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NOTE

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Solid-Phase Synthesis and Biological Evaluation of Joro Spider Toxin-4 from *Nephila clavata*

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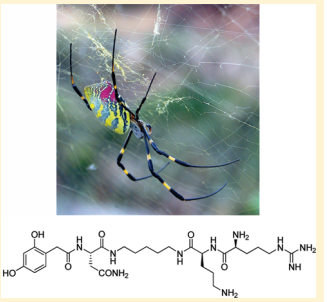
Supporting Information

ABSTRACT: Polyamine toxins from orb weaver spiders are attractive pharmacological tools particularly for studies of ionotropic glutamate (iGlu) receptors in the brain. These polyamine toxins are biosynthesized in a combinatorial manner, providing a plethora of related, but structurally complex toxins to be exploited in biological studies. Here, we have used solid-phase synthetic methodology for the efficient synthesis of Joro spider toxin-4 (JSTX-4) (**1**) from *Nephila clavata*, providing sufficient amounts of the toxin for biological evaluation at iGlu receptor subtypes using electrophysiology. Biological evaluation revealed that JSTX-4 inhibits iGlu receptors only in high μM concentrations, thereby being substantially less potent than structurally related polyamine toxins.



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. Dysfunctional iGlu receptor signaling is involved in a range of neurological and psychiatric diseases, and iGlu receptors are considered important drug targets for the treatment of brain diseases.[1] In particular, inhibition of iGlu receptors is a promising therapeutic strategy in neurodegenerative diseases such as stroke and Alzheimer's disease.[1] Very few drugs targeting iGlu receptors have been approved, among them memantine, an open-channel blocker of the *N*-methyl-D-aspartate (NMDA) subtype of iGlu receptors, used in the treatment of Alzheimer's disease.[2,3]

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.[4−6] Polyamine toxins have found valuable use as neuropharmacological tools based on their high affinity and selectivity for iGlu receptors,[4,5] in particular because of their ability to discriminate Ca^{2+} permeable from impermeable α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate receptor subtypes.[7] To date most structure−activity relationship (SAR) studies of polyamine toxins have used philanthotoxin-433 (PhTX-433, Figure 1), a wasp toxin identified by Nakanishi and co-workers,[8] as a template for synthetic analogues. In contrast, the structurally more complex polyamine toxins from spiders have generally been used in their native forms only. Polyamine toxins from spiders are biosynthesized in a combinatorial manner, providing a cocktail of structurally related compounds, the majority of which remain to be explored biologically.[9,10] The spider polyamine toxins have the general structure as depicted for the prototypical polyamine toxin argiotoxin-636 (ArgTX-636, Figure 1), composed of an aromatic headgroup, an optional amino acid linkage, a polyamine backbone, and an optional amino acid tail.[10]

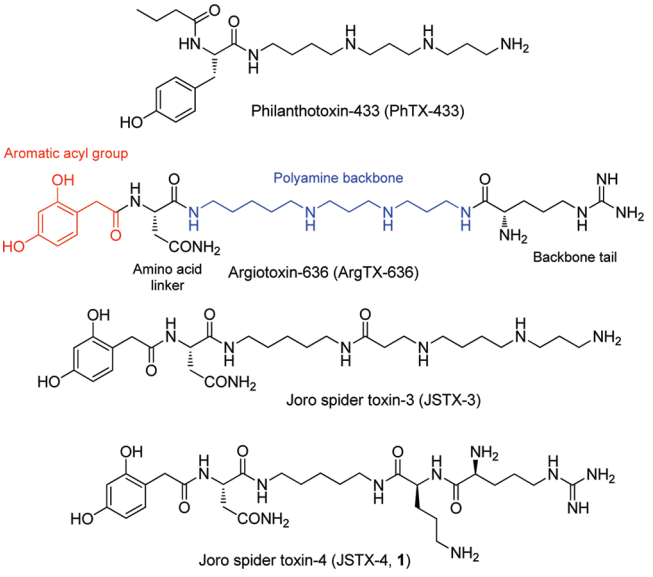


Figure 1. General structure of polyamine toxins found in wasps and spiders, illustrated with PhTX-433 and ArgTX-636 where the four variable regions are indicated. JSTX-3 and JSTX-4 (**1**) are structurally related toxins to ArgTX-636.

We recently developed a synthetic methodology for the preparation of ArgTX-636 and analogues using solid-phase synthesis.[11] However, the overall yield for the synthesis of ArgTX-636 was only around 2%, thus not rendering the method applicable for synthesis

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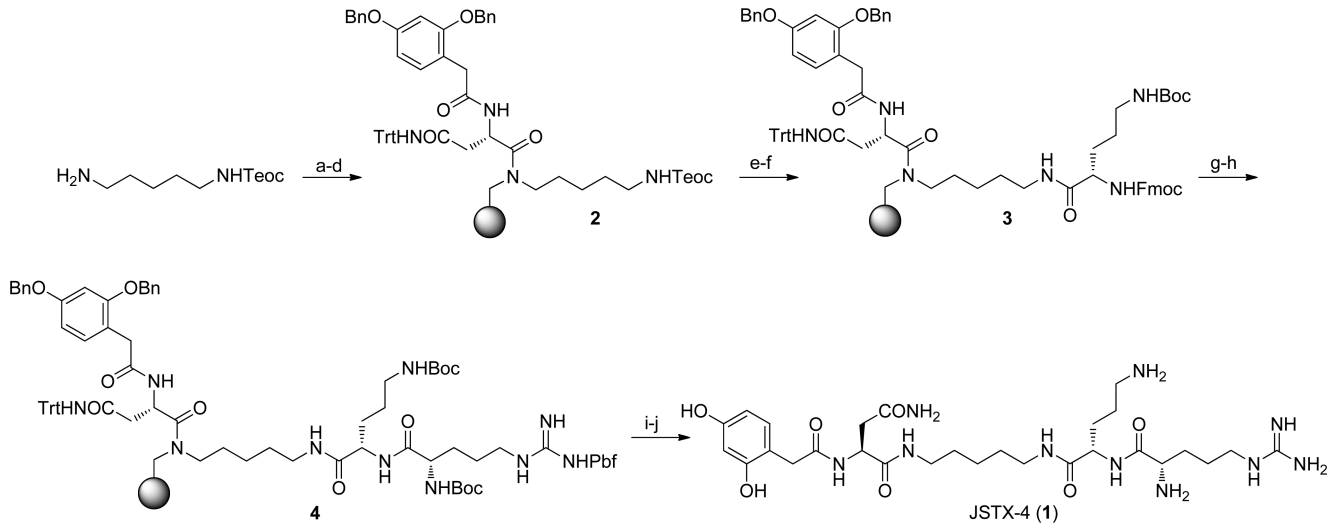
Scheme 1. Solid-Phase Synthesis of JSTX-4 (1)[a]



[a] Reagents and conditions: (a) (*i*) DFPE resin, DMF/acetic acid (9:1), (*ii*) $NaBH(OAc)_3$; (b) Fmoc-L-Asn(Trt)-OH, HATU, DIPEA, CH_2Cl_2/DMF (9:1); (c) 20% piperidine in DMF; (d) 2-(2,4-bis(benzyloxy)phenyl)acetic acid, HATU, DIPEA, CH_2Cl_2/DMF (9:1); (e) TBAF, THF, 55 °C; (f) Fmoc-L-Orn(Boc)-OH, HATU, DIPEA, CH_2Cl_2/DMF (9:1); (g) 20% piperidine in DMF; (h) Boc-L-Arg(Pbf)-OH, HATU, DIPEA, CH_2Cl_2/DMF (9:1); (i) TFA/CH_2Cl_2/TIPS/H_2O (75:20:2.5:2.5); (j) $Pd(OH)_2$/C, H_2, HOAc.

of larger amounts of toxins required for advanced biological studies or for generation of libraries of polyamine toxins in general. Thus, we wanted to apply the synthetic methodology to a related toxin, which could be prepared in a significantly higher overall yield and subsequently be used in chemical and biological studies. Initially, we explored the synthesis of JSTX-3 (Figure 1), which is also a prototypical polyamine toxin with potent inhibitory activity at iGlu receptors.[12,13] However the synthesis of JSTX-3 was even more challenging than for ArgTX-636, in particular introduction of the 3-aminopropanoic acid moiety (data not shown). Instead, we took a different approach and became inspired by the wealth of polyamine toxins isolated from the Joro spider *Nephila clavata*[10] and in particular JSTX-4 (**1**, Figure 1),[14] which can be considered a hybrid between JSTX-3 and ArgTX-636. Moreover, JSTX-4 had been neither synthesized nor biologically characterized before, and we envisioned that the synthesis of JSTX-4 could be efficiently achieved using our previously developed protocol.[11]

The synthetic strategy for obtaining JSTX-4 was related to that of ArgTX-636: Starting from a backbone amide linker (BAL) resin, 2-(trimethylsilyl)ethyl 5-aminopentylcarbamate was attached by reductive amination, and the aromatic headgroup was generated by subsequent couplings of protected asparagine and 2-(2,4-bis-(benzyloxy)phenyl)acetic acid[15] to furnish **2** (Scheme 1). Next, the *N*-Teoc protection group was selectively cleaved by treatment with tetrabutylammonium fluoride (TBAF), and appropriately protected ornithine was coupled to the free, primary amino group to give **3**. This was followed by Fmoc removal and coupling of protected arginine to the primary amine, giving fully protected, resin-bound JSTX-4 (**4**, Scheme 1). The target compound was obtained by treating resin **4** with trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water and subsequent deprotection of the aromatic *O*-benzyl groups with $Pd(OH)_2$/C and H_2 in solution. Gratifyingly the 10-step synthetic sequence and purification by preparative HPLC provided JSTX-4 (**1**) in >98% purity as determined by LC-MS and an overall yield of 39%, equaling >90% yield per step.

The potency of JSTX-4 to block NMDA- and AMPA-type iGlu receptors was evaluated using two-electrode voltage-clamp electrophysiology on *Xenopus* oocytes expressing the NMDA receptor subtype GluN1/2A and the AMPA receptor subtype GluA1. We tested the ability of increasing concentrations of JSTX-4 to inhibit agonist-evoked receptor currents at a membrane potential of −60 mV, which is close to the typical neuronal resting potential. Generally, we observed that high μM concentrations of JSTX-4 were needed to produce a weak inhibitory effect (Figure 2). The inhibitory effect of JSTX-4 at the two iGlu receptor subtypes was generally similar, and a 100 μM concentration of JSTX-4 produced approximately 25% inhibition at both receptor subtypes; however IC_{50} values could not be determined for the concentration range used. The low inhibitory potency of JSTX-4 at the GluN1/2A and GluA1 subtypes was in contrast to ArgTX-636, which has IC_{50} values of 74 and 77 nM at these subtypes, respectively, when tested under similar conditions;[11] thus clearly demonstrating a dramatic difference in the biological activity of ArgTX-636 and JSTX-4. The primary structural differences are the overall length of the molecule and the number of secondary amino groups; ArgTX-636 is a longer molecule and may therefore bind deeper in the ion channel region of iGlu receptors, as required for biological activity.[11] Moreover, in a previous study we demonstrated the importance of the secondary amino groups in ArgTX-636 for selectivity among AMPA and NMDA receptors, which could largely be explained by changes in their three-dimensional structure due to different internal hydrogen-bonding patterns.[11] We suggest that the surprisingly weak inhibitory activity of JSTX-4 at iGlu receptors is caused by the absence of secondary amino groups combined with a shorter distance between the aromatic headgroup and the terminal guanidinium groups.

In conclusion, we have developed a highly efficient solid-phase synthesis of JSTX-4, where the 10-step sequence provides the compound in a yield of 39%, hence being substantially more efficient than the previously reported synthesis for ArgTX-636. Biological evaluation of JSTX-4 revealed that the compound inhibits only iGlu receptors in high μM concentrations, thus being substantially less potent than structurally related polyamine toxins such as ArgTX-636.

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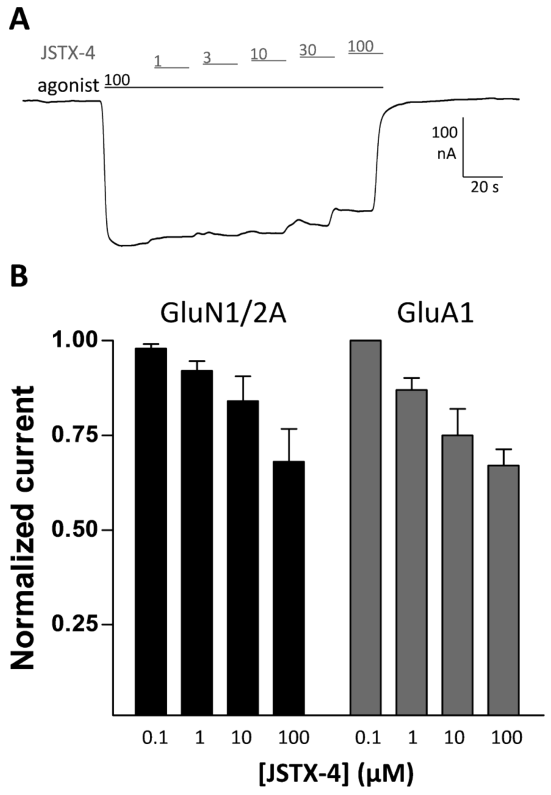

Figure 2. Biological evaluation of JSTX-4. (A) Representative current trace obtained from electrophysiological recording of the membrane current from a *Xenopus* oocyte expressing GluA1 receptors held at a membrane potential of −60 mV. The agonist-evoked response (Glu; 100 μM) is observed as a downward deflection of the membrane current. Co-application of increasing concentrations of JSTX-4 is indicated by gray bars. (B) Summary of the effect of various concentrations of JSTX at GluN1/2A (black) and GluA1 (gray) receptors. Data represent mean (±SEM) from experiments at 9 to 12 oocytes normalized to the agonist-evoked current response in the absence of JSTX-4.

EXPERIMENTAL SECTION

General Experimental Procedures. Unless otherwise stated, starting materials were obtained from commercial suppliers and were used without further purification. The BAL-resin used was 2-(3,5-dimethoxy-4-formylphenoxy)ethyl (DFPE) polystyrene with a loading of 0.5−1.0 mmol/g and was purchased from Novabiochem. 2-(2,4-Bis(benzyloxy)phenyl)acetic acid was prepared according to a published procedure,[15] and 2-(trimethylsilyl)ethyl 5-aminopentylcarbamate was prepared following procedures for related aliphatic diamines.[16] Tetrahydrofuran (THF) was distilled under N_2 from sodium/benzophenone immediately before use. *N*,*N*-Dimethylformamide (DMF) and dichloromethane were dried using AldraAmine trapping packets.

LC-MS analysis was performed on an Agilent 6410 Triple Quadrupole mass spectrometer with electron spray coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with autosampler and diode-array detector using a linear gradient of the binary solvent system of water/acetonitrile/formic acid (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 mL/min. During ESI-LC/MS analysis evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 light scattering detector, which were used for estimation of the purity of the final products. Preparative HPLC was performed on a Agilent 1100 system using a C18 reversed-phase column (Zorbax 300 SB-C18, 21.2 × 250 mm) with a linear gradient of the binary solvent system of water/acetonitrile/TFA (A: 95/5/0.086 and B: 5/95/0.086) with a flow rate of 20 mL/min and UV detection at 214 and 254 nm. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance AV-300 spectrometer. Solid-phase synthesis was carried out using Bohdan MiniBlock (Mettler-Toledo). Unless otherwise stated, reactions were carried out at room temperature, all washings were performed with 3 × 4 mL of solvent, and resins were dried in vacuo after washings.

2-(Trimethylsilyl)ethyl 5-aminopentylcarbamate:. yield 77%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.64 (bs, 1H), 4.16 (t, J = 8.3 Hz, 2H), 3.19 (q, J = 6.3 Hz, 2H), 2.71 (t, J = 6.6 Hz, 2H), 1.33−1.60 (m, 6H), 1.28 (bs, 2H), 1.00 (t, J = 8.3 Hz, 2H), 0.06 (s, 9H); EIMS m/z 247.1 $[M + H]^+$; purity (ELSD), 91%.

(*S*)-*N*^{1}-(5-((*S*)-5-Amino-2-((*S*)-2-amino-5-guanidinopentanamido)pentanamido)pentyl)-2-(2-(2,4-dihydroxyphenyl)acetamido)succinamide (JSTX-4, 1). DFPE resin (0.15 mmol) was swelled in DMF (4 mL) for 1 h. The resin was drained and treated with a solution of 2-(trimethylsilyl)ethyl-5-aminopentylcarbamate (1.50 mmol) in dry DMF/acetic acid (9:1, 4 mL), and after 2 min $NaBH(OAc)_3$ (1.50 mmol) was added. The mixture was agitated overnight, the solvents were drained, and the resin was washed successively with DMF, DIPEA (10% in DMF), DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2. A solution of Fmoc-L-Asn(Trt)-OH (0.75 mmol) and *N*-[(dimethylamino)-1*H*-1,2,3-triazol[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU, 0.75 mmol) in dry CH_2Cl_2/DMF (9:1, 4 mL) was added to the resin followed by addition of *N*,*N*-diisopropylethylamine (DIPEA, 1.50 mmol). The mixture was agitated overnight, and the resin was subsequently washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2. The resulting resin was treated with 20% piperidine in dry DMF (v/v, 4 mL) for 2 and 20 min and washed with DMF in between. The resin was washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2, drained, and treated with a solution of 2-(2,4-bis(benzyloxy)phenyl)acetic acid (0.75 mmol) and HATU (0.75 mmol) in dry CH_2Cl_2/DMF (9:1, 4 mL), followed by treatment with DIPEA (1.50 mmol). The mixture was agitated for 2 h and washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2 and subsequently suspended in THF (4 mL) for 30 min. Then it was heated to 55 °C before a solution of TBAF (1 M in THF, 0.60 mmol) was added slowly, and the mixture was agitated at 55 °C for 30 min. The resin was drained, washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2, and treated with a solution of Fmoc-L-Orn(Boc)-OH (0.75 mmol) and HATU (0.75 mmol) in dry CH_2Cl_2/DMF (9:1, 4 mL), followed by DIPEA (1.50 mmol). The mixture was agitated for 2 h, washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2, then treated with 20% piperidine in dry DMF (v/v, 4 mL) for 2 and 20 min, and washed with DMF in between. The resin was then washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2, treated with a solution of Boc-L-Arg(Pbf)-OH (0.75 mmol) and HATU (0.75 mmol) in dry CH_2Cl_2/DMF (9:1, 4 mL), followed by DIPEA (1.50 mmol), agitated for 2 h, and washed with DMF, CH_2Cl_2, CH_3OH, and CH_2Cl_2. Finally, the resin was treated with a mixture of TFA/CH_2Cl_2/TIPS/H_2O (75:20:2.5:2.5 v/v, 4 mL) for 2 h, drained, and washed with CH_3OH (2 mL) and CH_2Cl_2 (2 mL). The combined solvents were evaporated to give a sticky yellow solid (2,4-Di-*O*-Bn-JSTX-4), which was purified by preparative HPLC. The purified 2,4-Di-*O*-Bn-JSTX-4 was dissolved in glacial acetic acid (4 mL) and transferred to a screw cap vial, and $Pd(OH)_2$/C (10% w/w) was added. Hydrogen was bubbled through the solution for 1 min, and the mixture was stirred under an atmosphere of hydrogen until the reaction was completed. The reaction mixture was filtered and washed with CH_3OH, and water (10 mL) was added. The combined washings were removed by freeze-drying to give a colorless solid, which was purified by preparative HPLC to give the product as a colorless solid (56.9 mg, 39%): ^{1}H NMR (300 MHz, CD_3OD) δ 6.89 (d, J = 8.3 Hz, 1H), 6.31 (d, J = 2.2 Hz, 1H), 6.23 (dd, J = 8.0, 2.2, Hz, 1H), 4.64 (t, J = 6.3 Hz, 1H), 4.33 (t, J = 6.3 Hz, 1H), 3.95 (t, J = 6.1 Hz, 1H), 3.46 (d, J = 14.9 Hz, 1H), 3.38 (d, J = 15.1 Hz, 1H), 3.01−3.21 (m, 6H), 2.85−2.98 (m, 2H), 2.66 (d, J = 6.3 Hz, 2H), 1.83−1.96 (m, 2H), 1.51−1.83 (m, 6H), 1.33−1.51 (m, 4H), 1.15−1.33 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.2, 175.0, 173.2, 173.1, 169.9, 159.0, 158.6, 157.3,

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132.8, 114.0, 108.1, 103.9, 54.5, 53.9, 52.0, 42.0, 40.3, 40.4, 38.8, 37.6, 30.2, 29.9, 29.8, 29.8, 25.2, 24.9; EIMS m/z 637.3 $[M + H]^+$; purity (ELSD), 98.6%; HRMS m/z 637.3766 (calcd for $C_{28}H_{49}N_{10}O_7$, 637.3786).

Biology. In Vitro cRNA Transcription. The cDNA encoding rat $GluA1_i$ or GluN1/2A subunits was inserted into the vector pGEM-HE or pCI-IRES-BLAS, respectively, for preparation of high-expression cRNA transcripts. Plasmids were grown in Top10 *E. coli* bacteria (Invitrogen, Carlsbad, CA) and isolated by using column purification (Qiagen, La Jolla, CA). The cRNA was synthesized from the above cDNAs by in vitro transcription using the mMESSAGE mMACHINE T7 mRNA-capping kit (Ambion, Austin, TX) according to the protocol supplied by the manufacturer.

Oocyte Electrophysiology. Mature female *Xenopus laevis* (Nasco, Modesto, CA) were anaesthetized using 0.1% ethyl 3-aminobenzoate, and their ovaries were surgically removed. The ovarian tissue was dissected and treated with Collagenase IV (Worthington, Lakewood, NJ) in Ca^{2+}-free Barth's medium (2 mg/mL) for 1−2 h at room temperature. On the second day, oocytes were injected with 25 nL of cRNA (1 ng/nL $GluA1_i$ or 0.05 ng/nL GluN1 and GluN2A) and incubated at 17 °C in Barth's medium (in mM: 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaC1_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES; pH 7.4) with gentamicin (0.10 mg/mL). Oocytes were used for recordings 3−6 days postinjection and were voltage-clamped with the use of a two-electrode voltage clamp (OC-725C, Warner Instruments, Hamden, CT) with both microelectrodes filled with 3 M KCl. Recordings were made while the oocytes were continuously superfused with frog Ringer's solution (in mM: 115 NaCl, 2 KCl, 1.8 $BaC1_2$, 5 HEPES; pH 7.6). The test compounds were dissolved in frog Ringer's solution and added by bath application. Recordings were performed at room temperature at holding potentials of −60 mV. Inhibition of agonist-evoked currents was measured by recording the maximal current induced by a saturating concentration of agonist (300 μM glutamate for $GluA1_i$, 300 μM glutamate and 100 μM glycine for GluN1/2A) and then applying increasing concentrations of test compound in the presence of the appropriate agonist. For ArgTX-636, the concentration−inhibition data were fitted to the equation $I = I_{max}/[1 + 10^{(\log IC_{50} - \log[\text{antagonist}])n_H}]$, where I is the response observed at a given antagonist concentration and I_{max} is the response in the absence of antagonist. The parameters I_{max} (maximal current observed at infinite agonist concentration), n_H (Hill coefficient), and IC_{50} (concentration of antagonist producing 50% of I_{max}) were determined by an iterative least-squares fitting routine.

■ ASSOCIATED CONTENT

Supporting Information. Copies of ^1H and ^{13}C NMR spectra for JSTX-4 (**1**). This can be accessed free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

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