0, 2.5 Hz, 1H), 4.64 (t, J = 6.3 Hz, 1H), 3.86 (t, J = 6.3 Hz, 1H), 3.51 (d, J = 15.1 Hz, 1H), 3.41 (d, J = 15.1 Hz, 1H), 3.26–3.00 (m, 12H), 2.68 (d, J = 6.1 Hz, 2H), 2.61 (t, J = 6.3 Hz, 2H), 1.92–1.85 (m, 2H), 1.75–1.59 (m, 6H), 1.49–1.40 (m, 4H), 1.34–1.20 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 175.2, 175.0, 173.1, 171.9, 170.0, 159.1, 158.7, 157.3, 132.9, 114.3, 108.1, 103.9, 54.3, 52.1, 45.0, 41.9, 40.28, 40.25, 39.9, 38.9, 37.6, 31.8, 29.95, 29.88, 29.83, 27.4, 25.7, 24.9, 24.5. HRMS (EI) exact mass calcd for $C_{30}H_{53}N_{10}O_7$ [MH$^+$] 665.4099; found 665.4100. Purity (ELSD): >99%.

(*S*)-*N*^{1}-((6*S*,12*S*)-1,6-Diamino-1-imino-12-methyl-7,10,13,22-tetraoxo-2,8,11,14,19,23-hexaazaoctacosan-28-yl)-2-(2-(2,4-dihydroxyphenyl)acetamido)succinamide Tris(2,2,2-trifluoroacetate) (Clavamine, 4).[15] **4** was prepared in similar manner to JSTX-3. Instead of steps g and h, the following was performed first, acylation with Fmoc-L-Ala-OH, like step b, then step c, and acylation with Fmoc-L-Gly-OH like step b, then step c, and finally acylation with Boc-L-Arg(Pbf)-OH like step b. Yield: 21.6 mg (11.8%). ^{1}H NMR (300 MHz, CD_3OD) δ 6.92 (d, J = 8.3 Hz, 1H), 6.32 (d, J = 2.5 Hz, 1H), 6.25 (dd, J = 8.2, 2.1 Hz, 1H), 4.63 (t, J = 6.2 Hz, 1H), 4.29–4.19 (m, 1H), 4.04–3.88 (m, 3H), 3.49 (d, J = 15.0 Hz, 1H), 3.40 (d, J = 15.0 Hz, 1H), 3.26–3.16 (m, 7H), 3.14–2.95 (m, 5H), 2.67 (d, J = 6.1 Hz, 2H), 2.60 (t, J = 5.7 Hz, 2H), 2.00–1.85 (m, 2 H), 1.75–1.38 (m, 11H), 1.34 (d, J = 7.3 Hz, 3H), 1.25 (q, J = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 175.2 (2C), 175.0, 173.1, 171.9, 170.9, 170.6, 159.0, 158.7, 157.3, 132.9, 114.3, 108.1, 103.9, 54.2, 52.1, 51.1, 45.1, 43.3, 41.9, 40.3 (2C), 39.5, 38.8, 37.6, 32.0, 29.9, 29.9, 29.8, 29.7, 27.5, 25.3, 24.9, 24.4, 18.3. HRMS (EI) exact mass calcd for $C_{36}H_{61}N_{12}O_9$ [MH$^+$] 793.4685; found 793.4682. Purity (ELSD): >99%.

(*S*)-2-(2-(1*H*-Indol-3-yl)acetamido)-*N*^{1}-(5-(3-(4-(3-aminopropylamino)butylamino)propanamido)pentyl)-succinamide Tetrakis(2,2,2-trifluoroacetate) (NPTX-8, 5).[17] **5** was prepared in similar manner to JSTX-3 using 2-(1*H*-indol-3-yl)acetic acid in step c and without the final debenzylation step. Yield: 5.7 mg (3.3%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.54 (d, J = 7.7 Hz, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.21 (s, 1H), 7.12 (t, J = 8.0 Hz, 1H), 7.04 (t, J = 7.7 Hz, 1H), 4.70 (t, J = 7.2 Hz, 1H), 3.75 (s, 2H), 3.25–2.96 (m, 12H), 2.94–2.81 (m, 2H), 2.71–2.63 (m, 2H), 2.55 (t, J = 6.6 Hz, 2H), 2.12–1.97 (m, 2H), 1.76–1.63 (m, 4H), 1.50–1.31 (m, 4H), 1.29–1.15 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 174.9, 174.8, 173.1, 171.9, 138.1, 128.5, 125.2, 122.8, 120.2, 119.5, 112.6, 109.4, 52.1, 46.0, 45.0, 40.3, 40.1, 38.0, 33.9, 31.7, 30.0, 29.8, 25.6, 24.9, 24.4, 24.2. HRMS (EI) exact mass calcd for $C_{29}H_{49}N_8O_4$ [MH$^+$] 573.3877; found 573.3871. Purity (ELSD): 98%.

(*S*)-*N*^{1}-(5-(3-(4-(3-Aminopropylamino)butylamino)-propanamido)pentyl)-2-(2-(4-hydroxy-1*H*-indol-3-yl)-acetamido)succinamide Tetrakis(2,2,2-trifluoroacetate) (NPTX-1, 6). **6** was prepared in similar manner to JSTX-3 using 2-(4-(benzyloxy)-1*H*-indol-3-yl)acetic acid in step c. Yield: 2.3 mg (1.3%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.32 (d, J = 8.1 Hz, 1H), 7.56 (t, J = 5.8 Hz, 1H), 7.04 (s, 1H), 6.96–6.87 (m, 2H), 6.41 (dd, J = 1.6, 6.7 Hz, 1H), 4.66 (t, J = 6.3 Hz, 1H), 3.28–2.96 (m, 14H), 2.88–2.62 (m, 4H), 2.56–2.48 (t, J = 5.5 Hz, 2H), 2.12–2.01 (m, 2H), 1.83–1.70 (m, 4H), 1.55–1.24 (m, 6H), 1.10 (q, J = 7.8 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 176.8, 173.9, 173.6, 172.0, 170.8, 150.0, 143.9, 124.0, 121.6, 120.1, 110.2, 105.9, 102.1, 51.0, 44.6, 44.0, 39.2, 39.0, 37.4, 36.9, 32.7, 30.4, 28.6, 28.5, 24.3, 23.8, 23.3, 23.0. HRMS (EI) exact mass calcd for $C_{29}H_{49}N_8O_5$ [MH$^+$] 589.3826; found 589.3837. Purity (ELSD): 99%.

ASSOCIATED CONTENT

Supporting Information

Information on chemistry and synthesis of 2-(4-(benzyloxy)-1*H*-indol-3-yl)acetic acid, experimental procedures for biology, Scheme S1, and a table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ADDP, 1,1-(azodicarbonyl)dipiperidine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DIPEA, diisopropylethylamine; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; JSTX, Joro spider toxin; NMDA, *N*-methyl-D-aspartate; NPTX, *Nephila* polyamine toxin; Ns, 2-nitrobenzenesulfonamide; NSTX, *Nephila* spider toxin; Pbf, pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl; TBAF, tetra-*n*-butylammonium fluoride; Teoc, 2-(trimethylsilyl)ethyloxycarbonyl

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