Analogues of Neuroactive Polyamine Wasp Toxins That Lack Inner Basic Sites Exhibit Enhanced Antagonism Toward a Muscle-Type Mammalian Nicotinic Acetylcholine Receptor

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Philanthotoxin-433 (PhTX-433), a natural polyamine wasp toxin, is a noncompetitive antagonist of certain ionotropic receptors. Six analogues of PhTX-343 (a synthetic analogue of the natural product), in which the secondary amino groups are systematically replaced by oxygen or methylene groups, have been synthesized by coupling of *N*-(1-oxobutyl)tyrosine with 1,12-dodecanediamine, 4,9-dioxa-1,12-dodecanediamine, or appropriately protected di- and triamines, the latter being obtained by multistep syntheses. The resulting PhTX-343 analogues were purified and characterized, and their protolytic properties (stepwise macroscopic pK_a values) were determined by ^{13}C NMR titrations. All analogues are fully protonated at physiological pH. The effects of these compounds on acetylcholine-induced currents in TE671 cells clamped at various holding potentials were determined. All of the analogues noncompetitively antagonized the nicotinic acetylcholine receptor (nAChR) in a concentration-, time-, and voltage-dependent manner. The amplitudes of acetylcholine-induced currents were compared at their peaks and at the end of a 1 s application in the presence or absence of the analogues. Most of the analogues were equipotent with or more potent than PhTX-343. The dideaza analogue PhTX-12 [IC_{50} of 0.3 μM (final current value)] was the most potent, representing the highest potency improvement (about 50-fold) yet achieved by modification of the parent compound (PhTX-343). Thus, the presence of multiple positive charges in the PhTX-343 molecule is not necessary for antagonism of nAChR. In contrast, the compounds were much less potent than PhTX-343 at locust muscle ionotropic glutamate receptors sensitive to quisqualate (qGluR). The results demonstrate that the selectivity for different types of ionotropic receptors can be achieved by manipulating the polyamine moiety of PhTX-343.

Introduction

Philanthotoxin-433 [PhTX-433 (**1**), Figure 1] was isolated in 1988 from the venom of *Philanthus triangulum*,[1] an Egyptian digger wasp that preys on honeybees. PhTX-433 is the main venom constituent responsible for paralyzing the arthropod prey of this wasp through its antagonism of ionotropic glutamate receptors (GluR) present on skeletal muscle.[1−3] The synthetic analogue PhTX-343 (**2**) of the natural product also antagonizes these receptors.[1] Subsequently, a large number of analogues have been synthesized[4−14] and demonstrated to have a varying degree of noncompetitive antagonistic effect on insect muscle GluR,[1,5,13,15,16] insect and the *Torpedo* nicotinic acetylcholine receptors (nAChR),[6,11,14,17] and on native and cloned mammalian brain GluR.[6,18−22] The interest in philanthotoxins is primarily due to their potential as therapeutic agents, for example for neuroprotection,[23] and their importance as probes for receptor structure studies.

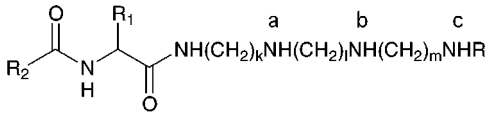

Figure 1. General structure of philanthotoxins PhTX-*klm*, where the numerals *klm* denote the number of methylene groups separating the nitrogen atoms (a, b, and c) in the polyamine side chain. PhTX-433 (**1**): R = H, R_1 = 4-hydroxybenzyl, R_2 = propyl, $k = 4$, $l = m = 3$. PhTX-343 (**2**): R = H, R_1 = 4-hydroxybenzyl, R_2 = propyl, $k = m = 3$, $l = 4$. **3**: R = H, R_1 = benzyl, R_2 = propyl, $k = 4$, $l = m = 3$. **4**: R = H, R_1 = benzyl, R_2 = propyl, $k = l = 3$, $m = 4$. **5**: R = $(CH_2)_4NH_2$, R_1 = 4-hydroxybenzyl, R_2 = hexyl, $k = l = m = 3$. **6**: R = $(CH_2)_3$-NH_2, R_1 = 4-hydroxybenzyl, R_2 = hexyl, $k = l = 3$, $m = 4$.

The term polyamine toxins, commonly used for philanthotoxins and also for the chemically and pharmacologically related class of compounds isolated from spider venoms,[24,25] implies the functional importance of the polyamine structural component. Indeed, it has been broadly accepted that the polyamine chain is essential for the antagonist effects exhibited by these compounds. In a proposed model of binding,[14,26] electrostatic interactions between the protonated amino groups and negatively charged residues in the interior of the receptor ion channel are expected to contribute to the binding.

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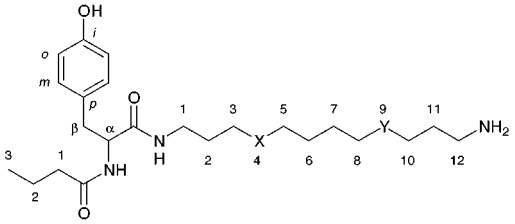
Chart 1



2 X = Y = NH (PhTX-343)
7 X = Y = CH_2 (PhTX-12)
8 X = Y = O (4,9-dioxa-PhTX-12)
9 X = NH, Y = CH_2 (PhTX-38)
10 X = CH_2, Y = NH (PhTX-83)
11 X = NH, Y = O (9-oxa-PhTX-38)
12 X = O, Y = NH (4-oxa-PhTX-83)

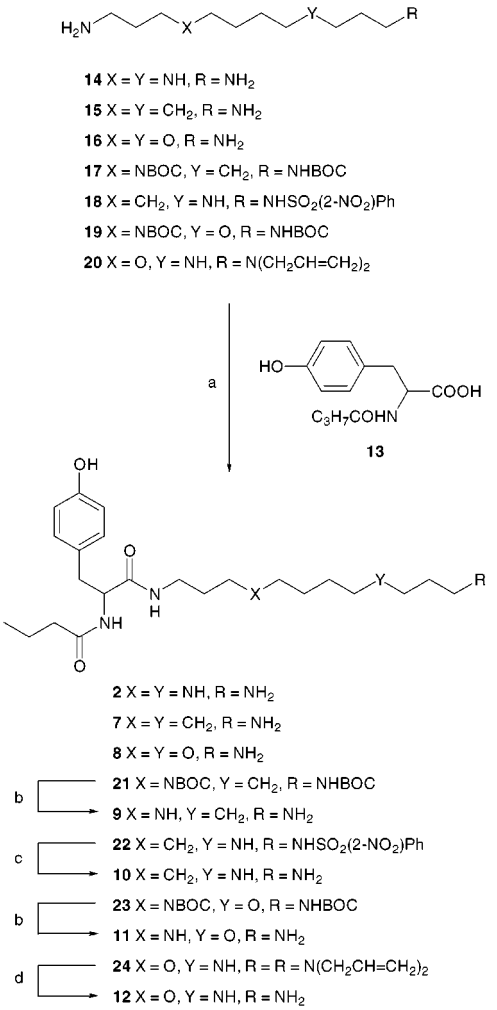
Consequently, differences in potencies of the analogues of **1**, which have different numbers of methylene groups between the nitrogen atoms (Figure 1), have been proposed to reflect the distribution of negatively charged groups at the binding site of the target macromolecule.[5,13] However, evidence for the electrostatic mode of binding of the polyamine chain to receptors is in fact rather limited. Acetylation of the primary amino group (N_c, Figure 1) in **2** greatly reduces antagonistic potency,[5,6,11] thus emphasizing the importance of the terminal electropositive site, but the assumed importance of the secondary amino groups is less clear.

The protolytic properties of PhTX-343 (**2**) have been studied using ^{13}C NMR spectroscopy.[27] This study showed that the inner nitrogen N_b is the first to be deprotonated when the pH is raised.[27] The extent of protonation of polyamine toxins in aqueous solution may be unrelated to their activities at ionotropic receptors, although it is interesting to note[27] that several analogues, which are predicted to be more easily deprotonated at the secondary amino groups than **2**, exhibited higher antagonistic activity. This is apparent when considering the relative potencies of philanthotoxins which have polyamine chains of the same length, but in which the separation of the secondary amino groups is changed from four to three methylene groups, resulting in reduced protonation.[27] Thus PhTX-433 (**1**) is less protonated, but more potent[5,6] than PhTX-343 (**2**). The same relationship was observed for the analogues of PhTX-433 (**1**) and PhTX-334 lacking the phenolic hydroxyl group, i.e., with **3** and **4**,[11] and also with **5** and **6** (Figure 1).[13] These observations prompted us to synthesize analogues of **2** in which the secondary amino groups are systematically replaced with either an oxygen atom or a methylene group, resulting in analogues lacking one or both of the inner basic sites (Chart 1). This paper presents the results of this work.

Results

Synthesis. The classical route to philanthotoxins is a coupling of appropriate amine with the *N*-acyl derivative of tyrosine using dicyclohexylcarbodiimide (DCC), with[13,28] or without[29] protection of the phenol group. Since DCC is expected to cause racemization of the product[30] and many of previously synthesized philanthotoxins have to be regarded as partially or fully racemized compounds, racemic *N*-(1-oxobutyl)tyrosine

Scheme 1[a]



14 X = Y = NH, R = NH_2
15 X = Y = CH_2, R = NH_2
16 X = Y = O, R = NH_2
17 X = NBOC, Y = CH_2, R = NHBOC
18 X = CH_2, Y = NH, R = $NHSO_2$(2-NO_2)Ph
19 X = NBOC, Y = O, R = NHBOC
20 X = O, Y = NH, R = $N(CH_2CH{=}CH_2)_2$

13

2 X = Y = NH, R = NH_2
7 X = Y = CH_2, R = NH_2
8 X = Y = O, R = NH_2
b → **21** X = NBOC, Y = CH_2, R = NHBOC → **9** X = NH, Y = CH_2, R = NH_2
c → **22** X = CH_2, Y = NH, R = $NHSO_2$(2-NO_2)Ph → **10** X = CH_2, Y = NH, R = NH_2
b → **23** X = NBOC, Y = O, R = NHBOC → **11** X = NH, Y = O, R = NH_2
d → **24** X = O, Y = NH, R = R = $N(CH_2CH{=}CH_2)_2$ → **12** X = O, Y = NH, R = NH_2

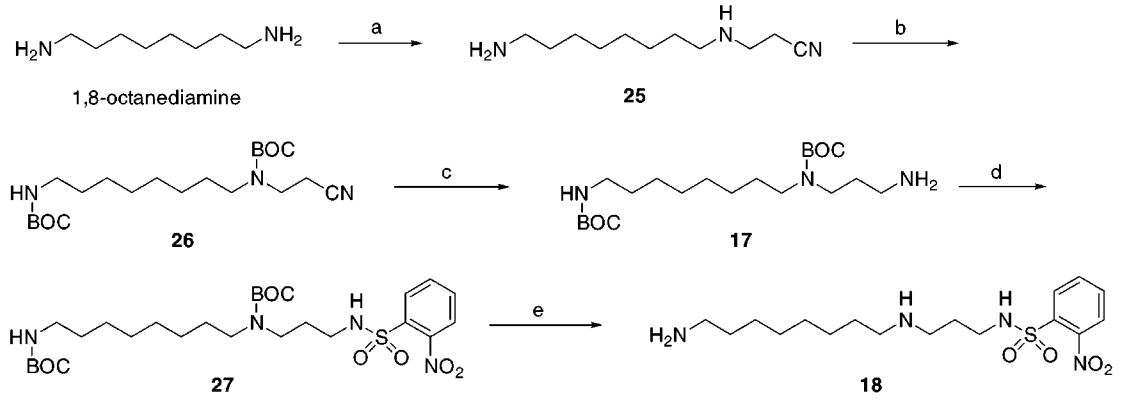
[a] Reagents: (a) DCC; (b) TFA; (c) 2-mercaptoethanol, DBU; (d) $Pd(PPh_3)_4$, *N*,*N*′-dimethylbarbituric acid.

(**13**) was used as a starting material for all coupling reactions. The latter, which to the best of our knowledge has not been appropriately characterized prior to this work,[29,31,32] was obtained by acylation of racemic tyrosine with an excess of butanoyl chloride and saponification of the O-acylated byproducts. PhTX-343 (**2**), PhTX-12 (**7**), and 4,9-dioxa-PhTX-12 (**8**) were prepared by acylation of commercially available amines **14**−**16** with **13** using standard coupling conditions[33] as outlined in Scheme 1.

For the synthesis of PhTX-38 (**9**), PhTX-83 (**10**), 9-oxa-PhTX-38 (**11**), and 4-oxa-PhTX-83 (**12**), a two step acylation−deprotection procedure was applied since the protected polyamines **17**−**20** were used in the coupling reactions (Scheme 1). The acylation was performed as described for **2**, **7**, and **8**, and the deprotection of the amino groups were carried out according to literature procedures. Thus, BOC groups were removed by treatment with TFA and 2-nitrobenzenesulfonamide group using 2-mercaptoethanol and 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU).[34,35] The allyl groups were removed using tetrakis(triphenylphosphine)palladium(0) and *N*,*N*′-dimethylbarbituric acid[36] (Scheme 1).

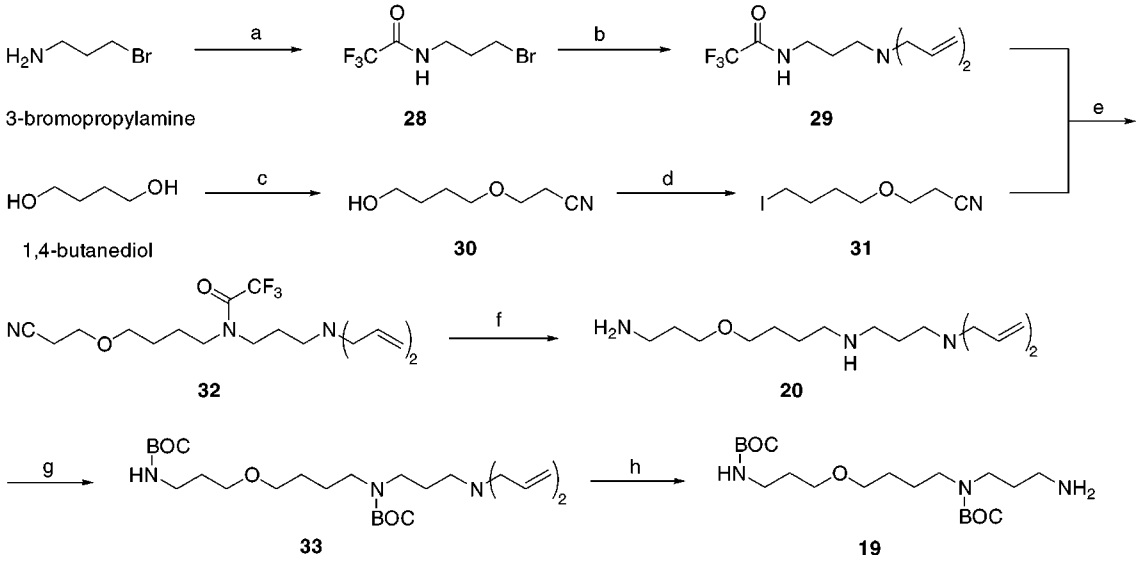
The protected polyamines **17** and **18** were obtained as shown in Scheme 2. Conjugate addition of 1,8-octanediamine to acrylonitrile gave **25**. It was not

Scheme 2[a]



[a] Reagents: (a) acrylonitrile; (b) $(BOC)_2O$; (c) H_2, Raney Ni; (d) $Ph(2\text{-}NO_2)SO_2Cl$, pyridine; (e) TFA.

Scheme 3[a]



[a] Reagents: (a) CF_3COOEt, Et_3N; (b) diallylamine; (c) acrylonitrile, Triton B; (d) $(PhO)_3(CH_3)P^+I^-$; (e) NaH; (f) $LiAlH_4$; (g) $(BOC)_2O$; (h) $Pd(PPh_3)_4$, *N*,*N*′-dimethylbarbituric acid.

possible to avoid some bis(cyanoethylation) in this reaction, but the desired product was easily purified by flash chromatography. The amino groups in **25** were protected using di(*tert*-butyl) dicarbonate, prior to reduction of the nitrile group using Raney nickel[37,38] resulting in the diprotected triamine **17**. The latter reaction is superior to the previously reported procedures using $LiAlH_4$,[4,9] which is expected to give some reduction of the BOC groups.[39,40] The diprotected triamine **17** was converted to the monoprotected triamine **18** in a protection−deprotection procedure. Thus, the primary amino group was protected using 2-nitrobenzenesulfonyl chloride[34] affording **27**, followed by removal of the BOC groups with TFA to give **18**.

The protected triamines **19** and **20** were prepared as shown in Scheme 3. The two key intermediates **29** and **31** were prepared as follows. Trifluoroacetylation of 3-bromopropylamine with ethyl trifluoroacetate followed by reaction with diallylamine afforded protected 1,3-propanediamine (**29**). 3-(4-Iodobutyloxy)propanenitrile (**31**) was obtained by cyanoethylation of 1,4-butanediol, followed by treatment of the resulting nitrile **30** with methyltriphenoxyphosphonium iodide.[41,42] Alkylation of **29** with **31** afforded the nitrile **32**. Conversion of the nitrile group in **32** to the amino group using $LiAlH_4$ and simultaneous removal of the trifluoroacetyl protecting group[43] afforded the diallyl protected polyamine **20**. The di-BOC protected polyamine **19** was prepared from **20** by treatment with di(*tert*-butyl) dicarbonate followed by cleavage of the allyl groups as described above.

Purity of the so-obtained philanthotoxins **2** and **7**−**12** was a major concern, since simple polyamines such as spermine modulate the function of neuronal ion channels,[44−47] and their presence as impurities of the final products had to be avoided. In the case of 9-oxa-PhTX-38 (**11**) and 4-oxa-PhTX-83 (**12**), flash column chromatography was sufficient to provide pure products. For the remaining philanthotoxins, satisfactory purity was achieved only by the use of preparative, reversed phase HPLC. Electrospray-ionization mass spectrometry recording the total ion current (TIC) was especially useful as the HPLC detection method and enabled a reasonable quantification of polyamine impurities which are not detectable by UV absorption. Thus, purity of the final products as assessed by HPLC-TIC was 98−100%. The yields of the purified philanthotoxins **2** and **7**−**12** were in the range 13−54%, based on **13** (Scheme 1). The structure of all intermediates and final products was confirmed by ^1H and ^{13}C NMR, and that for the final products also by HRMS.

NMR Spectroscopy. The protolytic properties of the PhTX-343 analogues **7**−**12** were determined by ^{13}C NMR titrations, similarly as previously described.[27] Thus, the ^{13}C NMR spectra were recorded as a function of the ionization state of the compounds in the pH range of 7.5−12.7. Since the acid−base equilibria are fast in the NMR time scale, the chemical shifts δ_i of each carbon atom of a compound with n ionizable hydrogens changes with pH according to the relationship

$$\delta_i = \frac{\delta_i^0 K_1 K_2 ... K_n + \delta_i^1 K_1 K_2 ... K_{n-1}[H^+] + \delta_i^2 K_1 K_2 ... K_{n-2}[H^+]^2 + ... + \delta_i^n [H^+]^n}{[H^+]^n + K_1[H^+]^{n-1} + K_1 K_2 [H^+]^{n-2} + ... + K_1 K_2 ... K_{n-1}[H^+] + K_1 K_2 ... K_n}$$

where δ_i^0, δ_i^1, ... δ_i^n are the intrinsic chemical shifts of ith site in nonprotonated, monoprotonated, and n-protonated (fully protonated) species, respectively, and K_1, K_2, ... K_n are the successive equilibrium constants for deprotonation. The pK_a values were obtained by simultaneous nonlinear fitting of a set of these equations (one for each of the carbon atoms in the molecule) to a set of experimental data (pH dependent chemical shifts of all carbons, δ_i). In the case of **8**−**12** the solvent was H_2O/D_2O 9:1. The amount of D_2O was sufficient for the internal field-frequency lock required by the spectrometer, but it was low enough to eliminate the necessity of corrections for solvent isotope effect.[48] To avoid uncertainties due to changes of the ionic strength of the solution during the NMR titration, the experiments were carried out at a high (1 M) ionic strength. The titration of **7** was carried out in 1:1 H_2O/CD_3OD because of the insolubility of the material in water.

Figure 2 shows an example of the ^{13}C NMR titration curves, and the macroscopic pK_a values determined in this way are shown in Table 1. Dissociation curves for **7**−**12** are shown in Figure 3. Unlike with **2**,[27] the analogues **7**−**12** start to dissociate only above pH 7.5, and hence are fully protonated at the physiological pH.

In Vitro Electrophysiology. Whole-cell patch-clamp recordings were used to investigate the effects of PhTX-343 (**2**) and **7**−**12** on human muscle-type nAChR of TE671 cells. This cell line is identical with the human rhabdomyosarcoma RD cell line[49] and expresses embryonic muscle-type nAChR, which contains a δ-subunit rather than an ϵ-subunit as is the case for nAChR of adult muscle.[50,51] The sensitivity of the nAChR of TE671 cells to acetylcholine was as described previously.[47] PhTX-343 (**2**) and analogues were co-applied with acetylcholine as 1 s pulses. The cells were clamped at a holding potential (V_H) of either −25 mV, −50 mV, or −100 mV. Peak current amplitudes and final current amplitudes (at the end of the 1 s pulse) obtained in response to acetylcholine were compared with those obtained during co-application of the agonist with philanthotoxins. Concentration−inhibition curves were constructed at each of the three V_H (Figure 4). The derived IC_{50} values for PhTX-343 (**2**) and the analogues tested are shown in Figure 5 (for exact values, see Experimental Section). In each case, the IC_{50} value decreased as V_H was made more negative. Recovery from antagonism was complete in about 30 s after removal of the inhibitor. Like with PhTX-343 (**2**), there was a small but significant potentiation of responses to acetylcholine (V_H = −25 mV) in some cells with **9** and **10** when they were applied at concentrations just below those that antagonize the nAChR.[47]

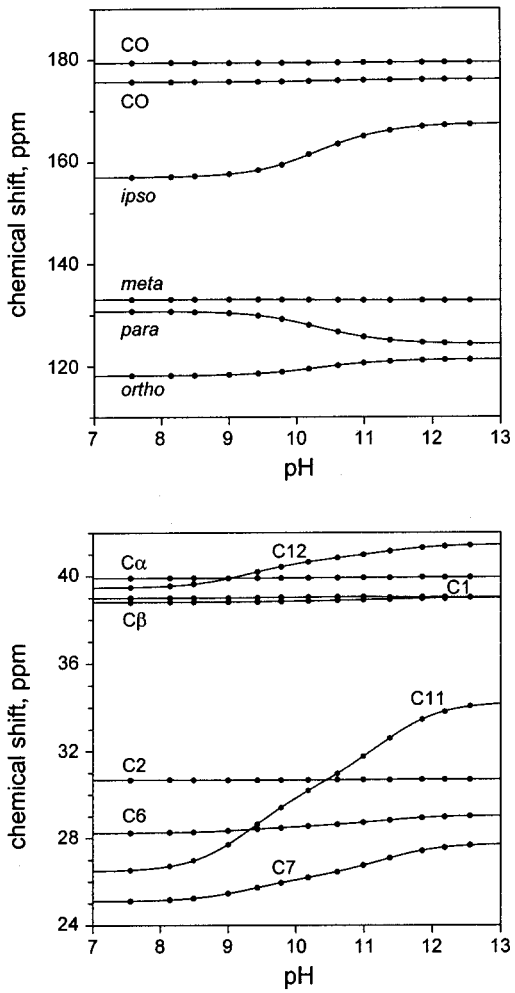


Figure 2. Selected ^{13}C NMR titration curves for **12** (H_2O/D_2O 9:1, 25 °C, ionic strength 1 M). The points represent experimental data, and the curves are the best fits through the data.

Table 1. Macroscopic Acidity Constants (pK_a Values) of PhTX-343 Analogues **7**−**12**[a,b]

compound	pK_1	pK_2	pK_3
PhTX-12 (**7**)[c]	10.28 ± 0.04	11.29 ± 0.06	
4,9-dioxa-PhTX-12 (**8**)	10.01 ± 0.02	10.86 ± 0.02	
PhTX-38 (**9**)	9.71 ± 0.03	10.43 ± 0.04	11.29 ± 0.02
PhTX-83 (**10**)	9.32 ± 0.02	10.24 ± 0.01	11.41 ± 0.01
9-oxa-PhTX-38 (**11**)	9.47 ± 0.06	10.25 ± 0.06	10.91 ± 0.04
4-oxa-PhTX-83 (**12**)	9.23 ± 0.01	10.26 ± 0.01	11.34 ± 0.02

[a] From ^{13}C NMR titrations in H_2O/D_2O 9:1, 25 °C, and 1 M ionic strength, unless stated otherwise. [b] The pK_a values were obtained by multiple nonlinear regression involving all carbon atoms of the molecule; standard deviations are from the regression analysis. [c] In H_2O/CD_3OD 1:1, 25 °C, and 1 M ionic strength.

PhTX-343 (**2**) is generally regarded as a noncompetitive inhibitor of ionotropic receptors. Preliminary determinations of concentration−response curves for acetylcholine (10^{-8}−10^{-3} M) obtained in the presence and absence of the inhibitors (10 μM **2**, 1 μM **7**, and 20 μM **11**) demonstrated similar behavior for the three compounds (data not shown). Thus, while the maximum response to acetylcholine was slightly reduced, as expected for a noncompetitive antagonism, there was a 2−3-fold increase in the EC_{50} value for acetylcholine, suggesting the presence of some competitive antagonism. This is in agreement with previous studies which

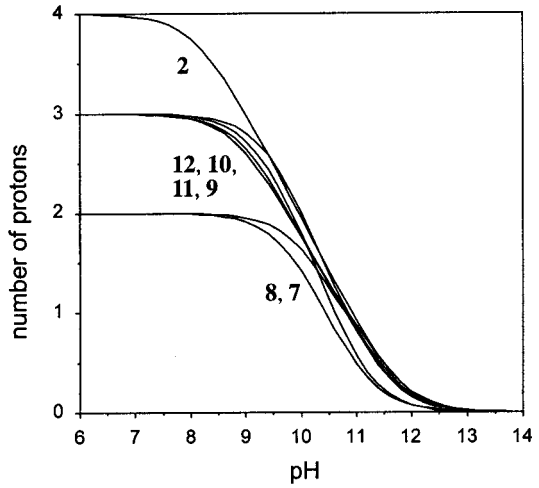


Figure 3. Dissociation curves for **2** (data from ref 27) and **7**−**12** (data in Table 1). The compounds are listed in the order of the appearance of the curves next to the numbers (from left to right).

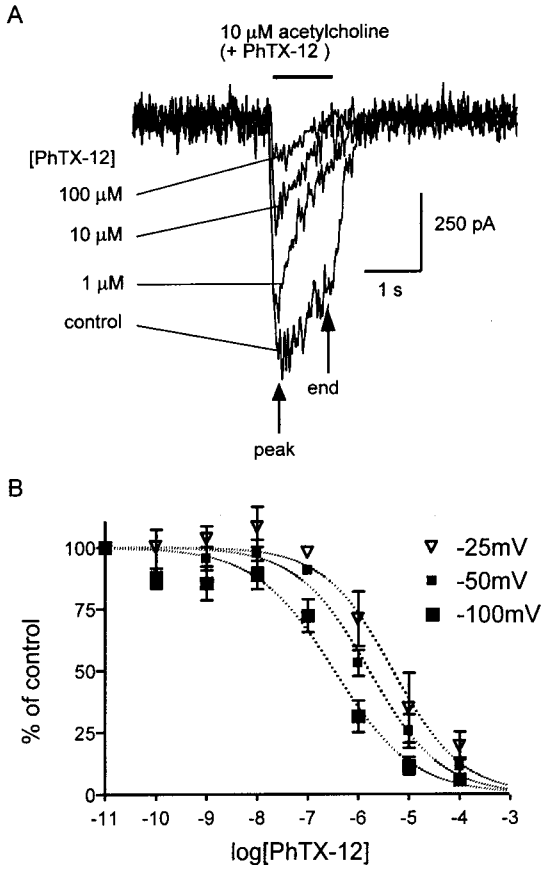


Figure 4. Effects of PhTX-12 (**7**) on nAChR of TE671 cells measured as reduction of the amplitude of acetylcholine-induced whole-cell currents. (A) Superimposed inward currents (holding potential $V_H = -100$ mV) elicited by a 1 s pulse of acetylcholine (10 μM; control) and by coapplication of acetylcholine (10 μM) with **7** (1, 10, and 100 μM) demonstrating concentration and time dependence of antagonism. (B) Concentration−inhibition data for **7** at V_H values of −25, −50, and −100 mV demonstrating voltage dependence of antagonism.

also suggested the presence of a competitive component in the action of PhTX-433 (**1**) on *Torpedo* nAChR.[52]

The compounds **7**−**12** were also tested on insect ionotropic GluR in the classical locust muscle assay.[53] In this assay, extensively used in the previous studies

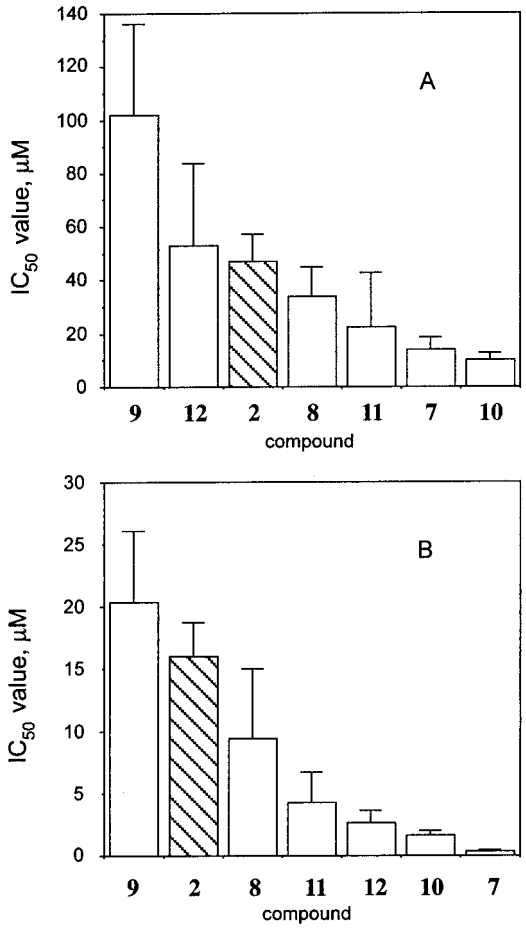


Figure 5. IC_{50} values for antagonism by PhTX-343 (**2**) and analogues **7**−**12** of acetylcholine-elicited inward currents in TE671 cells (holding potential $V_H = -100$ mV). Acetylcholine (10 μM) was coapplied with the antagonists in the concentration range 10^{-10} to 10^{-4} M. (A) IC_{50} values obtained from antagonism of peak acetylcholine-elicited current. (B) IC_{50} values obtained from antagonism of final acetylcholine-elicited current.

of philanthotoxins,[1,5,8,10,13,15,16] the analogues **7**−**12** had IC_{50} values greater than 100 μM. The IC_{50} value of PhTX-343 (**2**) in this assay was 23 μM.[10]

Discussion

Since PhTX-343 (**2**) is readily available, it has become a prototype polyamine toxin, widely used as a reference compound. The analogues **7**−**12** obtained in this work are structurally similar to **2**, except for the changes introduced to the polyamine chain that lead to diminished polycationic character. These changes result in a decreased charge interaction within the molecule leading to elevated pK_a values, with the remaining amine groups being fully protonated at physiological pH, as demonstrated by ^{13}C NMR. If the charge interactions between the PhTX-343 (**2**) molecule and the TE671 cell nAChR were primarily responsible for antagonism exhibited by this compound, the analogues having a decreased number of positive charges might be expected to be significantly less potent than **2**.

However, this is far from being the case (Figure 5). Like PhTX-343 (**2**), all of the analogues **7**−**12** antagonized acetylcholine-elicited responses of TE671 cells. IC_{50} values for data measured at the end of the acetylcholine-induced current were lower than those

obtained from measurements of current peaks (Figure 5). In other words, there was less antagonism during the initial phase (about 200 ms) of the response to acetylcholine than at its end. This suggests that the antagonists bind slowly to nAChR, possibly because antagonism is use-dependent. Since the IC_{50} values decreased as the holding potential V_H was made more negative, it seems likely that the analogues are open-channel blockers. The largely noncompetitive behavior known for PhTX-343 (**2**) was demonstrated with **7** and **11**.

The difference in the rank order of potency of the antagonists for the two sets of measurements (Figure 5) is insignificant, with possible exception of **12**. The analogues **7** and **10** were the most potent antagonists, whereas **9** was apparently the least active compound in the TE671 assay. Indeed, PhTX-12 (**7**), with an IC_{50} value of 0.3 μM (inhibition of final current), is about 50 times more potent than PhTX-343 (**2**). Such increase of potency on an ionotropic receptor upon chemical modification of **2** has never been observed before. On the other hand, the analogues **7**−**12** were inactive at the locust muscle ionotropic glutamate receptors sensitive to quisqualate (qGluR).

Earlier studies have demonstrated that the introduction of an aliphatic branch into the polyamine chain of PhTX-433 (**1**) resulted in increased activity on nAChR[6] and to a lesser extent on qGluR,[5] emphasizing the importance of the lipophilic interaction of the polyamine moiety with the binding site. PhTX-12 (**7**) was first synthesized by Piek et al. more than 10 years ago[17,54,55] and tested on the nAChR of the locust muscle, where it was twice as potent as PhTX-343 (**2**).[11] Our work now demonstrates systematically that the philanthotoxin analogues lacking inner basic sites not only retain but can have a strongly increased antagonist effect toward human muscle-type nAChR. This suggests that electrostatic interactions between electropositive sites on the polyamine moiety and complementary sites on the receptor are not the basis for binding of these compounds to nAChR. This is in contrast to the locust muscle qGluR and rat brain NMDA receptor (NMDAR), which may require the presence of the polycationic residues as suggested by the present and previous[11,56] results. This substantial difference opens up the possibility of developing philanthotoxin analogues that selectively target different ionotropic receptors.

Experimental Section

Chemistry. General Procedures. All chemicals were obtained from Aldrich Chemical Co., except for spermine (**14**) and 1,12-dodecanediamine (**15**) which were purchased from Sigma Chemical Co., and used without further purification, unless stated otherwise. Dimethylformamide (DMF), dichloromethane, and 1,2-dimethoxyethane (DME) were dried over molecular sieves. Flash column chromatography was carried out using Scharlau 60 230−400 mesh silica gel (Sorbil). Thin-layer chromatography (TLC) was performed on Merck 60 F_{254} 0.25 mm, 5 × 7.5 cm silica gel plates.

NMR spectra were recorded on a Varian Gemini 2000 spectrometer, a Bruker AMX 400 spectrometer, or a Bruker Avance DRX 500 spectrometer, operating for ^{1}H at 300.07, 400.13, and 500.13 MHz, respectively, using tetramethylsilane (TMS) or sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as internal standard for organic solvents and D_2O solutions, respectively. Coupling constants (^nJ) are expressed as numeric values in hertz.

Analytical and preparative HPLC were run on a system consisting of two Hitachi LC8A pumps. The UV trace was obtained with a Hitachi SPD10A detector operating at 274 nm. For HPLC-MS, a Perkin-Elmer API 150EX mass spectrometer equipped with Turboionspray (electrospray ionization) source recorded total ion current (TIC) in positive mode for m/z 100−1000. Analytical HPLC-MS was run injecting 10 μL samples to a 50 × 4.6 mm YMC RP18 column, with 2 mL/min of water/acetonitrile/TFA 90:10:0.05 raising to 10:90:0.05 during 7 min. Preparative HPLC-MS was run with 190 μL injections (50 mg samples in 1.0 mL MeOH), a 50 × 20 mm YMC RP18 column, and the same solvent gradient at 22.7 mL/min, with MS (TIC) detection using a split system.

Accurate mass measurements (±5 ppm) were performed at the Department of Chemistry, University of Odense, Odense, Denmark, on a Kratos MS50RF mass spectrometer equipped with a FAB source, using glycerol as matrix.

(*R,S*)-*N*-(1-Oxobutyl)tyrosine (13). (*R,S*)-Tyrosine (4.140 g, 22.85 mmol) was dissolved in 2 M NaOH (24 mL, 48 mmol) and the solution diluted with THF (50 mL). Butanoyl chloride (10 mL, 91 mmol) was added in small portions, and the reaction mixture was stirred overnight. Another portion of 2 M NaOH (10 mL, 20 mmol) and butanoyl chloride (5 mL, 46 mmol) was added and the stirring continued overnight. The solution was acidified to pH 1 and extracted with EtOAc (3 × 100 mL). The combined extracts were dried ($MgSO_4$) and evaporated in vacuo, and the residue dissolved in 2-propanol (80 mL) and stirred overnight with 40 mL of 2 M NaOH. The solution was acidified to pH 1 and extracted with EtOAc (3 × 100 mL). The combined extracts were dried ($MgSO_4$) and evaporated, and the crude product was crystallized from diethyl ether/light petroleum to give colorless crystals (4.17 g, 56%); mp 150−152 °C (corr.). ^{1}H NMR (400 MHz, CD_3OD): δ 0.84 (t, ^3J = 7.4, 4-CH_3), 1.54 (s, ^3J = 7.4, 3-CH_2), 2.13 (m, 2-CH_2), 2.83 and 3.10 (each dd, $^2J_{AB}$ = 14.1, $^3J_{AX}$ = 9.2, $^3J_{BX}$ = 5.0, β-CH_2), 4.50 (dd, α-CH), 6.64 and 7.03 (each 2H, AA′BB′ system, aromatic H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 13.9, 20.3, 37.7, 38.7, 55.2, 116.2 (2 C), 129.2, 131.3 (2 C), 157.4, 175.1, 176.0. Anal. ($C_{13}H_{17}NO_4$) C, H, N.

(*R,S*)-*N*-[3-[[4-[(3-Aminopropyl)amino]butyl]amino]-propyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide (2). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (0.500 g, 1.99 mmol) and DCC (0.452 g, 2.19 mmol) were dissolved in DME/CH_2Cl_2 (1:1, 15 mL) and stirred at room temperature for 30 min. The solution was added dropwise to a solution of spermine (**14**) (0.805 g, 3.98 mmol) in CH_2Cl_2 (5 mL), and the reaction mixture was stirred at room temperature for 20 h. The solvent was evaporated in vacuo, and the white, solid residue was purified by flash chromatography (CH_2Cl_2/MeOH/25% aqueous ammonia 4:2:1) to give a viscous, pale yellow oil (0.407 g). The oil was further purified by preparative HPLC with TIC detection to give 0.395 g (26%) of tris(trifluoroacetate) of **2** as a clear gum. The material was 100% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (500 MHz, CD_3OD): δ 0.86 (t), 1.55 (s) and 2.17 (m) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both ^3J = 7.3), 4.37 (t, ^3J = 8.0, α-CH), 6.71 and 7.05 (each 2 H, aromatic H), 1.79 (m, 2-, 6-, and 7-CH_2), 2.08 (p, 11-CH_2), 2.80−3.29 (m, 14 H, β-CH_2 and the remaining CH_2 of the polyamine moiety). ^{13}C NMR (100.6 MHz, H_2O/D_2O 9:1, pH 7.53): δ 15.3, 21.5, 25.5 (2 C), 26.6, 28.0, 38.7, 38.9, 39.5, 39.9, 47.3, 47.7, 49.7, 49.8, 58.3, 118.2 (2 C), 130.8, 133.2 (2 C), 157.1, 176.4, 179.7.[27] HRMS (FAB): $C_{23}H_{41}N_5O_3$ requires M + 1 at m/z 436.329, found 436.328.

(*R,S*)-*N*-(12-Aminododecyl)-4-hydroxy-α-[(1-oxobutyl)-amino]benzenepropanamide (7). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (1.314 g, 5.23 mmol) and DCC (1.187 g, 5.75 mmol) were dissolved in DMF (50 mL) and stirred at room temperature for 30 min. The solution was added dropwise to a solution of 1,12-dodecanediamine (**15**) (5.240 g, 26.15 mmol) in warm DMF (100 mL), and the reaction mixture was stirred at room temperature for 20 h. The solvent was evaporated in vacuo, and the white, solid residue was purified by flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 50:50:1] to give a viscous,

pale yellow oil (1.102 g). The oil was further purified by preparative HPLC with TIC detection to give 0.723 g (25%) of the trifluoroacetate of **7** as a clear gum. The material was 98.7% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (400 MHz, CD_3OD): δ 0.84 (t), 1.54 (s), and 2.15 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.3$), 2.77 and 2.95 (each dd, $^2J_{AB} = 13.7$, $^3J_{AX} = 8.2$, $^3J_{BX} = 7.2$, β-CH_2), 4.48 (dd, α-CH), 6.68 and 7.03 (each 2 H, aromatic H), 2.87−3.18 (m, 1- and 12-CH_2), 1.18−1.43 (m, 18 H) and 1.64 (m, 2 H) (remaining CH_2 of the diamine moiety). ^{13}C NMR (100.6 MHz, CD_3OD): δ 14.0, 20.3, 27.5, 27.9, 28.7, 30.2, 30.3, 30.4, 30.5, 30.6 (3 C), 38.5, 38.8, 40.5, 40.8, 56.5, 116.2 (2 C), 129.1, 131.3 (2C), 157.4, 173.8, 175.9. HRMS (FAB): $C_{25}H_{43}N_3O_3$ requires M + 1 at *m/z* 434.338, found 434.340.

(*R,S*)-*N*-(4,9-Dioxa-12-aminododecyl)-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide (8). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (0.250 g, 1.00 mmol) and DCC (0.226 g, 1.09 mmol) were dissolved in DME/CH_2Cl_2 (1:1, 10 mL) and stirred at room temperature for 30 min. The solution was added dropwise to a solution of 4,9-dioxa-1,12-dodecanediamine (**16**) (0.423 mL, 1.99 mmol) in CH_2Cl_2 (5 mL), and the reaction mixture was stirred at room temperature for 20 h. The solvent was evaporated in vacuo, and the oily residue was purified by flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 100:100:1] to give a viscous, clear oil (0.260 g). The oil was further purified by preparative HPLC with TIC detection to give 0.232 g (42%) of the trifluoroacetate of **8** as a clear gum. The material was 100% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (400 MHz, CD_3OD): δ 0.85 (t), 1.55 (s), and 2.15 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.4$), 2.78 and 2.95 (each dd, $^2J_{AB} = 13.9$, $^3J_{AX} = 7.9$, $^3J_{BX} = 7.4$, β-CH_2), 4.46 (dd, α-CH), 6.69 and 7.03 (each 2 H, aromatic H), 1.59−1.76 (m, 2-, 6-, 7-, and 11-CH_2), 3.10−3.51 (m, 1-, 3-, 5-, 8-, 10-, and 12-CH_2). ^{13}C NMR (100.6 MHz, CD_3OD): δ 14.0, 20.3, 27.6 (2 C), 30.3, 33.5, 37.8, 38.5, 38.8, 40.2, 56.5, 69.4, 70.1, 71.8 (2 C), 116.3 (2 C), 128.9, 131.3 (2 C), 157.6, 173.7, 175.9. HRMS (FAB): $C_{23}H_{39}N_3O_5$ requires M + 1 at *m/z* 438.297, found 438.299.

(*R,S*)-*N*-[3-[(8-Aminooctyl)amino]propyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide (9). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (0.111 g, 0.442 mmol) and **17** (0.177 g, 0.44 mmol) were dissolved in DME/CH_2Cl_2 (1:5, 30 mL), DCC (0.100 g, 0.49 mmol) was added, and the reaction mixture was stirred at room temperature for 20 h. The solution was filtered through Celite, the filtrate was concentrated in vacuo, and the residue was purified by flash chromatography (CH_2Cl_2/MeOH 50:1, 40:1, 30:1, and 20:1) to give **21** as a white, sticky solid (0.230 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.91 (t) and 2.19 (m) (respectively 4-CH_3 and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.4$), 4.62 (br m, α-CH), 6.74 and 7.04 (each 2 H, aromatic H), 1.19−1.67 [m, 2 × $C(CH_3)_3$, 2-, 6-, 7-, 8-, 9-, 10-, and 11-CH_2, 3-CH_2 of the 1-oxobutyl moiety], 2.82−3.18 (m, 1-, 3-, 5-, and 12-CH_2, β-CH_2). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 13.7, 19.0, 26.5, 26.6, 27.5, 28.4 (7 C), 28.6, 28.9, 29.1, 30.0, 35.6, 38.1, 38.6, 40.5, 43.0, 47.0, 54.4, 79.4, 79.7, 115.4 (2 C), 128.0, 130.5 (2 C), 155.4, 156.3, 156.5, 170.8, 172.7.

For deprotection of **21**, 0.209 g (0.33 mmol) was dissolved in CH_2Cl_2 (25 mL), TFA (2.5 mL, 32.5 mmol) was added, and the solution was stirred for 2 h at room temperature, concentrated, triturated with diethyl ether, and dried under high vacuum to give 0.194 g of a yellow, sticky solid. The product was purified by preparative HPLC with TIC detection to give the bis(trifluoroacetate) of **9** as a slightly yellow oil (0.143 g, 66%). The material was 99.5% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (400 MHz, CD_3OD): δ 0.87 (t), 1.56 (s), and 2.17 (m) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.4$), 4.37 (t, $^3J = 7.6$, α-CH), 6.71 and 7.05 (each 2 H, aromatic H), 1.40 (br s, 7-, 8-, 9-, and 10-CH_2), 1.62−1.70 (m, 6- and 12-CH_2), 1.78 (p, 2-CH_2), 2.81−3.28 (m, 1-, 3-, 5-, 12-, and β-CH_2). ^{13}C NMR (100.6 MHz, H_2O/D_2O 9:1, pH 7.40): δ 15.3, 21.5, 28.0 (2 C), 28.1 (2 C), 29.4, 30.5 (2 C), 38.7, 38.8, 40.0, 42.4, 42.5, 50.5, 58.3, 118.2 (2 C), 130.8, 133.1 (2 C), 157.1, 176.4, 179.7. HRMS (FAB): $C_{24}H_{42}N_4O_3$ requires M + 1 at *m/z* 435.333, found 435.333.

(*R,S*)-*N*-[8-[(3-Aminopropyl)amino]octyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide (10). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (0.173 g, 0.69 mmol) and DCC (0.157 g, 0.76 mmol) were dissolved in DMF (10 mL) and stirred at room temperature for 30 min. The solution was added dropwise to a solution of **18** (0.400 g, 1.04 mmol) in DMF (10 mL), and the reaction mixture was stirred at room temperature for 16 h. The solvent was evaporated in vacuo and the crude product purified by flash chromatography [EtOAc/heptane/$(CH_3)_2CHNH_2$ 5:5:1 and 3:3:1]. Second purification by flash chromatography (CH_2Cl_2/MeOH 1:1) gave **22** as a pale yellow oil (0.196 g, 46%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.92 (t), 1.63 (s), and 2.17 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.2$), 2.82 and 3.01 (each dd, $^2J_{AB} = 13.2$, $^3J_{AX} = 9.4$, $^3J_{BX} = 5.2$, β-CH_2), 4.54 (m, α-CH), 6.69 and 7.03 (each 2 H, tyrosine H), 1.17−1.30 (m, 8 H), 1.50 (p, 2 H) and 1.74 (p, 2 H) (2-, 3-, 4-, 5-, 6-, 7-, and 11-CH_2), 2.75 (t, 2 H, $^3J = 6.6$), 2.61 (t, 2 H, $^3J = 7.1$), 2.99 (m, 1 H) and 3.17−3.24 (m, 3 H) (1-, 8-, 10-, and 12-CH_2), 7.67−7.71 (m, 2 H), 7.79−7.81 (m, 1 H), and 8.08−8.10 (m, 1 H) (sulfonamide aromatic H), 1.04 (m, 2 H). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 13.5, 18.9, 26.2, 26.5, 28.3, 28.6, 28.7, 28.8, 29.2, 38.3, 39.2, 42.9, 47.8, 49.4, 50.6, 54.9, 115.6 (2 C), 125.0, 127.4, 130.2 (2 C), 130.9, 132.4, 133.3, 133.6, 148.0, 155.8, 170.7, 172.8. MS (ES): 630 (M + 1).

For deprotection of **22**, 0.475 g (0.77 mmol) was dissolved in DMF (50 mL), DBU (0.46 mL, 3.07 mmol) and 2-mercaptoethanol (0.11 mL, 1.53 mmol) were added, and the mixture was stirred at room temperature for 2 h. The solvent was evaporated in vacuo, and the residue was purified by flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$] to give a yellow oil (0.159 g), which was further purified by preparative HPLC with TIC detection to give the bis(trifluoroacetate) of **10** as a clear viscous oil (0.140 g, 28%). The material was 97.8% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (400 MHz, CD_3OD): δ 0.85 (t), 1.54 (s), and 2.15 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.4$), 2.78 and 2.95 (each dd, $^2J_{AB} = 13.8$, $^3J_{AX} = 8.2$, $^3J_{BX} = 7.2$, β-CH_2), 4.46 (dd, α-CH), 6.69 and 7.04 (each 2 H, aromatic H), 1.18−1.44 (m, 10 H) and 1.69 (p, 2 H) (2-, 3-, 4-, 5-, 6-, and 7-CH_2), 2.98−3.18 (m, 1-, 8-, 10-, and 12-CH_2). ^{13}C NMR (100.6 MHz, H_2O/D_2O 9:1, pH 7.64): δ 15.3, 21.7, 26.6, 28.1, 28.2, 28.3, 30.5, 30.6 (2 C), 39.1, 39.5, 39.9, 41.9, 47.2, 50.6, 58.3, 118.1 (2 C), 130.8, 133.1 (2 C), 157.1, 175.5, 179.5. HRMS (FAB): $C_{24}H_{42}N_4O_3$ requires M + 1 at *m/z* 435.333, found 435.334.

(*R,S*)-*N*-[3-[(5-Oxa-8-aminooctyl)amino]propyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide (11). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (0.087 g, 0.35 mmol) and DCC in DME/CH_2Cl_2 (1:1, 10 mL) were stirred at room temperature for 30 min, and the solution was added to a solution of **19** (0.140 g, 0.35 mmol) in CH_2Cl_2 (5 mL). After being stirred overnight at room temperature the solution was filtered, and the filtrate was concentrated in vacuo. The residue was purified by flash chromatography (CH_2Cl_2/MeOH 40:1 and 20:1) to give **23** as a clear oil (0.106 g, 48%). ^{1}H NMR [500 MHz, $(CD_3)_2SO$]: δ 0.73 (t), 1.36 (s), and 2.00 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both $^3J = 7.4$), 4.34 (m, α-CH), 2.61 and 2.80 (each dd, $^2J_{AB} = 13.7$, $^3J_{AX} = 9.3$, $^3J_{BX} = 4.7$, β-CH_2), 6.60 and 6.97 (each 2 H, aromatic H), 1.20−1.60 (m, 6 H) and 1.57 (p, 2 H) (2-, 6-, 7-, and 11-CH_2), 1.35 (s, 9 H) and 1.36 (s, 9 H) [2 × $C(CH_3)_3$], 2.92−3.09 (m, 1-, 3-, 5-, and 12-CH_2), 3.31 (apparent t, 8- and 10-CH_2). ^{13}C NMR [125.8 MHz, $(CD_3)_2SO$]: δ 67.7, 69.8, 77.4, 78.3, 114.8 (2 C), 128.7, 130.0 (2 C), 154.7, 155.6, 155.7, 171.3, 171.8, remaining signals at 13.5−40.1. MS (ES): 637 (M + 1), 537 [(M + 1) − $(CH_3)_2C{=}CH_2$ − CO_2], 437 [(M + 1) − $2(CH_3)_2C{=}CH_2$ − $2CO_2$].

Deprotection of **23** (0.082 g, 0.129 mmol) was carried out with TFA (1.25 mL, 12.9 mmol) in CH_2Cl_2 (20 mL) during 2 h at room temperature. Evaporation of the solvents and flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 50:50:1], fol-

lowed by another flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 10:10:1], afforded **11** (free base) as a clear, viscous oil (0.036 g, 64%). The material was 100% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (500 MHz, CD_3OD): δ 0.85 (t), 1.54 (s), and 2.15 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both ^3J = 7.4), 2.79 and 2.96 (each dd, $^2J_{AB}$ = 14.1, $^3J_{AX}$ = 8.0, $^3J_{BX}$ = 7.5, β-CH_2), 4.46 (t, α-CH), 6.69 and 7.04 (each 2 H, aromatic H), 1.54−1.64 (m, 6 H) and 1.75 (p, 2 H) (2-, 6-, 7-, and 11-CH_2), 2.48 (t, 2 H, ^3J = 7.1), 2.57 (t, 2 H, ^3J = 7.1), and 2.76 (t, 2 H, ^3J = 7.1) (3-, 5-, and 12-CH_2), 3.11−3.22 (m, 1-CH_2), 3.45 (t, 2 H, ^3J = 6.5) and 3.50 (t, 2 H, ^3J = 6.2) (8- and 10-CH_2). ^{13}C NMR (125.8 MHz, CD_3OD): δ 14.2, 20.5, 27.4, 28.8, 30.1, 33.2, 38.4, 38.7, 39.0, 40.3, 47.9, 50.6, 56.9, 70.3, 72.1, 116.6 (2 C), 129.3, 131.6 (2 C), 157.8, 174.2, 176.1. HRMS (FAB): $C_{23}H_{41}N_4O_4$ requires M + 1 at *m/z* 437.313, found 437.313.

(*R,S*)-*N*-[8-[(3-Aminopropyl)amino]-4-oxaoctyl]-4-hydroxy-α-[(1-oxobutyl)amino]benzenepropanamide (12). (*R,S*)-*N*-(1-Oxobutyl)tyrosine (**13**) (0.192 g, 0.77 mmol) and DCC (0.174 g, 0.84 mmol) were dissolved in DME/CH_2Cl_2(1:1, 10 mL) and stirred at room temperature for 30 min. The solution was added dropwise to a solution of **20** (0.217 g, 0.77 mmol) in CH_2Cl_2 (5 mL) and the reaction mixture stirred at room temperature for 20 h. The solvent was evaporated in vacuo and the crude **24** purified by flash chromatography [CH_2Cl_2/MeOH 1:1 and CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 10:10:1] to give a clear, viscous oil (0.175 g, 44%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.94 (t), 1.67 (s), and 2.19 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both ^3J = 7.4), 4.42 (dd, ^3J = 10.2 and 4.8, α-CH), 6.71 and 7.03 (each 2 H, aromatic H), 1.45−1.75 (m, 2-, 6-, 7-, and 11-CH_2), 2.51 (t, 2 H, ^3J = 7.1), 2.61 (m, 2 H) and 2.66−2.72 (m, 3 H) (8-, 10-, and 12-CH_2 and H_A proton of β-CH_2), 3.06−3.09 (m, 2 × allylic CH_2 and H_B proton of β-CH_2), 3.17 (t, 2 H, ^3J = 6.2) and 3.22−3.33 (m, 4 H) (1-, 3-, and 5-CH_2), 5.11−5.19 (m, 4 H) and 5.72−5.87 (m, 2 H) (olefinic H). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 13.7, 19.0, 26.2, 26.4, 27.2, 28.8, 37.4, 38.5, 39.1, 48.6, 49.5, 55.5, 56.8 (2 C), 68.9, 70.6, 116.2 (2 C), 117.6 (2 C), 127.1, 130.2 (2 C), 135.4 (2 C), 156.7, 170.6, 172.4. MS (ES): 517 (M + 1).

To a solution of **24** (0.070 g, 0.14 mmol) and *N,N′*-dimethylbarbituric acid (0.127 g, 0.81 mmol) in dry, degassed CH_2Cl_2 (2 mL) was added $Pd(PPh_3)_4$ (0.003 g, 2.7 μmol). The reaction mixture was stirred at 35 °C under nitrogen for 1.5 h. The reaction mixture was cooled to room temperature, the solvent was evaporated in vacuo, and the crude product was purified by flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 50:50:1], followed by another flash chromatography (CH_2Cl_2/MeOH 1:1), to give **12** (free base) as a clear viscous oil (0.059 g, 68%). The material was 100% pure according to HPLC with MS (total ion current) detection. ^{1}H NMR (400 MHz, CD_3OD): δ 0.85 (t), 1.55 (s), and 2.15 (t) (respectively 4-CH_3, 3-CH_2, and 2-CH_2 of the 1-oxobutyl moiety, both ^3J = 7.4), 4.46 (t, ^3J = 7.5, α-CH), 2.96 (dd, $^2J_{AB}$ = 14.1, $^3J_{BX}$ = 7.5, H_B proton of β-CH_2), 6.69 and 7.03 (each 2 H, aromatic H), 1.54−1.67 (m, 6 H) and 1.74 (p, 2 H) (2-, 6-, 7-, and 11-CH_2), 2.65−2.80 (m, 8-, 10-, and 12-CH_2 and H_A proton of β-CH_2), 3.10−3.45 (m, 1-, 3-, and 5-CH_2). ^{13}C NMR (100.6 MHz, CD_3OD): δ 14.2, 20.5, 27.0, 28.6, 30.6, 31.2, 38.0, 38.7, 39.0, 40.6, 48.4, 50.4, 56.8, 69.6, 71.8, 116.5 (2 C), 129.3, 131. 6 (2 C), 157.7, 173.9, 176.1. HRMS (FAB): $C_{23}H_{41}N_4O_4$ requires M + 1 at *m/z* 437.313, found 437.309.

3-[(8-Aminooctyl)amino]propanenitrile (25). Acrylonitrile (3.16 mL, 48.00 mmol) was added to a solution of 1,8-octanediamine (5.77 g, 40.0 mmol) in MeOH (15 mL) at 0 °C, and the reaction mixture was stirred for 16 h, protected from light. The solvent was evaporated in vacuo, and the residue was purified by flash chromatography (CH_2Cl_2, then CH_2Cl_2/MeOH 100:1) to give **25** as a clear oil (4.42 g, 56%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23−1.29 (m, 3′-, 4′-, 5′-, and 6′-CH_2), 1.33−1.48 (m, 2′- and 7′-CH_2), 2.47 (t, 2 H, ^3J = 6.6), 2.57 (t, 2 H, ^3J = 7.5), 2.63 (t, 2 H, ^3J = 6.0) and 2.88 (t, 2 H, ^3J = 6.9) (2-, 3-, 1′-, and 8′-CH_2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.5, 26.6, 27.0, 29.2, 29.3, 29.8, 33.6, 42.1, 45.0, 49.1, 118.8. MS (ES): 198 (M + 1).

Di-BOC Derivative of 3-[(8-Aminooctyl)amino]propanenitrile (26). The nitrile **25** (2.210 g, 11.20 mmol) was dissolved in CH_2Cl_2 (40 mL), and di(*tert*-butyl) dicarbonate (4.840 g, 22.18 mmol) was added. After 16 h the reaction mixture was poured into water and extracted with EtOAc (3 × 40 mL). The combined organic extracts were successively washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl. The organic phase was dried ($MgSO_4$) and concentrated in vacuo, the residue was dissolved in a 1:1 mixture of MeOH and 0.5 N NaOH, and the solution was stirred for 1 h. The solvent was removed by evaporation, the residue was dissolved in CH_2Cl_2 (100 mL), and the solution was washed with water (2 × 100 mL) and saturated aqueous NaCl (2 × 100 mL), dried ($MgSO_4$), and evaporated to give **26** as a clear oil (2.82 g, 64%), which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 (m, 3′-, 4′-, 5′-, and 6′-CH_2), 1.42−1.56 [m, 2′- and 7′-CH_2 and 2 × $C(CH_3)_3$], 2.60 (m, 2 H), 3.10 (m, 2 H), 3.25 (t, 2 H, ^3J = 7.8) and 3.46 (t, 2 H, ^3J = 6.6) (2-, 3-, 1′-, and 8′-CH_2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.1, 26.7, 26.8, 28.4 (3 C), 28.5 (3 C), 29.3 (2 C), 30.1 (3 C), 40.8, 43.9, 48.6, 79.1, 80.5, 118.6, 156.3 (2 C). MS (ES): 398 (M + 1).

Di-BOC Derivative of *N*-(3-Aminopropyl)-1,8-octanediamine (17). Raney nickel (0.600 g) was added to a solution of (**26**) (2.400 g, 6.04 mmol) and NaOH (0.600 g, 15.09 mmol) in EtOH (96%, 30 mL). The suspension was hydrogenated under 40 psi at room temperature for 28 h. The mixture was filtered through Celite, and the filtrate was concentrated in vacuo. The residue was taken up in water (100 mL), and the product was extracted with CH_2Cl_2 (4 × 30 mL). The organic phases were combined, dried ($MgSO_4$), and concentrated in vacuo to give **17** as a pale oil (1.900 g, 78%), which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (m, 3-, 4-, 5-, and 6-CH_2), 1.44−1.45 [m, 2- and 7-CH_2 and 2 × $C(CH_3)_3$], 1.66 (p, 2′-CH_2), 2.70 (t, 2 H, ^3J = 6.9) and 3.05−3.32 (m, 6 H) (1-, 8-, 1′-, and 3′-CH_2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 26.7, 26.8, 26.9, 28.4, 28.5 (3 C), 28.6 (3 C), 29.3, 29.4, 30.1, 40.7, 43.9, 47.0, 48.7, 79.1, 79.3, 155.2, 156.3. MS (ES): 402 (M + 1), 302 [(M + 1) − $(CH_3)_2C{=}CH_2$ − CO_2].

Di-BOC 2-Nitrobenzenesulfonyl Derivative of *N*-(3-Aminopropyl)-1,8-octanediamine (27). 2-Nitrobenzenesulfonyl chloride (0.426 g, 1.92 mmol) in CH_2Cl_2 (5 mL) was added slowly to **17** (0.644 g, 1.60 mmol) dissolved in CH_2Cl_2 (20 mL). The solution was cooled to 0 °C, pyridine (0.182 mL, 2.25 mmol) was added, and the reaction mixture was stirred at 0 °C for 1 h, followed by 1 h at room temperature. The solvent was removed in vacuo, and the crude product was purified by flash chromatography (EtOAc/heptane 1:2 and then 1:1) to give **27** as a slightly yellow oil (0.660 g, 70%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.22−1.33 (m, 3-, 4-, 5-, and 6-CH_2), 1.42 [m, 2 × $C(CH_3)_3$], 1.44−1.50 (m, 2- and 7-CH_2), 1.71 (m, 2′-CH_2), 3.01 (t, 2 H, ^3J = 7.1), 3.04 (t, 2 H, ^3J = 7.1), 3.12 (t, 2 H, ^3J = 7.5) and 3.21 (t, 2 H, ^3J = 7.1) (1-, 8-, 1′-, and 3′-CH_2), 7.80−7.82 (m, 2 H), 7.84−7.86 (m, 1 H) and 8.05−8.09 (m, 1 H) (aromatic H). ^{13}C NMR (125.8 MHz, CD_3OD): δ 40.0, 40.3, 43.7, 78.1, 79.3, 124.2, 129.9, 131.9, 133.2, 133.3, 148.0, 155.7, 156.9, remaining signals at 26.1−29.3. MS (ES): 587 (M + 1), 487 [(M + 1) − $(CH_3)_2C{=}CH_2$ − CO_2], 387 [(M + 1) − $2(CH_3)_2C{=}CH_2$ − $2CO_2$].

2-Nitrobenzenesulfonyl Derivative of *N*-(3-Aminopropyl)-1,8-octanediamine (18). The fully protected triamine **27** (0.660 g, 1.13 mmol) was dissolved in CH_2Cl_2 (20 mL), TFA (0.9 mL, 11.5 mmol) was added, and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated in vacuo, and the crude product was purified by flash chromatography [MeOH/CH_2Cl_2/$(CH_3)_2CHNH_2$ 20:20:1 and then 10:10:1] to give **18** as a clear oil (0.402 g, 92%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.34 (m, 3-, 4-, 5-, and 6-CH_2), 1.48 (m, 4 H) and 1.69 (p, 2 H) (2-, 7-, and 2′-CH_2), 2.52 (t, 2 H, ^3J = 7.5), 2.61 (p, 2 H, ^3J = 7.1), 2.64 (t, 2 H, ^3J = 7.1) and 3.09 (t, 2 H, ^3J = 6.6) (1-, 8-, 1′-, and 3′-CH_2), 7.78−7.81 (m, 2 H), 7.82−7.85 (m, 1 H) and 8.06−8.09 (m, 1 H) (aromatic H). ^{13}C NMR

(125.8 MHz, CD_3OD): δ 28.2, 28.6, 30.5, 30.6, 30.8, 30.9, 33.7, 42.7, 43.0, 48.1, 50.9, 126.1, 131.8, 133.8, 135.1, 135.5, 150.0. MS (ES): 387 (M + 1).

***N*-(3-Bromopropyl)trifluoroacetamide (28).** 3-Bromopropylamine hydrobromide (4.513 g, 20.61 mmol) was dissolved in MeOH (100 mL), triethylamine (3.33 mL, 22.68 mmol) was added, and the mixture cooled to 0 °C. Ethyl trifluoroacetate (2.70 mL, 22.68 mmol) was added dropwise, and the reaction mixture was stirred at 0 °C for 2.5 h. The solvent was removed in vacuo, and the residue was taken up in CH_2Cl_2 (100 mL) and washed successively with water (100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), and saturated aqueous NaCl (2 × 100 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo to give **28** as white crystals (4.190 g, 87%); mp 38−39 °C (corr.). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.18 (p, 2-CH_2), 3.45 (t, ^3J = 6.3, 3-CH_2), 3.56 (q, ^3J = 6.3, 1-CH_2), 6.70 (br s, NH). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 29.2, 31.2, 38.7, 115.7 (q, $^1J_{CF}$ = 288.3, CF_3), 157.6 (q, $^2J_{CF}$ = 36.5, CO). Anal. ($C_5H_7BrF_3NO$) C, H, Br, N.

***N*-[3-Di(2-propenyl)aminopropyl]trifluoroacetamide (29).** A solution of **28** (0.863 g, 3.69 mmol), diallylamine (1.37 mL, 11.06 mmol), and diisopropylamine (0.96 mL, 5.53 mmol) in toluene (15 mL) was stirred at 80 °C for 3 h. The mixture was allowed to cool to room temperature, water (15 mL) was added, the two layers separated immediately, and the aqueous layer was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were dried ($MgSO_4$), concentrated in vacuo, and the resulting brown oil was purified by flash chromatography (light petroleum/EtOAc 4:1 and 2:1) to give **29** as a pale yellow oil (0.622 g, 67%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.72 (m, 2-CH_2), 2.63 (m, 3-CH_2), 3.09 (dt, ^3J = 6.7, ^4J = 1.4, 2 × allylic CH_2), 3.46 (q, ^3J = 5.6, 1-CH_2), 5.17−5.22 (m, 4 H) and 5.75−5.83 (m, 2 H) (olefinic H), 9.32 (br s, NH). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.6, 41.0, 53.0, 56.8 (2 C), 116.2 (q, $^1J_{CF}$ = 288.5, CF_3) 119.5 (2 C), 134.1 (2 C), 157.2 (q, $^2J_{CF}$ = 36.5, CO).

3-(4-Hydroxybutyloxy)propanenitrile (30). To 1,4-butanediol (20.0 mL, 225.7 mmol) was added Triton B (0.5 mL) and acrylonitrile (7.4 mL, 112.8 mmol), and the mixture was stirred, protected from light, for 1.5 h. Concentration in vacuo, followed by purification by flash chromatography (light petroleum/EtOAc 2:1 and then 1:1) gave **30** as a clear oil (10.63 g, 66%).[57] ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.63−1.70 (m, 2′- and 3′-CH_2), 2.38 (br s, OH), 2.60 (t, ^3J = 6.2, 2-CH_2), 3.53 (t, 2 H, ^3J = 6.2), 3.64 (t, 2 H, ^3J = 6.2) and 3.65 (t, 2 H, ^3J = 6.2) (3-, 1′-, and 4′-CH_2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.9, 26.1, 29.4, 62.3, 65.3, 71.3, 118.3.

3-(4-Iodobutyloxy)propanenitrile (31). A solution of **30** (0.500 g, 3.49 mmol) and methyltriphenoxyphosphonium iodide (1.579 g, 3.49 mmol) in CH_2Cl_2 (5 mL) was stirred at room temperature for 1 h. Concentration in vacuo, followed by purification by flash chromatography (light petroleum/EtOAc 10:1 and then 4:1) gave **31** as a yellowish oil (0.722 g, 82%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.66−1.77 (m, 2 H) and 1.88−1.98 (m, 2 H) (2′- and 3′-CH_2), 2.60 (t, ^3J = 6.3, 2-CH_2), 3.23 (t, ^3J = 6.9, 4′-CH_2), 3.52 (t, 2 H, ^3J = 6.0) and 3.65 (t, 2 H, ^3J = 6.6) (3- and 1′-CH_2). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 6.8, 18.9, 30.1, 30.4, 65.4, 70.3, 118.1.

***N*-[4-(2-Cyanoethoxy)butyl]-*N*-[3-di(2-propenyl)aminopropyl]trifluoroacetamide (32).** To a suspension of NaH (0.320 g, 60% in mineral oil, 7.99 mmol) in DMF (15 mL) was added a solution of **29** (2.000 g, 7.99 mmol) in DMF (15 mL), and the mixture was stirred at room temperature under argon. After 45 min a solution of **31** (2.629 g, 10.39 mmol) in DMF (15 mL) was added, and the stirring continued for 2 h. The reaction was quenched by passing CO_2 through the solution for 10 min, the solvent was evaporated in vacuo, and the resulting brown solid was purified by flash chromatography (light petroleum/EtOAc 2:1 and then 1:1) to give **32** as a pale yellow oil (1.210 g, 40%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.56−1.80 (m, 2-, 3-, and 2′-CH_2), 2.43 (t, 2 H, ^3J = 7.5) and 2.59 (t, 2 H, ^3J = 6.4) (3′- and 2″-CH_2), 3.07 (m, 2 × allylic CH_2), 3.38−3.42 (m, 1- and 1′-CH_2), 3.51 (t, 2 H, ^3J = 6.1) and 3.64 (t, 2 H, ^3J = 6.5) (4- and 1″-CH_2), 5.11−5.20 (m, 4 H) and 5.77−5.87 (m, 2 H) (olefinic H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.7, 23.5, 25.5, 26.4, 45.3, 46.6, 50.1, 56.7 (2 C), 65.3, 70.7, 116.1 (q, $^1J_{CF}$ = 288.6), 118.0 (2 C), 118.1, 134.1 (2 C), 156.9 (q, $^2J_{CF}$ = 36.6). MS (ES): 376 (M + 1), 279 [(M + 1) − $COCF_3$].

***N*,*N*-Di(2-propenyl)-*N*′-[4-(3-aminopropyloxy)butyl]-1,3-propanediamine (20).** A solution of **32** (0.972 g, 2.59 mmol) in diethyl ether (25 mL) was added to a suspension of $LiAlH_4$ (0.393 g, 10.36 mmol) in diethyl ether (40 mL) at 0 °C. The mixture was stirred for 3 h at 0 °C and then for 1 h at room temperature. Water (0.5 mL) and EtOAc (50 mL) were added, and after 15 min the solution was filtered through Celite. Evaporation and flash chromatography [light petroleum/EtOAc/$(CH_3)_2CHNH_2$ 5:5:1] gave **20** as a brownish oil (0.613 g, 84%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.50−1.75 (m, 2-, 2′-, 3′-, and 2″-CH_2), 2.47 (t, 2 H, ^3J = 7.4), 2.60 (t, 2 H, ^3J = 7.2), 2.61 (t, 2 H, ^3J = 7.0) and 2.79 (t, 2 H, ^3J = 7.0) (1-, 3-, 1′-, and 3″-CH_2), 3.07 (d, ^3J = 6.5, 2 × allylic CH_2), 3.42 (t, 2 H, ^3J = 6.5) and 3.49 (t, 2 H, ^3J = 6.5) (4′- and 1″-CH_2), 5.10−5.18 (m, 4 H) and 5.80−5.88 (m, 2 H) (olefinic H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 26.8, 27.2, 27.6, 33.6, 39.7, 48.6, 49.8, 51.6, 56.8 (2 C), 69.0, 70.9, 117.4 (2 C), 135.7 (2 C). MS (ES): 387 (M + 1).

Di-BOC Derivative of *N*,*N*-Di(2-propenyl)-*N*′-[4-(3-aminopropyloxy)butyl]-1,3-propanediamine (33). The triamine (**20**) (0.386 g, 1.36 mmol) was dissolved in CH_2Cl_2 (5 mL), di(*tert*-butyl) dicarbonate (0.592 g, 2.71 mmol) was added, and the reaction mixture was stirred at room temperature for 16 h, poured into water (10 mL), and the mixture was extracted with EtOAc (3 × 10 mL). The combined extracts were washed with saturated aqueous $NaHCO_3$ (2 × 25 mL) and saturated aqueous NaCl (2 × 25 mL), dried ($MgSO_4$), and concentrated in vacuo to give a brown oil (0.674 g). The crude product was dissolved in a 1:1 mixture of MeOH and 0.5 M NaOH (10 mL) and stirred for 1 h. The solvent was evaporated in vacuo, the residue was dissolved in CH_2Cl_2 (10 mL), and the solution was washed with water (2 × 10 mL) and saturated aqueous NaCl (2 × 10 mL), dried ($MgSO_4$), and evaporated to give 0.419 g (64%) of **33** as a brown oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.44 (br s) and 1.45 (br s) [2 × $C(CH_3)_3$], 1.50−1.60 (m, 4 H), 1.68 (p, 2 H) and 1.74 (p, 2 H) (2-, 2′-, 3′-, and 2″-CH_2), 2.42 (t, ^3J = 7.4, 1-CH_2), 3.08 (dt, ^3J = 6.5, ^4J = 1.3, 2 × allylic CH_2), 3.13−3.24 (m, 3-, 1′-, and 3″-CH_2), 3.41 (t, 2 H, ^3J = 6.1) and 3.47 (t, 2 H, ^3J = 6.1) (4′- and 1″-CH_2), 5.10−5.19 (m, 4 H) and 5.80−5.88 (m, 2 H) (olefinic H). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 38.4, 45.2, 46.7, 50.5, 56.6 (2 C), 68.9, 70.4, 78.9 (2 C), 117.1 (2 C), 135.4 (2 C), 155.3, 155.8, remaining signals at 24.8−29.6. MS (ES): 484 (M + 1), 384 [(M + 1) − $(CH_3)_2C{=}CH_2$ − CO_2], 284 [(M + 1) − 2$(CH_3)_2C{=}CH_2$ − 2CO_2].

Di-BOC Derivative of *N*-[4-(3-Aminopropyloxy)butyl]-1,3-propanediamine (19). To a solution of **33** (0.204 g, 0.42 mmol) and *N*,*N*′-dimethylbarbituric acid (0.395 g, 2.53 mmol) in dry, degassed CH_2Cl_2 (1.5 mL) was added $Pd(PPh_3)_4$ (0.010 g, 8.4 μmol), and the reaction mixture was stirred at 35 °C under nitrogen for 1.5 h. The reaction mixture was chilled to room temperature, the solvent evaporated in vacuo, and the crude product was purified by flash chromatography (CH_2Cl_2/MeOH 1:1) and then by another flash chromatography [CH_2Cl_2/MeOH/$(CH_3)_2CHNH_2$ 50:50:1] to give **19** as a clear oil (0.112 g, 66%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.44 (br s) and 1.45 (br s) [2 × $C(CH_3)_3$], 1.50−1.62 (m, 4 H), 1.65 (p, 2 H) and 1.75 (p, 2 H) (2-, 2′-, 3′-, and 2″-CH_2), 2.69 (t, ^3J = 6.6, 1-CH_2), 3.13−3.31 (m, 3-, 1′-, and 3″-CH_2), 3.41 (t, 2 H, ^3J = 5.7) and 3.48 (t, 2 H, ^3J = 6.0) (4′- and 1″-CH_2). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 38.5, 39.4, 46.6, 69.0, 70.5, 78.8, 79.2, 155.9 (2 C), remaining signals at 25.3−29.7.

NMR Titrations. ^{13}C{^{1}H} NMR spectra for the NMR titration curves were obtained at 100.6 MHz and 25 °C. Digital resolution in the time domain was about 0.8 Hz/data point, and the spectra were accumulated for 0.5−6 h or until a satisfactory signal-to-noise ratio was obtained. Samples of **2** or **8**−**12** (11−24 mg) were dissolved in 0.6 mL of H_2O/D_2O 9:1, an equimolar amount of DSS was added for internal calibration of the resonances, and the solution was made up to 1 M ionic strength with solid KCl. The solutions were titrated in the NMR tubes with 0.1 or 0.5 M NaOH or HCl adjusted to

the ionic strength 1 M with KCl, pH being determined by means of a glass microelectrode before and after acquisition of the NMR spectrum; the average of the two readings, which usually differed by less than 0.05 pH unit, was used for calculations. Titration curves for each compound consisted of 13−16 points in the pH range of about 7.5−12.7. Because of the insolubility of **7** in water, the titration of this compound (17 mg) was carried out in H_2O/CD_3OD 1:1 with 0.1 or 0.5 M NaOH or HCl in H_2O/CD_3OD 1:1 (adjusted to 1 M ionic strength with KCl). Analysis of data by multiple nonlinear regression was carried out with GraFit v. 4.06 (Erithacus Software Ltd.).

Biology. nAChR Assay. TE671 cells originating from the American Type Culture Collection were maintained in Dulbeccos's modified Eagle's medium (4.5 g/L glucose) supplemented with 10% foetal calf serum, 1 mM pyruvic acid, 4 mM glutamine, 10 IU/mL penicillin, and 10 μg/mL streptomycin and incubated at 37 °C in a 5% CO_2 atmosphere. Cultures were divided when the cells reached ca. 80% confluence. For electrophysiological recordings, cells were grown on pieces of glass coverslip (3−4 × 20 mm) in 35 mm Petri dishes and transferred to a perfusion bath mounted on an inverted microscope.

Patch pipets were fabricated from borosilicate glass using a Sutter (p-97) programmable puller. Pipets were filled with 140 mM CsCl, 1 mM $CaCl_2$, 11 mM EDTA, and 5 mM HEPES (pH adjusted to 7.2 with 1 M CsOH) for whole-cell recording. Pipet resistances were about 5 MΩ. Cells were constantly perfused at 3−8 mL/min with saline containing 135 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH adjusted to 7.4 with 3 M NaOH). Ligands were applied using a DAD-12 superfusion system under computer control. At least 30 s was allowed between successive 1 s acetylcholine (10 μM) applications to allow the nAChR to recover from the desensitizing effect of agonist and the blocking effect of the antagonist. Whole-cell currents were monitored using a List Electromedical L/M-EPC7 patch-clamp amplifier that was controlled by pClamp software (Axon Instruments) run on a IBM-compatible computer. This second computer also provided appropriate trigger outputs to the DAD-12 interface via TTL channels. The output from the patch-clamp amplifier was recorded (after 10 kHz low-pass filtering) onto hard disk and analyzed using the pClamp software. Whole-cell recordings were performed at ambient laboratory temperature (17−23 °C).

Measurements of the peak current elicited by acetylcholine and the current amplitude obtained at the end (plateau current) of a 1 s application of agonist were made in the absence and in the presence of PhTX-343 and analogues. These measurements obtained in the presence of PhTX-343 and analogues were normalized and expressed as percentages of the equivalent currents obtained in the absence of these compounds. IC_{50} values (μM) were estimated from concentration−inhibition relationships (obtained using peak and end currents). The effects of PhTX-343 and analogues on the currents elicited by acetylcholine were examined at three holding potentials (V_H), −25 mV, −50 mV, and −100 mV. Data were analyzed using Graphpad Prism software. Prism was used for all statistical analysis. Concentration−inhibition curves were fitted using a four-parameter logistic equation.

The IC_{50} values (μM) determined at $V_H = -100$ mV for the peak current values were as follows: **2**, 46.8 ± 10.2; **7**, 14.1 ± 4.5; **8**, 33.8 ± 11.0; **9**, 102 ± 34; **10**, 10.0 ± 2.5; **11**, 22.6 ± 20.3; **12**, 52.9 ± 30.7. The IC_{50} values (μM) determined at $V_H = -100$ mV for the final current value were as follows: **2**, 16.0 ± 2.8; **7**, 0.33 ± 0.09; **8**, 9.4 ± 5.7; **9**, 20.4 ± 5.7; **10**, 1.64 ± 0.36; **11**, 4.24 ± 2.47; **12**, 2.61 ± 1.05.

Locust Muscle Assay. The assay was preformed essentially as previously described.[52] The locust (*Schistocerca gregaria*) retractor unguis muscle and its nerve were isolated and mounted in a Perspex perfusion chamber (volume 0.5 mL). The muscle was stretched to maximal body length and attached at its apodeme to a Grass FT 10-strain gauge. The preparations were continuously perfused with locust saline (180 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, pH 6.8). PhTX-343 analogues were applied for 10 min periods, and their effect on neurally evoked (0.22 Hz) muscle twitch contractions was recorded.

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