Synthesis and Pharmacological Activity of Philanthotoxin-343 Analogs: Antagonists of Ionotropic Glutamate Receptors

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Abstract: The synthesis of two classes of Philanthotoxin-343 analogs is described. Quantitative information on the antagonism of quisqualate-sensitive ionotropic glutamate receptors of insect muscle by these compounds is presented. © 1997 Elsevier Science Ltd.

INTRODUCTION

L-Glutamate, a major neurotransmitter in vertebrates[1,2] and invertebrates[3], interacts at neuronal and nerve-muscle synapses with postjunctional proteins, the glutamate receptors.[4] The latter group has attracted much attention because in the mammalian brain they are implicated in several higher neural functions such as memory and learning as well as degenerative brain diseases and ischemic damage[5,6]. Electrophysiological, pharmacological and molecular biological studies have resulted in the division of glutamate receptors into two groups, the ionotropic receptors that gate integral ion channels and the metabotropic receptors that are coupled to G-proteins.[4] Mammalian ionotropic glutamate receptors are divided into two classes according to their selective agonists, i.e. the N-methyl-D-aspartate receptors (NMDAR, NR1 and NR2A-D) and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) / kainate receptors (non-NMDAR, Glu1-6).[1] Invertebrate ionotropic glutamate receptors are of four subtypes: (i) quisqualate receptors (qGluR) that gate cation channels; (ii) ibotenate receptors that gate anion channels; (iii) kainate receptors; and (iv) NMDA receptors.[7] The availability of agonists and antagonists for ionotropic glutamate receptors is a prerequisite for clarifying the mode of action of these signaling proteins on a molecular structural basis and for the design of pesticides for harmful arthropods.

Philanthotoxins[8,9] are potent non-competitive antagoists of ionotropic glutamate receptors that have been isolated from the venom of the digger wasp, *Philanthus triangulum*.[10] δ-Philanthotoxin (PhTX-433 (**1**) (433 denotes the number of methylene groups in the polyamine moiety), a major component of this venom, was initially shown to antagonize qGluR of locust leg muscle in a noncompetitive manner.[11] It was also found to antagonize mammalian NMDAR and non-NMDAR[12,13] and nicotinic acetylcholine receptors (nAChR) of vertebrates and invertebrates.[14] The structure of PhTX-433 (**1**) can be modified at various sites to provide probes to study the tertiary structures of these receptor proteins.

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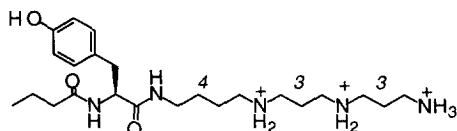
Figure 1. Structure of Philanthotoxin-433 (1)

In previous publications[11-13,16,17] we have reported structure-activity studies of ionotropic GluR and nAChR designed to obtain potent and selective analogs of PhTX-433 (1), including photoactive compounds.[18] In this article we present the results of further systematic studies of analogs that have evolved from the earlier work on polyamine-containing spider toxins[19] and philanthotoxins, i.e., analogs with different polyamine lengths including amines which are resistant to enzymatic hydrolysis are described.

PHARMACOLOGICAL ASSAYS AND SYNTHESIS OF PHILANTHOTOXIN-343 ANALOGS

In the following systematic synthetic modification, the philanthotoxin molecule is divided into five regions shown in Figure 2. Despite the slightly lower potency of PhTX-343 (2)[8,11,13] as compared to native PhTX-433 (50-70%), many of the analogs described contain the 343 polyamine chain in region **II** rather than the native 433 chain because of ease of synthesis with the symmetric 343 moiety.
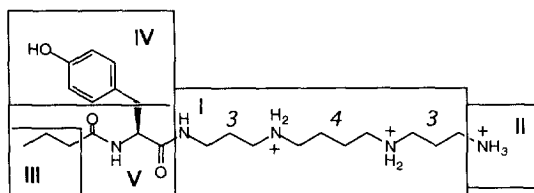
Figure 2. Five Regions of PhTX-343 (2)

We reported earlier that substitution of heptanoyl or decanoyl moieties for a butyl moiety in region **III** enhanced the antagonistic potency of a philanthotoxin, and also showed that elongation of the chain in region **II** generally resulted in activity enhancement.[11, 13] Based on these findings, four analogs (4-7) with an heptanoyl moiety and extended polyamine chains have been made. Assays of these compounds on the qGluR of locust muscle showed them to be more active than PhTX-343 (2). A comparison of C_7-PhTX-343 (3) with **4, 6** and **7** illustrates the importance of the length of the polyamine chain. However, compound **5** has a lower potency than **3** despite its longer polyamine chain; even more striking is the difference in activities of **4** and **5** even though they differ only in the spatial distributions of their polyamine methylene groups, i.e., 3334 vs. 3343, thus suggesting the subtle differences in the matching between the amine intervals and the distances between the spacing of hydrophilic groups in the antagonist binding site on qGluR. The high potency of the arginine analog **6**, which has structural characteristics in common with some polyamine-containing spider toxins, is probably due to delocalization of the positive charge within the guanidinium group over an area

which is much larger than that covered by the positive charge of a single primary amino group.[11] Namely, this analog can localize its positive charge leading to an efficient accommodation of the anionic charge at its binding site. A similar argument could account for the high potency of the lysine analog (**7**). All four analogs caused a use-independent depression of the muscle twitch, although at their IC_{50} concentrations this was accompanied by use-dependent inhibition. In these respects they were pharmacologically similar to PhTX-343 (**2**).


Figure 3. Activities of C_7-PhTX analogs against qGluR

Synthesis of the C_7-PhTX analogs followed the general procedure shown in Scheme 1. Treatment of O-benzyl-L-tyrosine (**8**) with heptanoyl chloride in aqueous alkaline condition gave **9** which is then coupled to various polyamines by DCC, followed by removal of its protective groups.

The synthesis of polyamine-3334 (**10**) utilized commercially available spermine (Scheme 2),[16,17] rather than the conventional stepwise extension of the polyamine chain. This shortened the synthetic pathway and also simplified the purification step of amine intermediates. Cbz protection of 3-amino-1-propanol (**11**) was followed by oxidation of alcohol to 3-Cbz-amino-propanal (**13**). Reductive amination of the aldehyde **13** and subsequent treatment with excess spermine yielded the crude polyamine N-Cbz-3343; after aqueous work to eliminate most of the free spermine, di-*tert*-butyl-dicarbonate was added to afford the fully protected

polyamine (**14**). The tedious purification protocol of the free polyamine is unnecessary in this scheme. Selective removal of Cbz group gave tetra-Boc-polyamine 3343 (**10**).
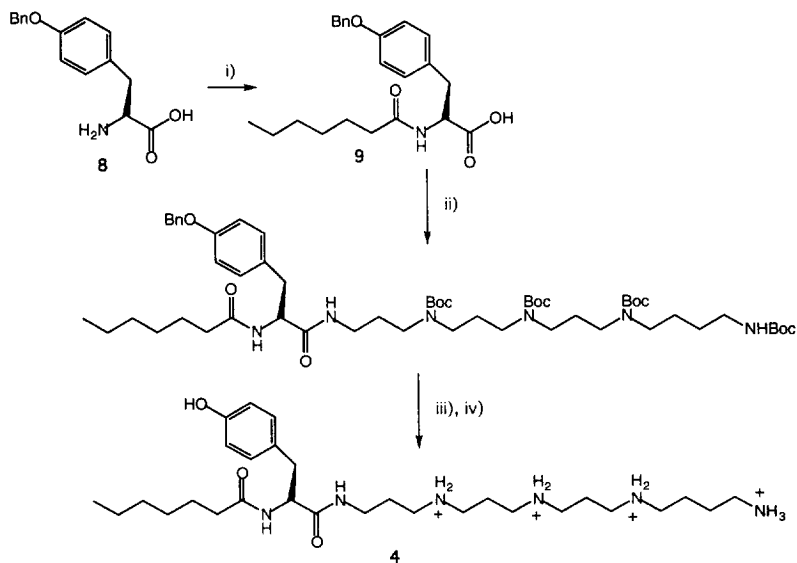
i) heptanoyl chloride, NaOH/aq. THF, 99%; ii) tetra-Boc-polyamine-3334, DCC, CH_2Cl_2, rt, 12 h, 79%; iii) H_2, Pd/C, MeOH, rt, 8 h, 70%; iv) TFA, CH_2Cl_2, rt, 2 h, 100%.

Scheme 1. Synthetic scheme of C_7-PhTX analogs
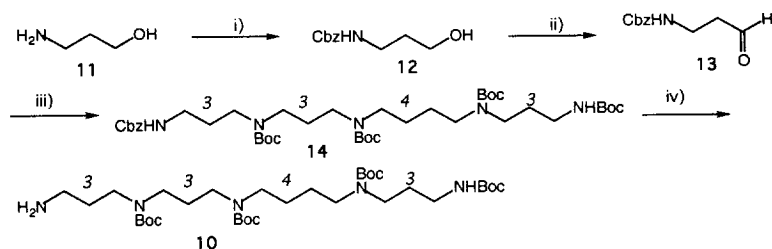
i) CbzCl, Et_3N, CH_2Cl_2, rt, 2 h, 90%; ii) $(CO)_2Cl_2$, DMSO, Et_3N, CH_2Cl_2, rt, 45 min, 86%; iii) a. spermine, Na_2SO_4, $NaBH_3CN$, EtOH, rt, 36 h; b. $(Boc)_2O$, CH_2Cl_2, rt, 2 h, 85%; iv) H_2, Pd/C, MeOH, rt, 6 h, 91%.

Scheme 2. Synthetic scheme of polyamine-3343

Spermine-arginine (**15**) and spermine-lysine (**16**) were prepared by coupling spermine to commercially available tri-Cbz-arginine-succinimide ester (**17**) and tri-Boc-lysine-succinimide ester (**18**), respectively (Scheme 3).
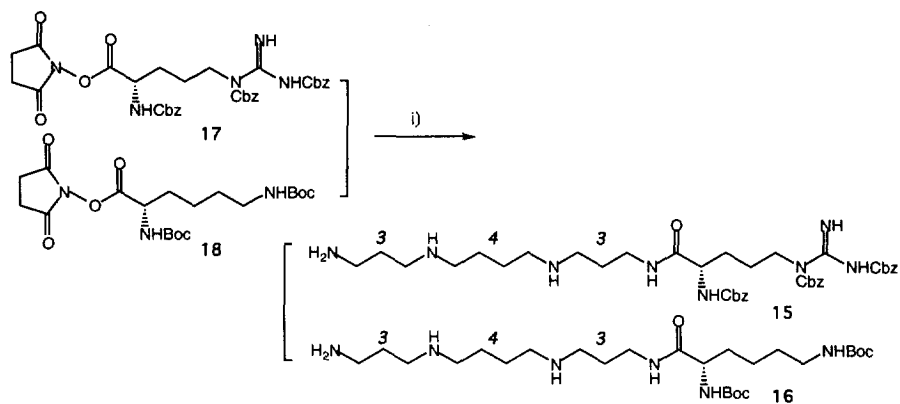
i) spermine, Et$_3$N, DMF, rt, overnight. **15**: 53%; **16**: 48%.

Scheme 3. Synthetic scheme of spermine-arginine and spermine-lysine

Figure 4. Activities of PhTX amine analogs against qGluR

Although past studies have been focused on modifications of regions **I-IV**, the amide in region **V** is of especial interest because amides are vulnerable to enzymatic cleavage. In order to increase the durability of the philanthotoxins as pesticides or drugs, analogs in which the amide bonds in region **I** and **V** were replaced by amines, thereby introducing resistance to enzymatic degradation, were prepared.

The activities of five "amine" analogs against locust muscle qGluR are shown in Figure 4. All five analogs are more active than PhTX-343 (**2**). Although compounds **20-23** are characterized by extended amine chains, surprisingly the activities of these compounds are not as high as that of "amine" **19** which contains the shorter spermine moiety. Antagonistic potency does not vary much when the length of the alkyl group is varied (compare **20** with **22** and **21** with **23**). Interestingly, masking of the hydrophilic tyrosine phenolic group led to increased potency as seen in pairs **2** vs. **19**, and **21** vs. **20**.

Many methods are available for preparing polyamines, including amine alkylation, reductive amination, and amide formation followed by reduction to the desired amine. The most direct way to make PhTX-amine analogs is through reduction of PhTX-343 (**2**) analogs. However, alternative approaches were explored since attempts to directly reduce Boc protected O-Bn-PhTX by BH_3-THF[20] or triethyloxonium fluoboroate in CH_2Cl_2[21] failed to give the desired compounds.
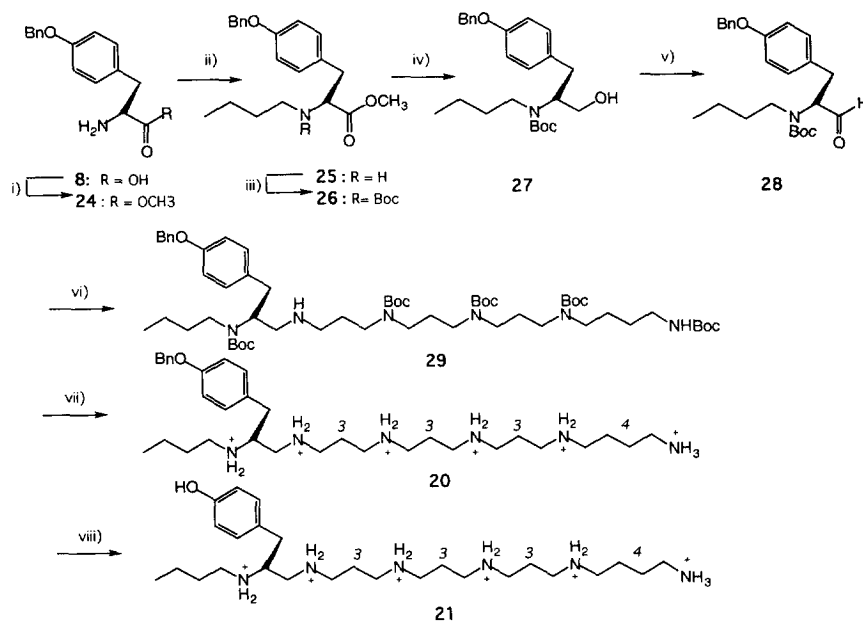
i) $SOCl_2$, MeOH, 40°C, 4 h, 64%; ii) BuBr, KF/Celite, CH_3CN, reflux, 10 h, 67%; iii) $(Boc)_2O$, Et_3N, CH_2Cl_2, rt, 3 h, 100%; iv) DIBAL, CH_2Cl_2, -78°C, 1 h, 94%; v) $(CO)_2Cl_2$, DMSO, Et_3N, CH_2Cl_2, 45 min, 98%; vi) tetra-Boc-amine 3334, Na_2SO_4, $NaBH_4$, EtOH, rt, 36 h, 88%; vii) TFA, CH_2Cl_2, rt, 1 h, 100%; viii) H_2, Pd/C, MeOH, rt, 8 h, 93%.

Scheme 4. Synthetic scheme for PhTX-amine analogs

The PhTX-amine analogs were prepared as shown in Scheme 4. Starting from O-benzyl-L-tyrosine (**17**), methylation with thionyl chloride in methanol yielded O-benzyl-L-tyrosine methyl ester (**24**), which was alkylated with bromobutane to produce **25**. The site-specific N-alkylation of the amine was performed by the method originally described by Ando and Yamawaki[22] and later utilized by Samejima et al.[23] and by Jasys et al. (for the synthesis of an unique hydroxylamine-containing polyamine from a spider venom)[24] was used. This involves alkylating **24** with alkyl bromide in the presence of neutral KF/Celite to produce the monoalkylated product in relatively high yield. Protection of the secondary amine with di-*tert*-butyl dicarbonate gave protected ester **26**. DIBAL reduction[25, 26] of the ester at -78°C, instead of yielding the desired aldehyde, gave alcohol **27**, which was submitted to Swern oxidation[27] to afford aldehyde **28** in high yield (98%). Reductive amination of aldehyde **28** with polyamine 3334 to give **20** followed by deprotection yielded the 3334 PhTX analog **20**.

CONCLUSION

PhTX analogs and PhTX-amine analogs with extended polyamine chains described above have been shown to be potent antagonists of qGluR of locust muscle. In addition to the pesticide area, these polyamine amides and further derivatives should be of interest in the biomedical area since past data on locust muscle qGluR have shown that they can be qualitatively extrapolated to mammalian NMDAR and GluR. The results of a preliminary photoaffinity cross-linking study with an analog with the photoactive azidophenyl group in region III coupled with SAR results showed regions III and IV to be in the intracellular side with the polyamine chain in the channel.[28] However, in these studies the analog was applied in a manner that it had access to both the extracellular and cytoplasmic sides of the receptor. On the other hand, experiments with philanthotoxin carrying porphyrin and other bulky aromatic groups (BIG derivatives) in regions III/IV[29] suggest bulky group entering from the extracellular side with the polyamine chains dangling into the channel. The mode of entry of philanthotoxin analogs and related spider toxins[30] into the nAChR is thus still unknown, and may depend on experimental conditions in which they are administered and the analog structure. A further complicating and unexpected observation is that philanthotoxin itself can efficiently produce a tunnel (or a "channel") when incubated with phosphtadidylcholine vesicles as measured by sodium NMR (unpublished, S. Matile). Although the spider and wasp polyamineamides represent a physiologically active intriguing group of compounds, further studies using synthetic analogs and receptors will be required to clarify their mode of entry into recepotrs and mode of action. Such knowledge is crucial for the synthesis of analogs which will interact specifically with the numerous receptor subtypes for use in biomedicine or pest control.[31]

EXPERIMENTAL SECTION

General

Reagents and starting materials purchased from common commercial suppliers were used as received. Solvent CH_2Cl_2, and reagent Et_3N were distilled at atmospheric pressure over CaH_2, THF was distilled over Na. MeCN, MeOH, and EtOH were dried over molecular sieves (4 Å). All reactions were performed in dry glassware under argon. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed, precoated silica gel plates (60 F_{254}) gel. Preparative TLCs (PTLC) were performed on Analtech (500 microns, 20 x 20 cm, silica gel) or Whatman TLC plates (K5F, 20 x 20 cm, silica gel 150 Å, 250 mm, UV_{254}). Column chromatography was carried out by using ICN silica gel (32-63 mesh). ^1H NMR and spectra were recorded on Varian VXR 400, and reported in parts per million (ppm) using residual proton solvent peaks of either $CDCl_3$ at 7.24 ppm or CD_3OD at 3.30 ppm as an internal standard, with coupling constants (J) in Hertz (Hz). MS (CI, NH_3) spectra were obtained on a NERMAG R10-10 while low and high resolution MS (FAB, 3-nitrobenzyl alcohol matrix) spectra were obtained with a JOEL JMS-DX 303 HF, MS was expressed as m/z. In the most cases the M + H^+ or M + NH_3 were the strongest peaks; only the former peaks were given here.

O-Benzyl-C_7-tyrosine (9): To a solution of O-benzyl-tyrosine (0.540 g, 1.99 mmol) in 1N NaOH (5 ml), heptanoyl chloride was added (0.462 ml, 2.99 mmol) in THF (10 ml), then the reaction mixture was stirred for 5 h. After THF was removed under reduced pressure, the mixture was redissolved in CH_2Cl_2, then was washed by 1N HCl (20 ml x 3), H_2O (15 ml x 2), and brine (25 ml). The organic layer was dried over Na_2SO_4, and evaporated to dryness to afford a white solid (0.755 g, 99%). R_f. 0.45 (EtOAc/Hexane 50:50); FAB-MS ($C_{23}H_{29}NO_4$): 401 (M+18)$^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 7.43-7.32 (*m*, 5H), 7.08 (*d*, 2H, *J*=8.8 Hz); 6.91 (*d*, 2H, *J*=8.8 Hz), 5.44 (*d*, 1H, *J*=7.6 Hz), 5.03 (*s*, 2H), 4.82 (*dd*, 1H, *J*=6.4 Hz), 3.12 (*dd*, 2H, *J*=5.2 Hz), 2.36 (*t*, 2H, *J*=7.2 Hz), 2.18 (*m*, 2H), 1.63-1.55 (*m*, 4H), 1.34-1.25 (*m*, 2H), 0.88 (*t*, 3H, *J*=6.8 Hz).

C_7-Tyrosyl-polyamine 3334 (4): To a CH_2Cl_2 (15 ml) solution containing **9** (88.5 mg, 0.221 mmol) and polyamine 3334 (145.8 mg, 0.221 mmol) was added DCC (52.4 mg, 0.254 mmol), the mixture was stirred for 12 h at rt under an atmosphere of Ar. The reaction was worked up by filtration through a pad of Celite and the filtrate was concentrated *in vacuo* to a light yellow oil. The oil was purified by column chromatography (CH_2Cl_2/MeOH 95:5) to yield the product (179.0 mg, 79%). R_f. 0.32; FAB-MS ($C_{56}H_{92}N_6O_{11}$): 1025 (M+1)$^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 7.54-7.30 (*m*, 5H), 7.10 (*d*, 2H, *J*=8.8 Hz), 6.88 (*d*, 2H, *J*=8.8 Hz), 5.01 (*s*, 2H), 3.31-2.95 (*br m*, 16H), 2.22-2.14 (*m*, 2H), 1.78-1.52 (*br m*, 10H), 1.46 (*s*, 36H), 1.30-2.18 (*m*, 8H), 0.88 (*t*, 3H, *J*=6.8 Hz). To a solution of the previous product (42.1 mg, 41.0 μmol) in MeOH (10 ml) was added catalytic amount of 10% Pd/C, and purged with H_2 3 times. The mixture was stirred under H_2 for 8 h. The black suspension was filtered through Celite and washed extensively with MeOH, and the filtrate was evaporated to dryness. An oily product (26.8 mg, 70%) was obtained after flash chromatography (CH_2Cl_2/MeOH 95:5). R_f. 0.53; FAB-MS ($C_{49}H_{86}N_6O_{11}$): 952 (M+18)$^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 7.07 (*d*, 2H, *J*=8.0 Hz), 6.26 (*d*, 2H, *J*=8.0 Hz), 3.35-3.03 (*m*, 16H), 2.22 (*t*, 2H, *J*=7.2 Hz), 1.78-1.62 (*m*, 10H), 1.58 (*s*, 9H), 1.56 (*s*, 9H), 1.55 (*s*, 9H), 1.47 (*s*, 9H), 1.42-1.26 (*m*, 8H), 0.88 (*t*, 3H, *J*=6.8 Hz). To a solution of this product (26.8 mg, 28.7 mmol) in CH_2Cl_2 (5 ml) was added TFA (0.265 ml, 3.446 mmol). The reaction mixture was stirred for 2 h and concentrated to give the desired product (15.3 mg, 100%) as a white

salt. FAB-MS ($C_{29}H_{54}N_6O_3$): 534 (M+1)$^+$; ^1H NMR (400 MHz, CD$_3$OD): δ 7.07 (d, 2H, J=8.0 Hz), 6.26 (d, 2H, J=8.0 Hz), 3.35-3.03 (m, 16H), 2.22 (t, 2H, J=7.2 Hz), 1.78-1.62 (m, 10H), 1.42-1.26 (m, 8H), 0.88 (t, 3H, J=6.8 Hz).

C$_7$-Tyrosyl-polyamine 3343 (5): FAB-MS ($C_{29}H_{54}N_6O_3$): 534 (M+1)$^+$; ^1H NMR (400 MHz, CD$_3$OD): δ 7.07 (d, 2H, J=8.0 Hz), 6.26 (d, 2H, J=8.0 Hz), 3.37-3.01 (m, 16H), 2.22 (t, 2H, J=7.2 Hz), 1.80-1.60 (m, 10H), 1.45-1.23 (m, 8H), 0.88 (t, 3H, J=6.8 Hz).

C$_7$-PhTX-343-arginine (6): FAB-MS ($C_{32}H_{60}N_9O_4$): 634 (M+1)$^+$; ^1H NMR (400 MHz, CD$_3$OD): δ 7.01 (d, 2H, J=8.0 Hz), 6.68 (d, 2H, J=8.0 Hz), 4.33 (t, 1H, J=7.4 Hz), 3.84 (t, 1H, J=7.4 Hz), 3.41-3.35 (m, 1H), 3.22-3.16 (m, 4H), 3.08-2.97 (m, 4H), 2.98-2.90 (m, 2H), 2.86-2.74 (m, 2H), 2.18-2.10 (m, 1H), 1.93-1.80 (m, 10H), 1.69-1.60 (m, 2H), 1.49-1.31 (m, 2H), 1.28-1.14 (m, 6H), 0.89 (t, 3H, J=7.2 Hz).

C$_7$-PhTX-343-lysine (7): FAB-MS ($C_{32}H_{59}N_7O_4$): 606 (M+1)$^+$; ^1H NMR (400 MHz, CD$_3$OD): δ 7.02 (d, 2H, J=8.4 Hz), 6.68 (d, 2H, J=8.4 Hz), 4.35 (t, 1H, J=6.8 Hz), 3.82 (t, H, J=7.0 Hz), 3.42-3.34 (m, 1H), 3.08-2.72 (m, 12H), 2.18-2.08 (m, 2H), 1.93-1.61 (m, 12H), 1.50-1.38 (m, 4H), 1.29-1.15 (m, 8H), 0.87 (t, 3H, J=7.2 Hz).

N-Cbz-3-amino-1-propanol (12): To a solution of 3-amino-1-propanol (11) (1.127 g, 15.03 mmol) in CH$_2$Cl$_2$ (50 ml) at 0°C was injected CbzCl (2.57 ml, 18.04 mmol), followed by Et$_3$N (10.5 ml, 75.15 mmol). The reaction mixture was stirred for 2 h, and concentrated. 12 (2.827 g, 90%) was obtained after purification by flash chromatography (CH$_2$Cl$_2$/MeOH 95:5). Rf. 0.62; MS (CI): 210; ^1H NMR (400 MHz, CDCl$_3$): δ 7.36 (s, 5H), 5.12 (s, 2H), 5.03 (br s, 1H), 3.72-3.64 (m, 2H), 3.40-3.32 (m, 2H), 2.55 (m, 1H), 1.74-1.58 (m, 2H).

N-Cbz-3-amino-1-propanal (13): To an oven-dried flask containing CH$_2$Cl$_2$ (40 ml) under Ar at -50°C, was injected oxalyl chloride (0.30 ml, 3.44 mmol), followed by addition of DMSO (0.49 ml, 6.88 mmol). The reaction mixture was stirred for 5 min, then the alcohol (12) (0.653 g, 3.13 mmol) in CH$_2$Cl$_2$ was injected over 5 min. The mixture was kept at low temperature for 35 min. After Et$_3$N (2.18 ml, 15.63 mmol) was added, the mixture was allowed to warm to rt after 10 min. The mixture was washed by H$_2$O (20 ml x 2), and the organic layer was dried over Na$_2$SO$_4$. The product (0.556 g, 86%) was obtained after flash chromatography (CH$_2$Cl$_2$). Rf. 0.2; MS (CI): 308; ^1H NMR (400 MHz, CDCl$_3$): δ 9.81 (s, 1H), 7.26 (br s, 5H), 5.02 (s, 2H), 3.46-3.40 (m, 2H), 2.81-2.65 (m, 2H).

N^1-Cbz-N^5, N^9, N^{14}, N^{18}-tetra-Boc-polyamine 3343 (14): To a mixture of aldehyde (13) (556.4 mg, 2.662 mmol) and spermine (1.347 g, 6.660 mmol) in dry EtOH (30 ml), Na$_2$SO$_4$ (1.89 g, 13.31 mmol) was added as drying agent. The mixture was stirred for 24 h, NaBH$_3$CN (1.338 g, 21.30 mmol) was added in portions. The reaction mixture was stirred overnight. To the reaction mixture, H$_2$O (10 ml) was added, then the aqueous solution was extracted with CH$_2$Cl$_2$ (25 ml x 3). The organic layer was dried over Na$_2$SO$_4$ and concentrated

to afford a crude product which was dissolved in CH_2Cl_2 (20 ml), followed by the addition of di-*tert*-butyl-dicarbonate (5.647 g, 25.85 mmol). The mixture was stirred for 2 h. After solvent was removed, the crude mixture was applied to flash chromatography (CH_2Cl_2/MeOH 98:2), product **14** (1.803 g, 85%) was obtained. Rf. 0.32; MS (CI): 794; ¹H NMR (400 MHz, CDCl$_3$): δ 7.50-7.32 (m, 5H), 5.10 (s, 2H), 3.32-2.96 (*m*, 16H), 1.77-1.55 (*m*, 10H), 1.62 (*s*, 36H).

N⁵, N⁹, N¹⁴, N¹⁸-Tetra-Boc-polyamine 3343 (10): To the protected amine (**14**) (89.2 mg, 0.113 mmol) in MeOH (8 ml) was added catalytic amount of 10% Pd/C. The mixture was purged with H_2 3 times, and stirred under H_2 for 6 h. The black suspension was filtered through Celite, washed extensively with MeOH, the filtrate was evaporated to dryness. An oily product **10** (67.3 mg, 91%) was obtained after flash chromatography (CH_2Cl_2/MeOH/*i*-PrNH$_2$ 95:5:1). MS (CI): 660; ¹H NMR (400 MHz, CDCl$_3$): δ 3.40-2.92 (*m*, 16H), 1.82-1.54 (*m*, 10H), 1.46 (*s*, 36H).

Spermine-lysine (16): To 5 ml DMF solution of spermine (152 mg, 0.751 mmol) was added Nα, Nω-di-Boc-L-lysine-N-hydroxysuccinimide ester (**18**) (184 mg, 0.375 mmol). This solution was stirred overnight at rt. After evaporation of the solvent, the residue was dissolved in 50 ml CH_2Cl_2 and was extracted with sat. NaHCO$_3$ (30 ml x 3). The organic layer was washed with brine (50 ml), then dried over MgSO$_4$ before filtered through a pad of Celite. The filtrate was concentrated and the oily residue was purified on a silica gel flash column with a step gradient of CH_2Cl_2/MeOH 9:1 and CH_2Cl_2/MeOH/*i*-PrNH$_2$ 4:4:1 which eluted the desired product (**16**) (104 mg, 48 %). MS (CI): 578; ¹H NMR (400 MHz, CDCl$_3$): δ 4.53-4.49 (*m*, 1H), 3.96-3.92 (*m*, 2H), 3.28-3.18 (*m*, 12H), 1.78-1.56 (*m*, 8H), 1.46-1.42 (*m*, 18H), 1.33-1.13 (*m*, 6H).

Spermine-arginine (15): Yield: 53%. MS (CI): 818; ¹H NMR (400 MHz, CDCl$_3$): δ 7.28-7.18 (*m*, 15H), 5.20-4.95 (*m*, 6H), 4.15 (*m*, 1H), 3.20-2.40 (*m*, 14H), 1.58-1.52 (*m*, 12H).

O-Benzyl-tyrosine methyl ester (24): To a solution of MeOH (20 ml) in 0°C was injected SOCl$_2$ (0.289 ml, 3.965 mmol), followed by the addition of O-benzyl-tyrosine (**8**) (715.4 mg, 2.64 mmol). The reaction mixture was stirred at 40°C for 4 h, and was concentrated. The residue was washed by 2N NaOH (10 ml), extracted by CH_2Cl_2 (25 ml x 3), dried over MgSO$_4$. The product (482.5 mg, 64%) was used without purification. Rf. 0.65 (90:10 CH_2Cl_2/MeOH); MS (CI): 287 (M+1)⁺; ¹H NMR (400MHz, CDCl$_3$): δ 7.45-7.31 (*m*, 5H), 7.0 (*d*, 2H, *J*=8.8 Hz), 6.90 (*d*, 2H, *J*=8.8 Hz), 5.02 (*s*, 2H), 3.68 (*s*, 3H), 3.62-3.61 (*m*, 1H), 3.10-2.72 (*m*, 2H).

O-Benzyl-butyl-tyrosine methyl ester (25): To a solution of MeCN (20 ml) containing **24** (0.940 g, 3.284 mmol) and 0.64g of KF/Celite was injected 1-bromobutane (0.317 ml, 2.955 mmol). The reaction mixture was refluxed for 10 h, and was filtered through Celite. The filtrate was removed by evaporation. After column chromatography purification (EtOAc/Hexane 35:65), the product (0.677 g, 67%) was obtained. Rf. 0.65; MS (CI): 342 (M+1)⁺; ¹H NMR (400 MHz, CDCl$_3$): δ 7.46-7.30 (*m*, 5H), 7.08 (*d*, 2H, *J*=8.8 Hz), 6.88 (*d*, 2H, *J*=8.8 Hz), 5.02 (*s*, 2H), 3.61 (*s*, 3H), 3.51-3.40 (*m*, 3H), 2.91-2.82 (*m*, 2H), 2.60-2.38 (*m*, 2H), 1.51-1.38 (*m*, 2H), 1.32-1.25 (*m*, 2H), 0.84 (*t*, 3H, *J*=7.2 Hz).

O-Benzyl-N-Boc-butyl-tyrosine methyl ester (26): To a solution of **25** (98.0 mg, 343.5 mmol) in CH_2Cl_2 (10 ml) was added di-*tert*-dicarbonate (150.1 mg, 687.0 mmol) and dry Et_3N (0.192 ml, 1.37 mmol). The mixture was stirred for 3 h, concentrated and purified by silica gel column (EtOAc/Hexane 35:65), product (132.3 mg, 100%) was yielded. *Rf.* 0.80; MS (CI): 442 (M+1)$^+$; ^1H NMR (400 MHz, CDCl$_3$): δ 7.46-7.30 (*m*, 5H), 7.16-7.09 (*br s*, 2H), 6.88 (*d*, 2H, *J*=8.8 Hz), 5.02 (*s*, 2H), 3.90-3.81 (*m*, 1H), 3.62 (*s*, 3H), 3.50-3.00 (*m*, 2H), 2.72-2.51 (*m*, 2H), 1.49 (*s*, 9H), 1.32 (*m*, 2H), 1.24-1.12 (*m*, 2H), 0.86 (*t*, 3H, *J*=7.2 Hz).

O-Benzyl-N-Boc-butyl-tyrosine alcohol (27): To CH_2Cl_2 (10 ml) solution of ester **26** (574.9 mg, 1.30 mmol) was injected 1.0M DIBAL (2.6 ml) in Hexane at 0°C under Ar protection. The reaction was stirred over 1 h until starting material disappeared by TLC analysis. The reaction was quenched by 1N HCl (15 ml), the aqueous solution was extracted with CH_2Cl_2. After the solvent was removed, the residue was applied to silica gel column (EtOAc/Hexane 35:65) to give **27** (609 mg, 94%). *Rf.* 0.5; MS (CI): 414 (M+1)$^+$; ^1H NMR (400 MHz, CDCl$_3$): δ 7.44-7.30 (*m*, 5H), 7.18-7.06 (*br s*, 2H), 6.89 (*d*, 2H, *J*=8.7 Hz), 5.05 (*s*, 2H), 3.88-3.55 (*m*, 4H), 3.51-3.46 (*m*, 1H), 3.15-3.02 (*m*, 2H), 2.95-2.70 (*m*, 2H), 1.45 (*s*, 9H), 1,39-1.18 (*m*, 4H), 0.84 (*t*, 3H, *J*=7.2 Hz).

O-Benzyl-N-Boc-butyl-tyrosine aldehyde (28): To an oven-dried flask containing CH_2Cl_2 (25 ml) under Ar at -50°C was added oxalyl chloride (25.3 ml, 0.29 mmol), followed by the addition of DMSO (41.3 ml, 0.58 mmol). The reaction mixture was stirred for 5 min, alcohol (**27**) (0.109 g, 0.26 mmol) in CH_2Cl_2 was added dropwise over 5 min, with continuous stirring at low temperature for 35 min. Et_3N (183.6 ml, 1.32 mmol) was added. The mixture was stirred for another 10 min, and then allowed to warm up to rt. The mixture was washed with H_2O (20 ml x 2) and the organic layer was dried over Na_2SO_4. The product **28** (116.8 mg, 98%) was obtained after flash chromatography (EtOAc/Hexane 35:65). *Rf.* 0.9; MS (CI): 412 (M+1)$^+$; ^1H NMR (400 MHz, CDCl$_3$): δ 9.58 (*d*, 1H, *J*=10 Hz), 7.42-7.28 (*m*, 5H), 7.05 (*d*, 2H, *J*=8.8 Hz), 6.88 (*d*, 2H, *J*=8.8 Hz), 5.02 (*s*, 2H), 3.60-3.50 (*m*, 1H), 3.40-3.12 (*m*, 2H), 3.10-2.90 (*m*, 1H), 2.42-2.24 (*m*, 2H), 1.45 (*s*, 9H), 1.36-1.14 (*m*, 4H), 0.82 (*t*, 3H, *J*=7.2 Hz).

O-Benzyl-C$_4$-PhTX-amine-3334 (20): To a mixture of aldehyde (**28**) (100 mg, 0.243 mmol) and polyamine-3334 (123 mg, 0.473 mmol) in dry EtOH (10 ml) was added Na_2SO_4 (341 mg, 2.40 mmol) as drying agent. The mixture was stirred for 24 hr, $NaBH_4$ (92.0 mg, 2.433 mmol) was then added in portions. The reaction mixture was stirred overnight. It was quenched with H_2O (2 ml), the aqueous solution was extracted with CH_2Cl_2 (15 ml x 3). The organic layer was then dried over Na_2SO_4. After purified by silica gel column (CH_2Cl_2/MeOH/*i*-PrNH$_2$ 85:10:5), the pure product (225.6 mg, 88%) was yielded. To a solution of this product in CH_2Cl_2 (5 ml) was added excess of TFA. The mixture was stirred for 1 h and was evaporated to dryness. This afforded final product (118.4 mg, 100%). FAB-MS ($C_{33}H_{58}N_6O$): 554 (M+1)$^+$; ^1H NMR (400 MHz, CD$_3$OD): δ 7.42-7.28 (*m*, 5H), 7.10-7.00 (*br s*, 2H), 6.84 (*d*, 2H, *J*=8.4 Hz), 5.02 (*s*, 2H), 3.72-3.70 (*br s*, 1H), 3.20-3.00 (*br m*, 10H), 2.95-2.51 (*br m*, 10H), 1.72-1.60 (*m*, 2H), 1.30-1.02 (*br m*, 4H), 0.88 (*t*, 3H, *J*=7.2 Hz).

C$_4$-PhTX-amine-3334 (21): To a solution **20** (27.6 mg, 50.0 mmol) in MeOH (5 ml) was added catalytic amount 10% Pd/C, purged with H$_2$ 3 times. The mixture was stirred under H$_2$ for 8 h. The black suspension was filtered through Celite and washed extensively with MeOH. The filtrate was evaporated to dryness. An oily product (22.4 mg, 99%) was obtained. MS (CI): 453(M+1)$^+$; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.10-7.00 (*br s*, 2H), 6.84 (*d*, 2H, *J*=8.4 Hz), 5.02 (*s*, 2H), 3.72-3.70 (*br s*, 1H), 3.20-3.00 (*br m*, 10H), 2.95-2.51 (*br m*, 10H), 1.72-1.60 (*m*, 2H), 1.46 (*m*, 45H), 1.30-1.02 (*br m*, 4H), 0.88 (*t*, 3H, *J*=7.2 Hz).

Pharmacological assays

These were undertaken on retractor unguis nerve-muscle preparations isolated from the hind legs of adult locusts (females)[11]. The preparations were continuously perfused with locust saline (in mM - NaCl, 180; KCl, 10; CaCl$_2$ 2: HEPES, 10, pH 6.8, adjusted with 1N NaOH) at rt (21-23°C). PhTX-343 (**2**), PhTX analogs and PhTX-amine analogs were applied at a range of concentrations for 10 min periods. The influence of these compounds on the amplitude of the neurally-evoked twitch contraction of the retractor unguis muscle was determined. Each concentration of a compound was tested at 3 times and a minimum of 6 concentrations of each compound was assayed. Dose-response curves derived from the recorded data gave the concentration of compound required to cause a 50% reduction in amplitude of the twitch contraction (IC$_{50}$ value).

Acknowledgments

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