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The Effects of Conformational Constraints in the Polyamine Moiety of Philanthotoxins on AMPAR Inhibition

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*Philanthotoxin-433 (PhTX-433) is a known potent inhibitor of ionotropic glutamate receptors and analogues have been synthesized to identify more potent and selective antagonists. Here, we report on the synthesis of four PhTXs with a cyclopropane moiety introduced into their polyamine chain, and on their inhibition of an AMPA receptor subtype by using two-electrode voltage-clamp on *Xenopus* oocytes expressing the GluA1flop subunit. All analogues were more potent than PhTX-343 with trans-cyclopropyl-PhTX-343 being the most*

potent (~28-fold) and cis-cyclopropyl-PhTX-343 least potent (~4-fold). Both cis- and trans-cyclopropyl-PhTX-444 had intermediate potency (both ~12-fold). Molecular modelling indicates that a cyclopropane moiety confers a favourable steric constraint to the polyamine part but this is compromised by a cis conformation due to enhanced intramolecular folding. Elongated PhTX-444 analogues alleviate this to some extent but optimal positioning of the amines is not permitted.

Introduction

Philanthotoxin-433 (PhTX-433, **1**; the numerals indicate the number of methylene groups spacing the nitrogens in the polyamine moiety; Figure 1) is a toxin found naturally in the venom of the solitary wasp, *Philanthus triangulum*.^[1,2] PhTX-433 and many of its synthetic analogues have been shown to have non-competitive inhibitory effects at both ionotropic glutamate receptors and nicotinic acetylcholine receptors.^[3-6] In that respect PhTXs are attractive molecules to investigate further given that both of these receptor types are accepted as valid drug targets for a variety of neurodegenerative and other disorders of the central nervous system.^[7] The modular butyryl-tyrosyl-thermospermine composition of **1** has allowed for efficient generation of many synthetic analogues demonstrating the importance of all of these structure segments.^[4,6,8-11] PhTXs **1**, **2** and an array of other analogues have been shown to produce powerful voltage-dependent inhibition of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) currents suggesting a binding mode with the polyamine inserted deeply within the pore region of the ion channel.^[3] This hypothesis is supported by the observation that AMPARs containing the GluA2 subunit with arginine at the "Q/R" site in the selectivity filter of the pore exhibit drastically reduced inhibition by PhTX-343 and other polyamine-containing molecules.^[12]

In the last two decades advanced methodologies for solid-phase synthesis (SPS) of polyamines have been developed,^[13] however, no examples of SPS of cyclopropane-containing polyamine derivatives have been reported. The commonly used solution-phase method for obtaining polyamines displaying a cyclopropane moiety is alkylation of mesitylenesulfonamides with mesitylenesulfonates of cyclopropane diols, but this is not readily transferred into an SPS protocol due to the harsh conditions

required for deprotonation of the sulfonamide and the risk of cross-linking the resin due to the bifunctional building block.^[14] Cyclopropane-*trans*-1,2-dicarboxylic acid^[15] may be readily obtained from the corresponding ethyl diester and the *cis*-anhydride 3-oxabicyclo[3.1.0]hexane-2,4-dione was commercially available, and therefore, we chose an approach involving on-resin reduction of the diamide corresponding to the desired 4,4'-dimethoxytrityl-protected polyamine.^[16]

In the present work we focus on incorporating unprecedented structural variations of the polyamine moiety present in both **1** and its well-studied close structural analogue, PhTX-343 (**2**), and examine how these influence the inhibitory effects of the resulting PhTX analogues on a specific subunit, GluA1flop, present in members of the AMPAR subdivision of the ionotropic glutamate receptor family. This subunit is characteristic of a calcium-permeable and polyamine-sensitive subtype of AMPARs, with the flop splice variant (a 38 amino acid region upstream of the fourth transmembrane region) being upregulated in place of the flip splice variant during early development to become dominating in adult CNS. Constraints were introduced in the central region of the polyamine moiety in an attempt to assess the importance of rigidity in this region. This was achieved by solid-phase synthesis incorporating cyclopropane moieties into

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the polyamine chain to give *trans*- and *cis*-analogues with 3-4-3 or 4-4-4 (i.e. PhTX-343- or PhTX-444-like) spacing between the nitrogens (**3-6** = *trans*-343, *trans*-444, *cis*-343, *cis*-444, respectively; Figure 1). Analogues **2-6** were tested for inhibitory potency against homomeric rat GluA1flop AMPARs expressed in *Xenopus* oocytes by measurement of their reduction of currents evoked by stimulation with kainic acid (KA; 100 μ M) by using a two-electrode voltage-clamp at a holding potential of -80 mV. Although KA is a weaker agonist of AMPARs it is often used instead of AMPA or L-Glu in these assays to avoid the problem of rapid receptor desensitization.

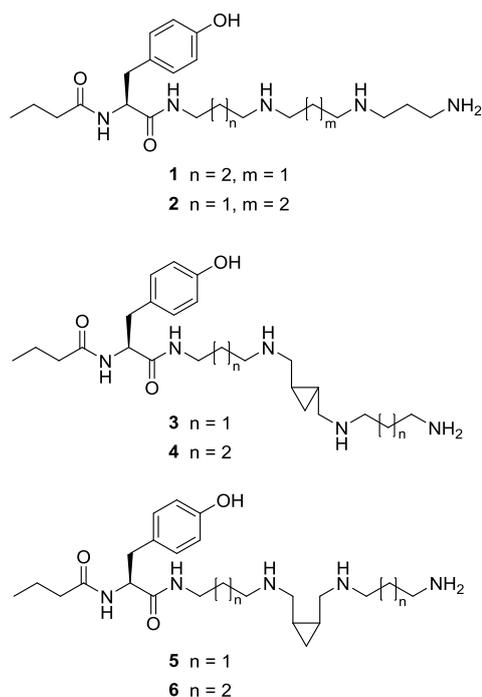


Figure 1. Natural (1) and synthetic (2-6) target philanthotoxins (PhTXs).

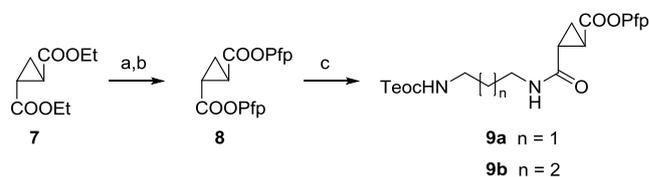
Results and Discussion

Solid-phase synthesis of philanthotoxin analogues

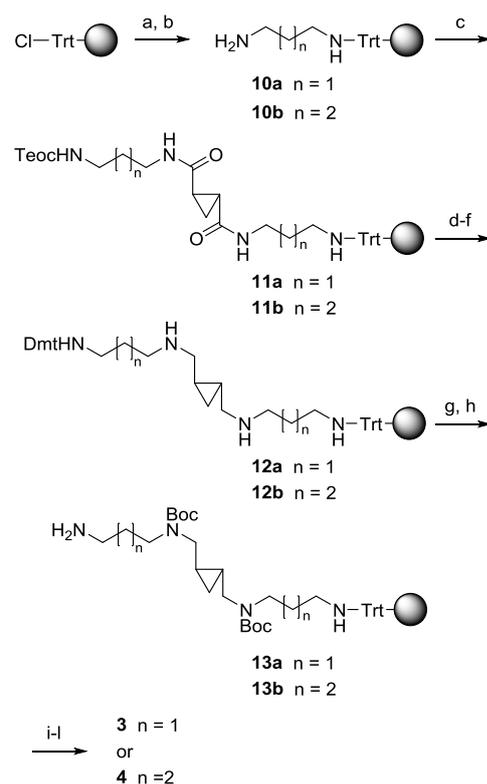
First, cyclopropane-*trans*-1,2-dicarboxylic acid diethyl ester (**7**) was converted into the corresponding pentafluorophenyl diester **8** (Scheme 1) via alkaline hydrolysis and subsequent transesterification by using pentafluorophenyl trifluoroacetate-pyridine (1:1) in DMF.^[15,17] Building blocks **9a** and **9b** were obtained upon condensation of pentafluorophenyl diester **8** with one equivalent of the appropriate 2-(trimethylsilyl)ethoxycarbonyl (Teoc)-monoprotected diamine.^[16b]

In order to minimize cross-linking of the 2-chlorotrityl chloride resin, it was loaded with the appropriate Teoc-monoprotected diamine (Scheme 2). A prolonged reaction time of two days enabled a cost-efficient use of these selectively protected diamines as only 10% excess was required. The resulting resin-bound diamines **10a/10b** were then coupled with building blocks **9a/9b** to give Teoc-protected diamide intermediates **11a/11b** that were subjected to exchange of the N-protecting group followed by borane reduction to yield the 4,4'-dimethoxytrityl (Dmt)-protected polyamine intermediates **12a/12b**. The thus formed secondary amino functionalities were Boc-protected, and then the Dmt group on the terminal primary amine was removed under weakly acidic conditions to give selectively

protected resin-bound polyamines **13a/13b**. Successive acylation with the activated ester, Fmoc-Tyr(^tBu)-OPfp, Fmoc deprotection, acylation with pentafluorophenyl butanoate,^[18] and cleavage from the linker afforded the crude *trans*-cyclopropane-containing target philanthotoxins that readily were purified by reversed-phase preparative HPLC to give PhTXs **3** and **4** as the tris(TFA) salts.



Scheme 1. Reagents and conditions: a) NaOH, H₂O–EtOH; b) CF₃COOPfp (2.5 equiv), pyridine (2.5 equiv), DMF, 19 h; c) TeocNH(CH₂)₃NH₂ or TeocNH(CH₂)₄NH₂ (1 equiv), DIPEA (2 equiv), CH₂Cl₂, 19 h. DIPEA = diisopropylethylamine, Pfp = pentafluorophenyl, Teoc = 2-(trimethylsilyl)ethoxycarbonyl.



Scheme 2. Reagents and conditions: a) TeocNH(CH₂)₃NH₂ or TeocNH(CH₂)₄NH₂ (1.1 equiv), DIPEA (5 equiv), CH₂Cl₂, 2 days then CH₂Cl₂–MeOH–DIPEA (85:15:5), 2 \times 10 min; b) TBAF (3 equiv), DMF, 50 $^{\circ}$ C, 1 h then room temperature for 3 h; c) Compound **9a** or **9b** (2 equiv), HODhBt (1 equiv), DIPEA (2 equiv), DMF, 16 h; d) TBAF (5 equiv), DMF, 55 $^{\circ}$ C, 2 \times 15 min; e) Dmt-Cl (6 equiv), DIPEA (6 equiv), CH₂Cl₂, 3.5 h then CH₂Cl₂–MeOH–DIPEA (85:15:5); f) 1M BH₃·THF (20 equiv), THF, reflux for 16 h; g) Boc₂O (10 equiv), DIPEA (10 equiv), CH₂Cl₂, 16 h; h) 0.1M chloroacetic acid, CH₂Cl₂, 4 \times 30 min; i) Fmoc-Tyr(^tBu)-OPfp (3 equiv), HODhBt (1 equiv), DIPEA (3 equiv), DMF, 2 \times 16 h; j) 20% piperidine–DMF, 2 \times 10 min; k) C₃H₇COOPfp (3 equiv), HODhBt (1 equiv), DIPEA (3 equiv), DMF, 16 h; l) TFA–CH₂Cl₂ (1:1), 2 h. TBAF: tetrabutylammonium fluoride; Dmt-Cl: 4,4'-dimethoxytrityl chloride; HODhBt: 3-hydroxy-1,2,3,4-benzotriazin-4(3H)-one.

Modelling studies on philanthotoxins

It is well known that the potency of a philanthotoxin analogue is influenced by changes in the length of the polyamine moiety as well as by the number and distribution of the positively charged amino groups as these factors determine the overall shape and electronic properties of the resulting molecule via intramolecular H-bonding.^[3, 4, 19, 20]

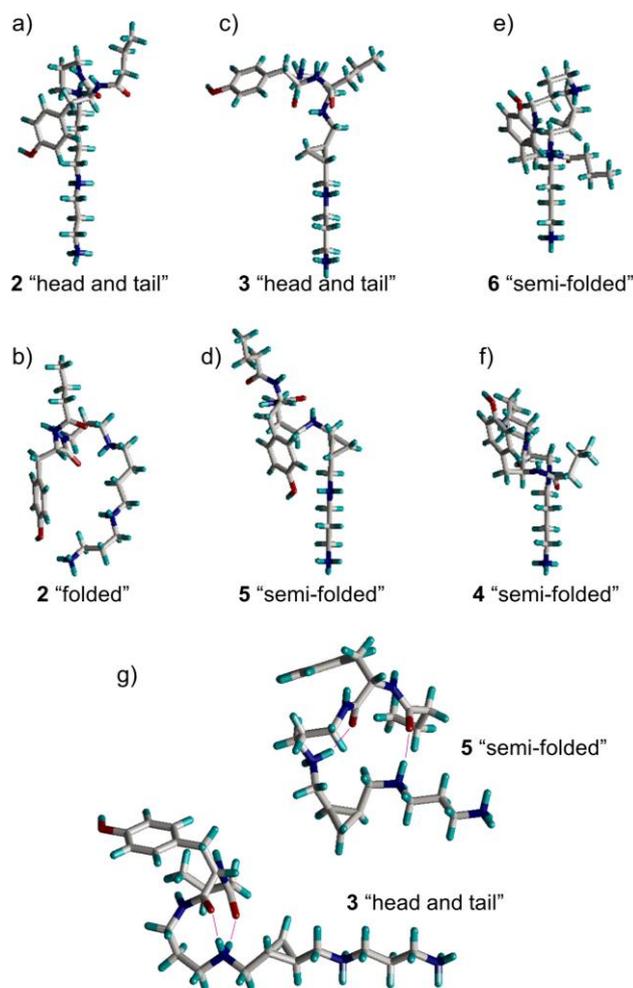


Figure 3. Structures of philanthotoxin analogues 2-6 visualizing the three general conformations that can be adopted: "head and tail" for 2 (A) and 3 (C), "semi-folded" for 4, 5 and 6 (D-F) and "folded" for 2 (B). G) A clearer view of H-bonding (pink lines) between amide oxygens and amine hydrogens in 3 for an HT conformation and in 5 for a SF conformation.

PhTX analogues can adopt three general low-energy structures, "head and tail" (HT; extended), "semi-folded" (SF) and "folded" (F) (as depicted in Figure 3), depending on whether H-bonding involves the first, second, or third amino group, respectively. The HT structure is believed to be the most active at AMPARs due to previous observations showing that the mono-cationic analogues are virtually inactive at AMPARs.^[4,14] In terms of general shape mono-cationic analogues are "folded" because they do not possess a "tail" at all. Our modelling studies show that for all compounds the vast majority (99.8%) of low-energy conformations possessed at least one intramolecular H-bond. Ensembles of low-energy conformations obtained for the PhTX analogues studied contained all three types of structures (HT, SF and F) within 3 kcal mol⁻¹ from the apparent energy minimum

(0.5-1.0 kcal mol⁻¹). To reveal the conformational preferences of the compounds we compared the relative numbers of conformations belonging to the different types within these ensembles. Compound 2 was found to be distributed as 85% HT (Figure 3A), 13% SF and 2% F (Figure 3B). This was unchanged for 3 so the additional hydrocarbon bulk, which in fact becomes part of the head group and relocating the tyrosyl group (Figure 3C), appears to be responsible for the increased potency of 3. This is reminiscent of analogues where short hydrocarbon chains were introduced to the central section of the polyamine moiety resulting in more potent analogues at insect quisqualate sensitive ionotropic glutamate receptors.^[9] The *cis*-analogue of 3 (i.e compound 5) was less potent as the *cis* arrangement caused the molecule to adopt a greater proportion of SF structure (44%) (Figure 3D) at the expense of HT (54%). The cause of this effect is that in the *cis* form both amino groups occur at the same side of the bulk and rigid cyclopropane ring. While the *trans* configuration dictates a rather large distance between amino groups (6.2 Å in the lowest-energy structure), the *cis* configuration results in a significantly smaller distance (4.6 Å). As a result, the probability that the second amino group is involved in intramolecular H-bonding (and thus the structure is semi-folded) is larger for the *cis* than the *trans* configuration (Figure 3G).

PhTX analogues 4 and 6 showed intermediate potency between 3 and 5 since the additional carbon in the chain between the head group and the first amine functionality allowed for more flexibility and hence a higher proportion of SF structures (Figure 3; E-F). The SF structures for these analogues are likely to be more active because of the longer chain between the second and terminal amine groups (giving it a more HT-like structure).

Conclusion

Introduction of a cyclopropane moiety between the first and second amino groups of PhTX-343 proved beneficial for AMPAR antagonism, but less so for the *cis*-analogue due to its greater tendency to become semi-folded as inferred by modeling studies. The positive (for *trans*-analogues) and negative (for *cis*-analogues) effects of this structural alteration are to some extent obscured by the increased spacing of the amide and the first amine functionality as well as of the second and third amines, such that both PhTX-444 analogues display similar potency.

Experimental Section

General procedures. Unless otherwise stated, starting materials and solvents were purchased from commercial suppliers (H-Tyr(^tBu)-OH from Novabiochem, reagents and solvents from Sigma-Aldrich, Fluka, or Lancaster) and used as received. CH₂Cl₂ was distilled from P₂O₅ and stored over 4 Å molecular sieves. THF was distilled from Na/benzophenone immediately before use. ¹H NMR and ¹³C NMR spectra were recorded at 400.14 MHz and 100.62 MHz, respectively, on a Bruker AMX 400 spectrometer, or at 300.06 MHz and 75.45 MHz, respectively, on a Varian Mercury Plus spectrometer, using CDCl₃ or CD₃OD as solvents and tetramethylsilane (TMS) as internal standard. Coupling constants (*J* values) are given in hertz (Hz), and multiplicities of ¹H NMR signals are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; sx, sextet; m, multiplet; br, broad. High-resolution mass spectrometry (HRMS) measurements were performed on a Bruker APEX Qe Fourier transform mass spectrometer equipped with a 9.4 tesla superconducting cryomagnet, and an external electrospray ion source (Apollo II source). The spectra were externally calibrated with an arginine cluster in positive

mode. The samples were dissolved in MeOH, introduced into the electrospray ion source using a syringe pump with a flow of 2 μ L/min. Solid-phase reactions were performed in Teflon filter vessels on a Scansys PLS 4 \times 6 Organic Synthesizer equipped with a heating block. Preparative HPLC system consisted of an Agilent 1100 system with 2 preparative pump units, a UV detector, and a Phenomenex Luna C18(2) (5 μ m) column (25 \times 2.12 cm). Linear elution gradients were composed by mixing solvent A (MeCN–H₂O–TFA 5:95:0.1) and B (MeCN–H₂O–TFA 95:5:0.1) at a flow rate of 20 mL/min. Analytical HPLC was performed on a Shimadzu system consisting of an SCL-10A VP controller, an SIL-10AD VP autosampler, an LC-10AT VP pump, an SPD-M10A VP diode array detector, and a CTO-10AC VP column oven, using a Phenomenex Luna C18(2) (3 μ) column (150 \times 4.6 mm). The HPLC system was controlled by Class VP 6 software; elution was performed with two different solvent systems (total flow of 0.8 mL/minute). Solvent A = MeCN–H₂O–TFA 10:90:0.1 and solvent B = MeCN–H₂O–TFA 90:10:0.1; t = 0–5 min 0% B, t = 5–30 min 0–40% B, t = 30–35 min 40–100% B). The purities of target compounds **3–6** were determined (from UV absorption integration at λ = 215 nm) and were within the ranges 97–99%.

Dipentafluorophenyl Cyclopropane-*trans*-1,2-dicarboxylate (8): Diethyl *trans*-1,2-cyclopropanedicarboxylate (**7**, 5.15 g, 27.7 mmol) was subjected to hydrolysis with NaOH (5.16 g, 4.66 \times 27.7 mmol) in H₂O–EtOH (2:1, 75 mL) for 4 h at room temperature. The reaction mixture was diluted with H₂O (200 mL), and then washed with Et₂O (100 mL), which was extracted back with H₂O (50 mL). The combined aqueous phases were concentrated to dryness *in vacuo*. The residue was partially dissolved in EtOAc (100 mL), which then was filtered through a layer of Na₂SO₄. The filtrate was evaporated to give the crude diacid (2.98 g, 83%). The diacid (1.40 g, 10.8 mmol) was treated with CF₃COOPfp (4.64 mL, 2.5 \times 10.8 mmol) and pyridine (2.18 mL, 2.5 \times 10.8 mmol) in dry DMF (15 mL) for 19 h under N₂. The reaction mixture was diluted with EtOAc (250 mL), and then extracted successively with 0.1 M HCl (3 \times 150 mL), saturated aqueous NaHCO₃ (3 \times 150 mL), and brine (150 mL). Upon drying (Na₂SO₄) the solvent was removed to give **8** (4.86 g, 98%). ¹H NMR (300 MHz, CDCl₃): δ = 1.81 (t, *J* = 7.5 Hz, 2H), 2.65 (t, *J* = 7.5 Hz, 2H); ¹³C NMR (75 MHz, CDCl₃): δ = 17.6, 22.3 (2C), 124.5, 137.7 (2C), 139.7, 141.0 (2C), 167.0. Anal. calcd for C₁₇H₄F₁₀O₄: C 44.18, H 0.87, found: C 44.31, H 0.58.

Protected Monoamides (9a/9b) from Dipentafluorophenyl Cyclopropane-*trans*-1,2-carboxylate: The diPfp ester (1.78 g, 3.85 mmol) was dissolved in dry CH₂Cl₂ (25 mL), and then the appropriate Teoc-protected diamine (3.85 mmol) and DIPEA (1.34 mL, 2 \times 3.85 mmol) in dry CH₂Cl₂ (10 mL), were added. After 19 h, the reaction mixture was diluted with EtOAc (150 mL), and then washed with 0.1 M HCl–brine (3:1, 3 \times 100 mL), H₂O (100 mL), satd NaHCO₃–brine (3:1, 3 \times 100 mL), and brine (75 mL). The organic layer was dried (Na₂SO₄) and *concd*. The residue was dissolved in CH₂Cl₂ (15 mL) and loaded onto a VLC column (5.5 \times 6 cm), which was eluted with hexane and then hexane–Me₂CO 20:1 to 4:1. This afforded **9a** (1.24 g, 65%) or **9b** (1.10 g, 56%). **9a**: ¹H NMR (300 MHz, CDCl₃): δ = 0.03 (s, 9H), 0.97 (br t, *J* = 8.5 Hz, 2H), 1.52 (ddd, *J* = 9.2, 5.5, 4.1 Hz, 1H), 1.61–1.71 (br m, 3H), 2.20 (ddd, *J* = 9.2, 6.0, 3.9 Hz, 1H), 2.48 (ddd, *J* = 9.1, 5.5, 3.9 Hz, 1H), 3.35 (m, 2H), 3.24 (m, 2H), 4.14 (br t, *J* = 8.5 Hz, 2H), 5.05 (br s, 1H), 6.84 (br s, 1H) ppm; ¹³C NMR (75 MHz, CDCl₃): δ = –1.4, 16.2, 17.8, 20.3, 25.6, 30.1, 36.3, 37.5, 62.3, 157.5, 168.7, 169.1 ppm. HRMS *m/z* [M+Na]⁺ calcd for C₂₀H₂₅F₅N₂NaO₅Si: 519.13451, found 519.13431. **9b**: ¹H NMR (300 MHz, CDCl₃): δ = 0.02 (s, 9H), 0.96 (br t, *J* = 8.5 Hz, 2H), 1.51 (ddd, *J* = 9.0, 5.5, 3.9 Hz, 1H), 1.56 (m, 4H), 1.77 (ddd, *J* = 9.0, 6.1, 3.9 Hz, 1H), 2.16 (ddd, *J* = 9.0, 6.1, 3.9 Hz, 1H), 2.47 (ddd, *J* = 9.0, 5.5, 3.9 Hz, 1H), 3.32 (m, 2H), 3.29 (m, 2H), 4.13 (br t, *J* = 8.5 Hz, 2H), 4.79 (br s, 1H), 6.43 (br s, 1H) ppm; ¹³C NMR (75 MHz, CDCl₃): δ = –1.4, 16.1, 17.9, 20.3, 25.5, 26.5, 27.8, 39.7, 40.4, 63.1, 156.9, 168.7, 168.9 ppm. HRMS *m/z* [M+Na]⁺ calcd for C₂₁H₂₇F₅N₂NaO₅Si: 533.15016, found 533.14995.

Preparation of resin-bound diamines 10a/10b: N¹-[2-Trimethylsilyl)ethoxycarbonyl]-1,3-propanediamine was prepared (in

74% yield) as earlier reported for N¹-[2-trimethylsilyl)ethoxycarbonyl]-1,4-butanediamine.^[16b] ¹H NMR (300 MHz, CDCl₃): δ = 0.02 (s, 9H), 0.96 (t, *J* = 8.5 Hz, 2H), 1.67 (p, *J* = 6.6 Hz, 2H), 2.82 (t, *J* = 6.6 Hz, 2H), 3.26 (m, 2H), 4.12 (br t, *J* = 8.5 Hz, 2H) ppm; ¹³C NMR (75 MHz, CDCl₃): δ = –1.2, 17.9, 38.8, 39.4, 62.9, 157.0 ppm. Trityl chloride resin (1.89 g, –2.2 mmol/g) prewashed with 10% DIPEA–CH₂Cl₂ was added to a gently stirred solution of Teoc-protected diamine (1.1 \times 4.16 mmol) and DIPEA (3.6 mL, 5 \times 4.16 mmol) in dry CH₂Cl₂ (20 mL). After 2 days, the resin was transferred to a 20 mL syringe, and then washed with CH₂Cl₂ (2 \times 15 mL) and subsequently end-capped with CH₂Cl₂–MeOH–DIPEA (80:15:5, 15 mL, 2 \times 10 min). The resin was then washed with CH₂Cl₂, MeOH, dry CH₂Cl₂ (each 3 \times), dried overnight (to give 2.52 g and 2.57 g, respectively of resin-bound Teoc-protected 1,3-propanediamine and 1,4-butanediamine, respectively). Then the Teoc groups were removed by treatment with TBAF (3 equiv) in dry DMF (45 mL; in a round-bottomed flask) at 50 °C for 1 h and then at room temperature for 3 h. The resins were then washed with CH₂Cl₂, MeOH, dry CH₂Cl₂ (each 3 \times), dried overnight (to give ~2.0 g, **10a/10b**). The loadings were determined to be ~1.8 mmol/g based on the mass gain and coupling with Fmoc-Gly-OH.

SPS of Protected *trans*-Cyclopropane PhTX-Analogues: Resin-bound diamines (**10a/10b**, 0.44 mmol) were swelled in dry DMF for 30 min. The solvent was removed by suction, and the resin was treated with a cyclopropane building block (**9a** or **9b**, 2 equiv), HODhBt (1 equiv) and DIPEA (2 equiv) in dry DMF (10 mL/mmol resin). The mixture was shaken for 16 h at room temperature. The resin was drained and then washed with DMF, MeOH, CH₂Cl₂ and DMF (3 \times 12 mL/mmol resin with each solvent). The Teoc group was removed by treatment with TBAF (5 equiv) in DMF (7.5 mL/mmol resin) at 55 °C for 15 min. Upon draining and wash with DMF, this deprotection step was repeated. The resin was drained and then washed with DMF, MeOH, and CH₂Cl₂ (3 \times). Dmt-Cl (6 equiv) in dry CH₂Cl₂ (9 mL/mmol resin) and DIPEA (6 equiv) were added to the resin. After 3.5 h, the resin was drained and washed with CH₂Cl₂, CH₂Cl₂–MeOH–DIPEA (80:15:5), DMF, CH₂Cl₂, and THF (each 3 \times). The resin was transferred to a 25 mL flask with a minimum of THF, and then 1M BH₃·THF in THF (20 equiv) was added. The flask was heated to reflux under N₂ in an oil bath (70 °C) for 16 h. The resin was transferred back to a teflon reactor using THF. Upon draining, the resin was washed with MeOH (3 \times). Then the resin was treated with piperidine (9 mL/mmol resin) at 60 °C for 1.5 h. After washing with MeOH (12 mL/mmol), this decomplexation procedure was repeated twice. The resin was treated with Boc₂O (10 equiv) and DIPEA (10 equiv) in dry CH₂Cl₂ (6 mL/mmol resin) for 16 h followed by draining and wash with DMF, MeOH, DMF, and CH₂Cl₂ (each 3 \times). Dmt deprotection with 0.1M ClAcOH in CH₂Cl₂ (14 mL/mmol resin) for 30 min followed by wash with CH₂Cl₂ was repeated three times. The resin was drained and washed with DMF, MeOH, CH₂Cl₂, and dry DMF (each 3 \times). The resulting resin was coupled with Fmoc-Tyr(Bu)-OPfp (3 equiv), HODhBt (1 equiv), and DIPEA (3 equiv) in dry DMF (7 mL/mmol resin) for 16 h. Upon draining, the resin was washed with DMF, MeOH, CH₂Cl₂, and dry DMF (each 3 \times). Coupling with Fmoc-Tyr(Bu)-OPfp was repeated (due to a positive Kaiser test) using DMF–CH₂Cl₂ (3:1) as solvent. Fmoc deprotection with 20% piperidine–DMF (11 mL/mmol resin) for 10 min was repeated after a DMF wash. Upon draining, the resin was washed with DMF, MeOH, CH₂Cl₂, and dry DMF (each 3 \times). The resulting resin was treated with pentafluorophenyl butanoate (3 equiv), HODhBt (1 equiv), and DIPEA (3 equiv) in dry DMF (8 mL/mmol resin) for 16 h. Upon draining, the resin was washed with DMF, MeOH, and CH₂Cl₂ (each 3 \times). Cleavage of the product from the resin was performed with TFA–CH₂Cl₂ (1:1, 9 mL/mmol resin) for 2 h. The filtrate was collected by suction, and the resin eluted further with CH₂Cl₂ and MeOH (each 2 \times 9 mL/mmol resin). The combined filtrates were concentrated, and the residue purified by preparative HPLC (>95% purity): **3** (46 mg) and **4** (115 mg). **3**: ¹H NMR (300 MHz, methanol-*d*₄): δ = 0.85 (br t, *J* = 6.6 Hz, 2H), 0.87 (t, *J* = 7.4 Hz, 3H), 1.22 (m, 2H), 1.56 (sx, *J* = 7.4 Hz, 2H), 1.81 (br p, *J* = 6.5 Hz, 2H), 2.10 (m, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 2.82–3.30 (br m, 14H), 4.49/4.50 (t, *J* = 7.7 Hz, 1H), 6.71 (d, *J* = 8.3 Hz, 2H), 7.06 (d, *J* = 8.3 Hz, 2H); ¹³C NMR (75 MHz, methanol-*d*₄): δ = 10.8, 14.0, 15.5^{***}, 15.6^{*}, 20.3, 25.4, 27.5/27.4^{*}, 36.7, 37.8, 38.0,

38.6, 45.6, 46.0/45.9, 52.1, 52.2, 57.1, 116.2, 117.6, 128.7, 131.1, 157.2, 162.1, 175.1/175.0, 176.0; ¹H signals with half intensity (splitting due to presence of diastereomers); ¹³C two distinct close signals. HRMS *m/z* [M+H]⁺ calcd for C₂₄H₄₂N₅O₃: 448.32822, found 448.32830. **4**: ¹H NMR (300 MHz, methanol-d₄): δ = 0.84 (t, *J* = 7.4 Hz, 3H), 0.84 (obsc., 2H), 1.22 (m, 2H), 1.53 (sx, *J* = 7.4 Hz, 2H), 1.42–1.66 (br m, 4H), 1.67–1.85 (br m, 4H), 2.16 (t, *J* = 7.4 Hz, 2H), 2.79 (dd, *J* = 13.7 and 8.5 Hz, 1H), 2.87–3.15 (br m, 13H), 4.52 (dd, *J* = 8.5 and 6.9 Hz, 1H), 6.69 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H) ppm; ¹³C NMR (75 MHz, methanol-d₄): δ = 11.0, 14.0, 15.4 (2C), 20.3, 24.2, 24.3, 25.6, 27.3, 38.2, 38.7, 39.3, 40.0, 47.9, 48.2, 51.8, 52.0, 56.8, 116.1, 117.7, 128.9, 131.1, 157.1, 162.4, 173.9, 175.8. HRMS *m/z* [M+H]⁺ calcd for C₂₆H₄₆N₅O₃: 476.35952, found 476.35956.

SPS of Protected *cis*-Cyclopropane PhTX-Analogues: Resin-bound diamines (**10a/10b**, 0.55 mmol) were swelled in dry DMF for 30 min. The solvent was removed by suction, and a solution of 3-oxabicyclo[3.1.0]hexane-2,4-dione (4 equiv) and DIPEA (2 equiv) in dry DMF (7 mL/mmol resin) was added to the resin. After 16 h, the resin was drained and washed with DMF, MeOH, DMF, and dry CH₂Cl₂ (each 3 × 12 mL/mmol resin). Then the resin was treated with Teocmonoprotected diamine (4 equiv), HOBT (4 equiv), and DIC (3 equiv) in dry DMF (8 mL/mmol resin) for 16 h. The resin was drained and washed with DMF, MeOH, DMF, and dry CH₂Cl₂ (each 3 ×). Successive Teoc deprotection, introduction of Dmt protecting groups, borane reduction/decomplexation, and Boc protection were performed as described above for *trans*-analogues. Upon draining and wash with DMF, MeOH, DMF, and CH₂Cl₂ (each 3 ×), Dmt groups were removed with 0.1M ClAcOH in CH₂Cl₂ (11 mL/mmol resin, 4 × 30 min). The resin was drained and washed with DMF, MeOH, CH₂Cl₂, and dry DMF (each 3 ×). The resulting resin was elongated with the Tyr residue, and subsequently terminated by introduction of the *N*-butyryl group as described above (using single couplings for 16 h). Cleavage of the product from the resin was performed with TFA–CH₂Cl₂ (1:1, 7 mL/mmol resin) for 2 h. The crude products were purified by preparative HPLC (>95% purity): **5** (220 mg) and **6** (64 mg). **5**: ¹H NMR (300 MHz, methanol-d₄): δ = 0.56/0.58^{*} (q, *J* = 5.8 Hz, 1H), 0.85/0.86^{*} (t, *J* = 7.5 Hz, 3H), 1.11 (m, 1H), 1.39 (m, 2H), 1.54/1.56^{*} (sx, *J* = 7.5 Hz, 2H), 1.82 (p, *J* = 6.6 Hz, 2H), 2.15–2.22 (br m, 4H), 2.79–3.30 (br m, 14H), 4.39/4.40^{*} (t, *J* = 7.7 Hz, 1H), 6.72 (d, *J* = 8.4 Hz, 2H), 7.06/7.07^{*} (d, *J* = 8.4 Hz, 2H) ppm; ¹³C NMR (75 MHz, methanol-d₄): δ = 10.2/10.5^{*}, 13.8^{**}/13.9^{**}, 14.0^{**}, 20.3, 25.4, 27.4, 36.7/36.8^{*}, 37.8, 38.0^{**}, 38.6^{**}, 45.7^{**}/45.8^{**}, 46.0^{**}, 48.4 (2C), 57.0/57.2^{*}, 116.1/116.2^{*}, 118.0, 128.8, 131.1/131.2^{*}, 157.2^{**}, 162.9, 174.9/175.1^{*}, 176.0^{**} ppm; ¹H signals with half intensity (splitting due to presence of diastereomers); ¹³C two distinct close signals. HRMS *m/z* [M+H]⁺ calcd for C₂₄H₄₂N₅O₃: 448.32822, found 448.32802. **6**: ¹H NMR (300 MHz, methanol-d₄): δ = 0.60 (q, *J* = 5.5 Hz, 1H), 0.85 (t, *J* = 7.4 Hz, 3H), 1.12 (m, 1H), 1.39 (m, 2H), 1.45–1.68 (br m, 4H), 1.54 (sx, *J* = 7.4 Hz, 2H), 1.77 (m, 4H), 2.16 (t, *J* = 7.4 Hz, 2H), 2.80 (dd, *J* = 13.7 and 8.5 Hz, 1H), 2.89–3.38 (br m, 13H), 4.42 (dd, *J* = 8.5 and 6.9 Hz, 1H), 6.70 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 8.5 Hz, 2H) ppm; ¹³C NMR (75 MHz, methanol-d₄): δ = 10.6, 13.8^{**}, 14.0, 20.3, 24.2, 24.3, 25.6, 27.3, 38.2, 38.7, 39.3, 40.0, 48.0, 48.2, 48.3 (2C), 56.9, 116.1, 118.0, 128.9, 131.1, 157.1, 162.7, 173.9, 175.9 ppm; ¹³C two distinct close signals. HRMS *m/z* [M+H]⁺ calcd for C₂₆H₄₆N₅O₃: 476.35952, found 476.35936.

AMPA Inhibition Assay. A two-electrode voltage-clamp (TEVC) was used to record responses to 100 μM KA of *Xenopus laevis* oocytes expressing AMPARs containing GluA1flop subunits. *X. laevis* oocytes were injected with cRNA encoding GluA1flop and incubated at 18 °C for at least three days. Single oocytes were transferred to a perfusion bath and continuously washed with saline containing 96 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES (pH adjusted 7.5 with NaOH). Microelectrodes were pulled from borosilicate glass capillaries (GC150TF-10, Harvard Apparatus) using a Sutter P-97 programmable puller and had resistances of ~0.5 MΩ when filled with 3.0 M KCl. The oocytes were voltage clamped at –80 mV using an Axoclamp 2B (Axon Instruments) and output currents were transferred to a PC via a NI PCI-6221 A-D interface (National

Instruments, UK) and WinEDR software (Dr John Dempster, University of Strathclyde, UK). Responses of AMPAR were elicited by perfusion of 100 μM KA until a stable control current was obtained (60–90 s) then **2-6** were co-applied until a new plateau current was obtained (60–90 s). This was repeated for **2-6** concentrations in the range 10⁻⁹–10⁻⁵ M (+10⁻⁴ M for **2**). Percent of control response was plotted against concentration of PhTX analogue and fit with a Hill equation to estimate the IC₅₀. Analysis was performed using GraphPad Prism 6.

Molecular Modelling. Calculations were performed by using ZMM (ZMM Software, Inc., Flamborough, Ontario, Canada). The non-bonded interactions were calculated by using the AMBER force field^[21] with a cut-off distance of 8 Å. The hydration energy was calculated by using the implicit solvent method.^[22] Electrostatic interactions were calculated by using the distance-dependent dielectric function ε = 4r where (r) is the atom-atom distance. No specific energy terms were used for cation-π interactions, which were accounted for with partial negative charges at the aromatic carbons.^[23] Bond lengths and valent angles were assigned standard values. Atomic charges at ligands were calculated by using the AM1 method in the MOPAC program. For each ligand, 100 starting conformations were generated by randomizing torsion angle values. The Monte Carlo minimization (MCM) method^[24] was used to optimize the structures. MCM of each model was performed until 600 consecutive energy minimizations did not decrease the energy of the apparent global minimum. For statistical analysis, the 1000 lowest energy conformations were selected.

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Keywords: Glutamate receptors · Ion channels · Natural products · Polyamine toxins · Solid-phase synthesis

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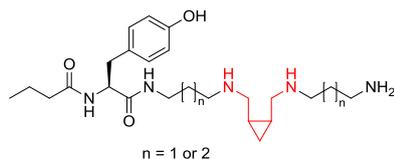
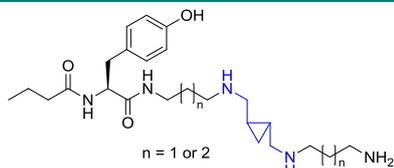
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In the search for improved inhibitors of GluA1 flop AMPA receptors – a question is “may incorporation of cyclopropane moieties be beneficial – and what configuration is superior – *cis* or *trans*?” Synthesis, test and modelling of four analogues revealed the structure-activity features of this unprecedented subclass of inhibitors.



*Henrik Franzyk, John W. Grzeskowiak, Denis B. Tikhonov, Jerzy W. Jaroszewski, and Ian R. Mellor**

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