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Structure–Binding Relation of Philanthotoxins from Nicotinic Acetylcholine Receptor Binding Assay

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Abstract—Philanthotoxins are noncompetitive inhibitors of the nicotinic acetylcholine receptor and the various glutamate receptors. Analogues carrying photoaffinity labels, fluorine atoms for solid-state NMR studies of ligand/receptor interaction, and large head groups such as porphyrins and planar bulky aromatic rings (BIG analogues) for clarifying mode of entry and orientation of analogues in receptors have been synthesized, assayed against the nicotinic acetylcholine receptor, and brief comments are given for the assay results. © 1997 Elsevier Science Ltd.

Introduction

Over 80 neurotoxins generically called polyamine amides have been isolated from the venoms of funnel-web spiders.[1–4] The major venom of the female Egyptian wasp *Philanthus triangulum*, philanthotoxin-433 (PhTX-433, numbers denote the methylene groups between nitrogens), is also a polyamine amide with butyryl/tyrosyl/polyamine moieties (Fig. 1).[5,6] These toxins, having similar structures and biological functions, employ similar molecular strategies to paralyze their victims, that is, inhibit ionic conductance of cation channels gated by nicotinic acetylcholine receptors (nACh-R) and ionotropic glutamate receptors (Glu-R) located at postsynaptic neurons. Both belong to a superfamily of ligand-gated ion-channel receptors, which also include serotonin receptors, glycine receptors (Gly-R), and γ-aminobutyric acid receptors (GABA-R).[7] Over 100 analogues of PhTX analogues have been prepared for structure–activity studies by dividing the molecule into four regions (Fig. 1) and performing systematic changes. Structure–activity relations (SAR) based on assays with quisqualate-sensitive Glu-R (qGlu-R, non-NMDA subtype) using neurally evoked twitch contraction of locust skeletal muscle,[8,9] and with nACh-R from the Torpedo electric organ by displacement of the channel blocker [^{3}H]-dodecahistrionicotoxin[8,10] are obtained. The results for nACh-R are summarized in Figure 1. There is an overall similarity in the activity trends between the qGlu-R and nACh-R, suggesting that the topology of binding of polyamine amides with the intermixed hydrophilic and hydrophobic moieties to the transmem-

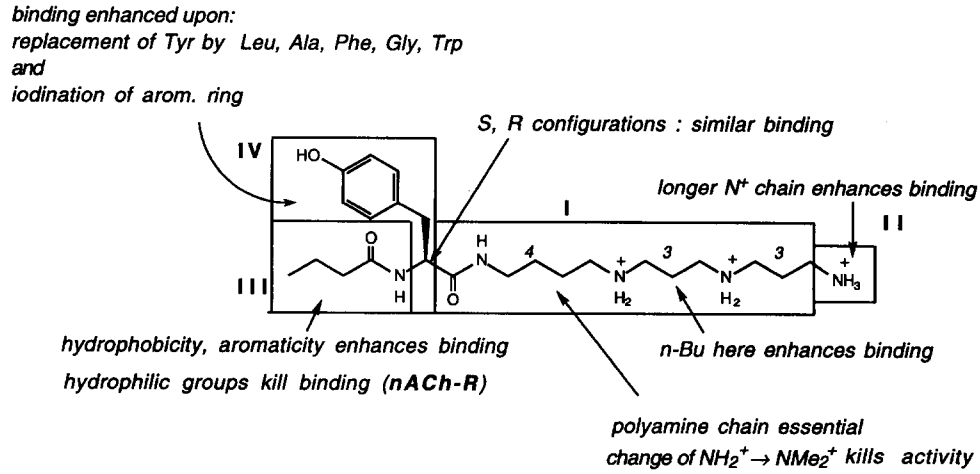


Figure 1. Summary of structure–binding relation from nACh-R binding assay. (The molecule shown is philanthotoxin-433.)

brane segments of the various receptors is similar. The assay of 18 photolabile PhTX analogues with qGlu-R before photoactivation has also been performed in preparation to perform photo-crosslinking experiments with other subtypes of Glu-Rs,[11] one of the principal excitatory amino acid receptors in the vertebrate central nervous system which have been implicated in learning/memory and a host of neurodegenerative diseases.

The nACh-R consisting of five subunits (Fig. 2A, top view of α, α, β, γ, δ subunits) is accompanied by a cytoplasmic 43 kDa protein. Each subunit consists of four membrane-spanning regions, M1–M4, with the α-helical M2 segments lining the channel. The receptor has a large hydrophilic extracellular region which connects, via the transmembrane region, to a hydrophobic cytoplasmic moiety (Fig. 2). The channel lined by five M2 segments opens on binding of the neurotransmitter acetylcholine to its two binding sites on the extracellular surface close to the two α subunits. The M2 segments form several well-defined rings which directly affect the channel functions (Fig. 2B) there is a negatively charged ring (**1**) at the top, then a large hydrophobic region (**2**), a leucine ring (**3**) which forms a constriction, two hydrophilic rings (**4**), two anionic rings (**5**), and finally a hydrophobic domain (**6**) in the cytoplasmic interior. A 43 kDa protein resides near the receptor in the cytoplasmic interior (Fig. 2A).[12–15]

A preliminary crosslinking experiment was performed with pure nACh-R without the 43 kDa protein and the membrane-bound nACh-R with the 43 kDa protein isolated from the electric organ of *Torpedo marmorata*. using the radioactive and photolabile PhTX analogue,[16] N_3-Ph-$^{125}I_2$-PhTX-343-Lys (Fig. 2A). The analogue photo-crosslinked to all five subunits; however, in experiments using receptor-enriched membranes (i.e., in the presence of the cytoplasmic 43 kDa protein) the ligand preferentially labeled one α-subunit and the 43 kDa protein. This indicates that the cytosolic protein must be asymmetrically disposed with respect to nAChR and that the hydrophobic 'head' of the analogue is in the cytoplasmic side (Fig. 2A). Most SAR results can be rationalized by this orientation. However, in the photo-crosslinking studies, the ligand had access to both the extracellular and intracellular sides in these in vitro experiments. The SAR comments are thus equally applicable to a head-up orientation with the hydrophobic regions III/IV of the analogue in the hydrophobic cavity 2 between rings 1 and 3 in Figure 2B.

Our current understanding of the major mode of noncompetitive antagonism is that the toxins enter and plug the open cation channels gated by Glu-R and nACh-R, and sterically inhibit the ion flow. However, the interactions between the polyamine amides and channels are not understood at the molecular level because of the complex detergent properties of polyamines and the diversity in cellular actions. In order to correlate the structures of PhTX analogues and their channel blocking potencies at these receptors, we had performed SAR and preliminary photo-crosslinking studies as the first step in understanding this aspect (mentioned above). Subsequent steps regarding ligand/receptor interaction will be directed towards securing experimental data regarding the mode of entry of the ligand into the receptor, the orientation of the ligand in the receptor, the tertiary structure of the receptor, etc. Regarding the mode of entry of PhTX into the receptor (R), five possibilities are conceivable (Fig. 3).

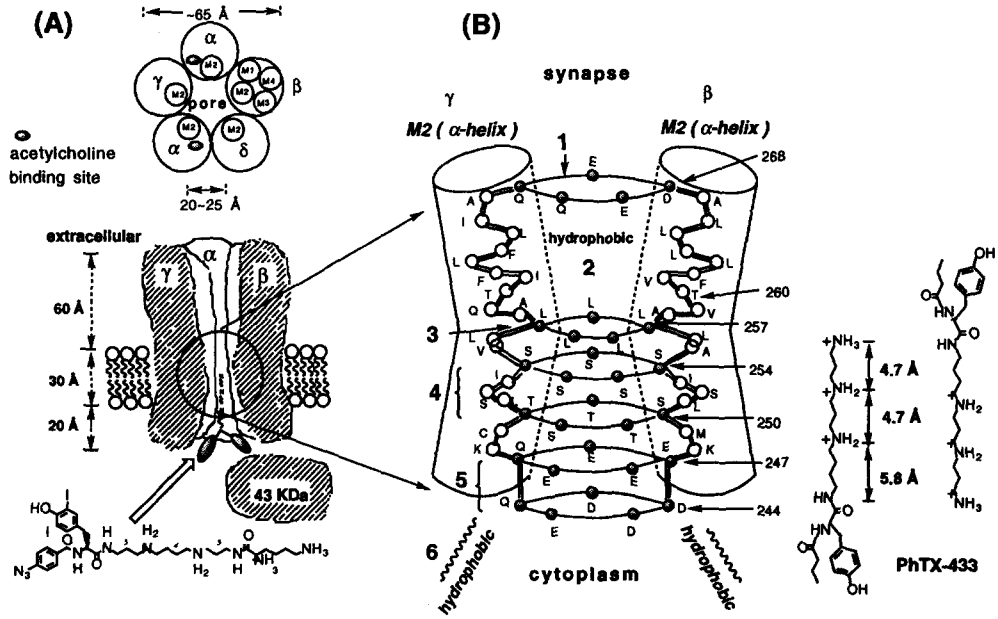


Figure 2. (A) Top and side view of nACh-R with 43 kDa protein showing orientation of PhTX analogue in receptor. (B) Cross-section of receptor and two orientations of PhTX molecule.

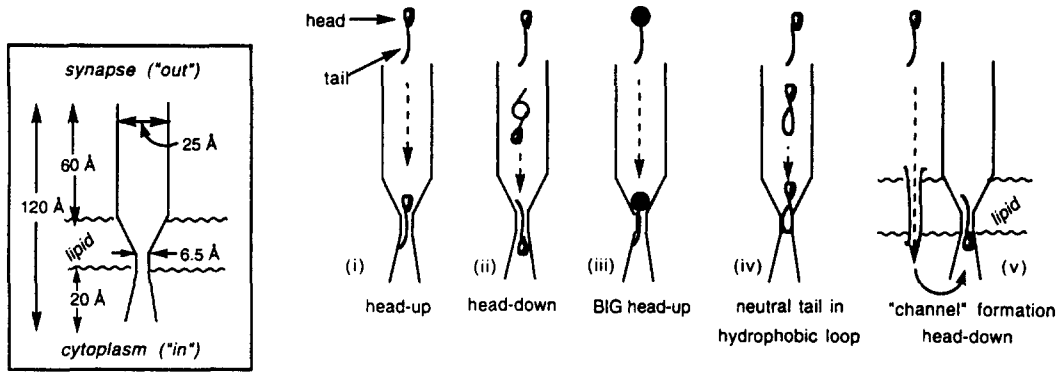


Figure 3. Modes of entry of PhTX analogues into nACh-R.

(i) PhTX enters R from the synapse and settles in head-up direction.

(ii) PhTX enters R from the synapse, flips over, and settles head-down.

(iii) PhTX with bulky head (Tables 4 and 5) cannot pass constriction and settles head-up.

(iv) PhTX enters R from the synapse, the positively charged polyamine complexes with Cl^- anion, or the neutral polyamine chelates with Na^+ or other cations and settles head-up.

(v) PhTX penetrates the lipid bilayer into the cytoplasm and settles head-down. Ongoing studies show that PhTX can efficiently pass sodium ions through phosphatidylcholine vesicles; the mechanism of this penetration is still not clear.

Clarification of the ligand/receptor interaction, mode of entry, etc., on a molecular structural basis is indispensable for the design of drugs that will interact specifically with the various receptor subtypes which are actively being sequenced.

Results and Discussion

In the following we report the synthesis and nACh-R binding assay results of PhTX analogues which have been designed to perform future experiments. The analogues contain: (a) photoaffinity labels for planned photo-crosslinking studies, Table 2; (b) fluorine atoms for solid-state NMR measurements of ligand/receptor complex, Table 3; and (c) and porphyrin rings and BIG head groups for studies of binding orientation, Tables 4 and 5. Short comments regarding the respective analogues are included in the tables.

The binding assay, as performed, measures binding of [^{3}H]-H_{12}-HTX to activated nAChR.[19] In the past short incubation periods (30 s) were used with single filter assays performed one at a time. With the current, high-volume performance equipment and protocols, the incubation interval was increased to 5 min to allow sufficient time to reach steady-state binding in all 96 samples in a microtiter plate. This change from the original protocol has no effect on the final outcome. [^{3}H]-H_{12}-HTX binds to activated nAChR with much higher affinity and faster kinetics. Binding kinetics indicated that steady state, in presence of 100 μM carbamylcholine, is reached in 5 min.[19] At the same time interval, [^{3}H]-H_{12}-HTX binding in absence of 100 μM carbamylcholine is less than 5% of that observed in presence of carbamylcholine. Potency of PhTX 343 to inhibit [^{3}H]-H_{12}-HTX in this study is 2.5-fold lower than previously reported. This is partially due to the changes in binding assay. Primarily, because of the exhaustive washing of samples after transfer on the filter mats as well as using a different species of Torpedo (i.e., *Torpedo nobiliana*). Potency of PhTX 343 was considered one and potencies of the other PhTxs were calculated by dividing the IC_{50} for PhTX 343 over the IC_{50} for the compound. Thus, values higher than one, means higher potency and vice versa. Compounds that failed to produce any effect on [^{3}H]-H_{12}-HTX binding at 100 μM were given $IC_{50} > 500$ (Tables 1–5).

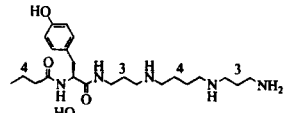
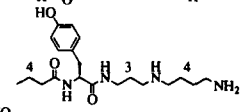
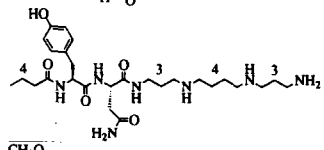
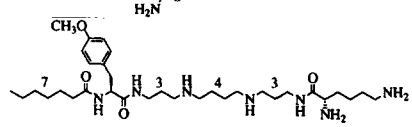
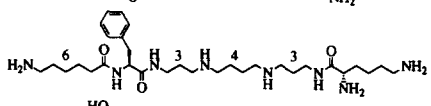
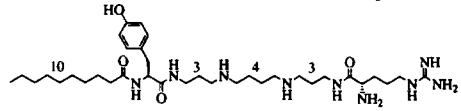
The data suggested that certain modifications of the PhTX structure produce compounds that have higher potencies as blockers of the nAChR. Compounds which have a C_{10} structure instead of the C_4 of the natural toxin have higher potencies (compounds **6**, **7**, **8**, and **10**). Porphyrin containing PhTX (e.g., compounds **19**, **20**, **21**, and **22**) had equal or higher potency than the parent PhTX 343. The porphyrin-PhTX solutions were highly colored especially at the higher concentrations. Torpedo membranes incubated with these compounds became colored. It is possible that color quenching may have contributed to their higher potencies. On the other hand, biotinylated PhTX (compound **23**) had lower potency. Interestingly, the two big 343 analogues (compounds **26** and **27**) also had higher potency than the parent PhTX 343.

Experimental

General

Reagents and starting materials purchased from common commercial suppliers were used as received.

Table 1. Simple analogues

	Compound	IC_{50} (μM)	Relative potency	Remarks	Reference
1		50 ± 0.67	1	Reference compound, relative potency taken as 1	5
2		>500	<0.1	Shorter polyamine chain reduces binding[10]	—*
3		>500	<0.1	Bulky side-chain inhibits fit into opened gate[16]	9
4		50 ± 0.55	0.1	Lys in II and C7 in III enhances binding; unclear why methoxylation in IV reduces binding	—*
5		350 ± 4.2	0.1	Hydrophilic amino group in III kills binding[5]	—*
6		4 ± 0.06	12.5	Arg in II and C10 in III enhances binding[10]	21

*New compounds, synthesis described in this paper.
I, II, etc., in remarks column refer to regions I, II, etc. in Fig. 1.

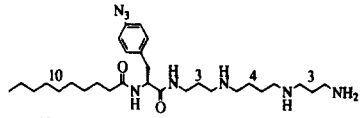
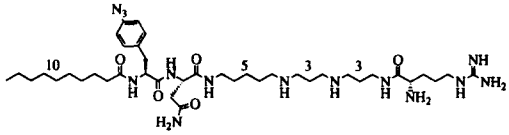
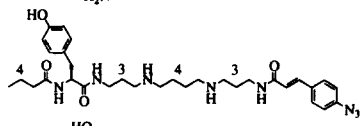
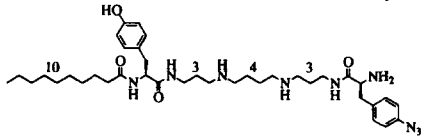
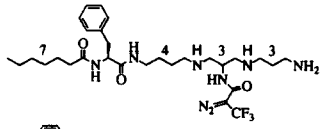
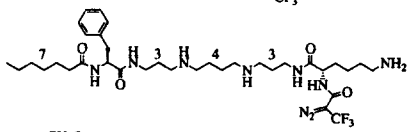
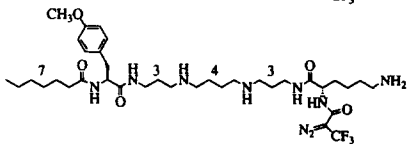
Solvent CH_2Cl_2 and reagent Et_3N were distilled at atmospheric pressure over CaH_2, THF was distilled over Na. MeCN, MeOH, and EtOH were dried over molecular sieves (4 Å). Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed, precoated silica gel plates (60 F_{254}) gel. Preparative TLCs (PTLC) were performed on Analtech (500 μm, 20 × 20 cm, silica gel) or Whatman TLC plates (K5F, 20 cm × 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 200 300 and 400, and reported in parts per million (ppm) using residual proton solvent peaks of either $CDCl_3$ at 7.26 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*.

***N*-butyryl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester (2b).** To a solution of 2.95 g (6.0 mmol) of *N*-Boc-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester in 30 mL of dry CH_2Cl_2 was added 15 mL of trifluoroacetic acid (TFA) and this mixture was stirred at rt for 2 h and the solution was evaporated to dryness. The resulting solid was dissolved in 10 mL of CH_2Cl_2 and to which was added 0.75 mL (7.20 mmol) of butyryl chloride and 2.5 mL (18.0 mmol) of Et_3N. The slightly yellow solution was stirred at rt. After 1.5 h the solution was evaporated to a slightly yellow solid and recrystallized with EtOH or chromatographed on silica gel with CH_2Cl_2 yielded 2.0 g (72%) of the desired product. ^1H NMR (250 MHz, $CDCl_3$) δ 8.25 (d, 2H, J = 8 Hz), 5.15 (m, 1H), 3.3 (m, 2H), 2.58 (t, 2H, J = 6 Hz), 1.80 (m, 2H), 1.05 (t, 3H, J = 6 Hz); CI-MS ($C_{26}H_{26}N_2O_6$) 463 $(M + 1)^+$.

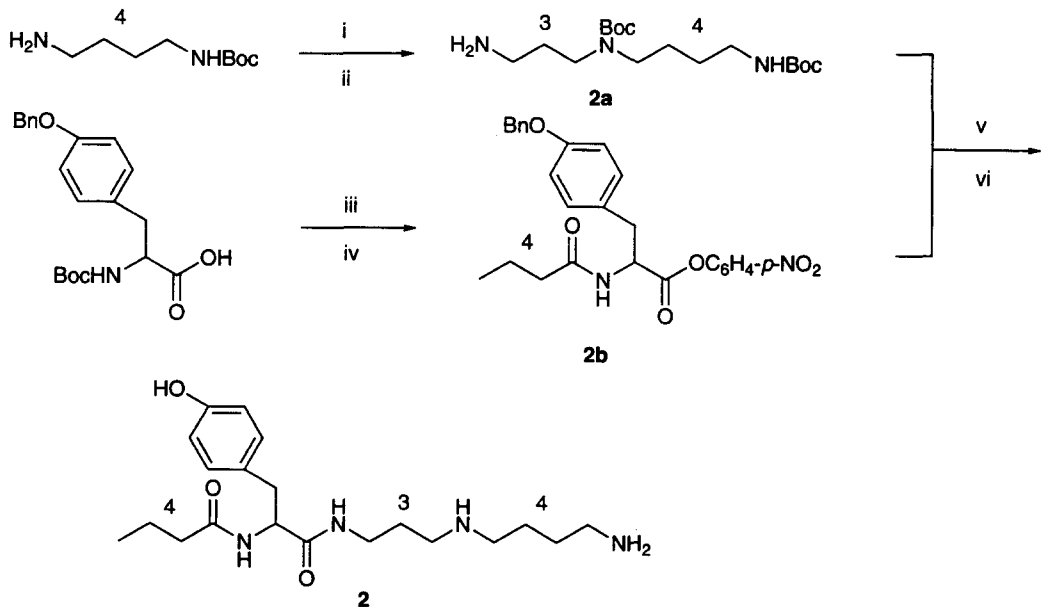
PhTX-34 (2). To a solution of 300 mg (0.65 mmol) of *N*-butyryl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester in 5 mL of MeOH was added dropwise 224 mg (0.65 mmol) of *N,N'*-di-Boc-polyamine-34 in 5 mL of MeOH while stirring at rt. After 12 h, the reaction mixture was evaporated to a slightly yellow solid residue, which was taken up in 50 mL of CH_2Cl_2. The organic phase was washed with 0.5 M NaOH (30 mL × 2), citric acid (30 mL × 2) and brine (30 mL), and was dried with $MgSO_4$ followed by evaporation of the solvent which yielded a pale-yellow solid. The crude product was purified with silica gel flash column eluted with CH_2Cl_2 and CH_2Cl_2:MeOH (95:5). The product was obtained in 88% yield (382 mg). R_f (CH_2Cl_2:MeOH, 95:5) 0.25.

To 6 mL of dry CH_2Cl_2 was dissolved 360 mg (0.54 mmol) of *O*-benzyl-PhTX-34 $(Boc)_2$ and to which was added 3 mL of TFA. This solution was stirred under argon for 12 h and then the solvent was removed. The resultant slightly yellow oil was dissolved in 20 mL of MeOH, to which was added 0.10 g of 10% Pd–C. This suspension was purged three times with H_2, and then stirred under H_2 at rt for 12 h. The reaction was terminated by filtration through celite, followed by

Table 2. Analogues with photoaffinity labels

	Compound	IC_{50} (μM)	Relative potency	Remarks	Reference
7		5 ± 0.08	12.0	Good candidate for photo-crosslinking	21
8		9 ± 0.11	5.6	Compared to **7**, the enhanced binding due to longer chains in II and III are counterbalanced by bulky side-chain in I	9
9		>500	<0.1	Binding is weak because there is no free amino group in II	21
10		7 ± 0.09	7.1	Polyamine chain ends with amino group (cf. **10**); good candidate for photo-crosslinking studies	21
11		>500	<0.1	Bulky polar branching in I kills binding; when this branching is n-Bu, activity in enhanced 5.2-fold[5]	—*
12		30 ± 0.65	2.0	Branching in II is bulky but bindng preserved since terminal is hydrophilic	—*
13		120 ± 2.16	0.4	Prepared as intermediate for preliminary solid-state affinity studies. Methoxylation weakens binding (cf. **12**; **4**/**5**).	—*

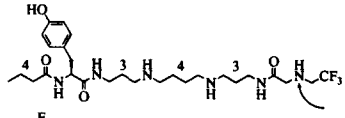
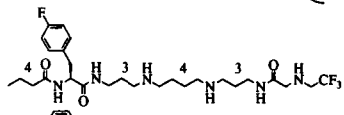
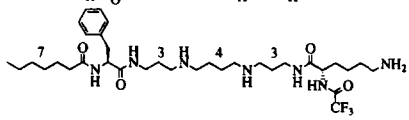
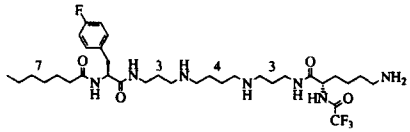
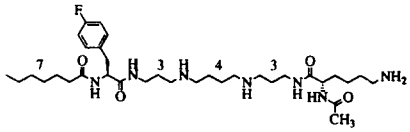
*New compounds, synthesis described in this paper.
I. II, etc., in remarks column refer to regions I, II, etc. in Fig. 1.



(i) a. acrylonitrile, MeOH, rt; b. $(Boc)_2O$, Et_3N, CH_2Cl_2; (ii) $LiAlH_4$, Et_2O; (iii) DCC, *p*-nitrophenol, EtOAc; (iv) a. TFA/CH_2Cl_2, 0°C, 6 h; b. butyryl chloride, Et_3N, CH_2Cl_2; (v) MeOH; (vi) a. TFA, CH_2Cl_2, 0 °C, 6 h; b. H_2, Pd/C, MeOH

Scheme 1.

Table 3. Analogues containing fluorine, prepared for solid-state (SS) ^{19}F–NMR studies

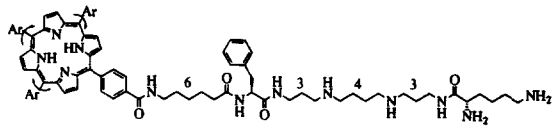
	Compound	IC_{50} (μM)	Relative potency	Remarks	Reference
14		>500	<0.1	Amino group in II (shown by arrow) is not protonated due to CF_3, hence less binding	—*
15		>500	<0.1	Reduced binding for same reason as above.	—*
16		50 ± 0.72	1.0	The hydrophobic branching in II decreases binding (cf. **12**) .	—*
17		60 ± 0.85	0.8	Aromatic fluorination has little effect on binding (cf. **16**). This analogue with two F groups can be used for F–F distance measurements in SS-NMR.	—*
18		28 ± 0.62	1.9	Less bulky group in II increases binding (cf. **17**)	—*

*New compounds, synthesis described in this paper.
I, II, etc., in remarks column refer to regions I, II, etc. in Fig. 1.

washing with MeOH. After evaporation of the solvent, the clear oil was chromatographed on silica gel with CH_2Cl_2:MeOH (95:5) and CH_2Cl_2:MeOH:*i*PrNH$_2$ (4:4:1), which gave 0.29 g of PhTX-34 as TFA salt (90%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.05–7.02 (d, 2H, J = 8.4 Hz), 6.69–6.67 (d, 2H, J = 8.4 Hz), 4.47–4.43 (dd, 1H, J = 6.8, 8.0 Hz), 3.21–3.11 (m, 2H), 2.97–2.92 (dd, 1H, J = 6.8, 13.6 Hz), 2.81–2.75 (dd, 1H, J = 8.0, 13.6 Hz), 2.70 (s, 2H), 2.56 (s, 2H), 2.48–2.44 (t, 2H, J = 7.2 Hz), 2.17–2.13 (t, 2H, J = 7.2 Hz), 1.61–1.51 (m, 8H), 0.86–0.82 (t, 3H, J = 7.2 Hz).

C_7-*O*-benzyl-tyrosine (4a). To a solution of *O*-benzyl-tyrosine (1.08 g, 4 mmol) in 1 N NaOH (10 mL), was added heptanoyl chloride (0.93 mL, 6 mmol) in THF (20 mL) and the mixture was stirred at rt for 4 h. Then THF was removed under reduced pressure. And the remaining mixture was redissolved in

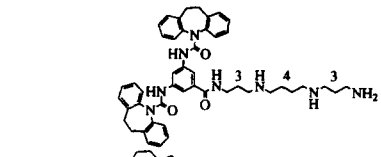
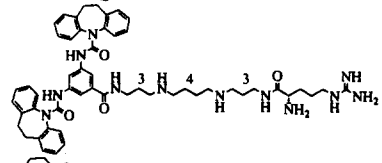
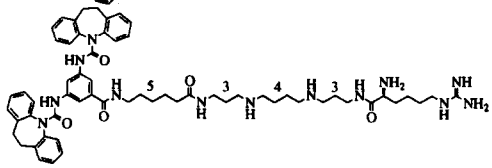
Table 4. Porphyrin PhTX analogues[17]. Ar (aromatic) planes are orthogonal to porphyrin skeletal plane[22]

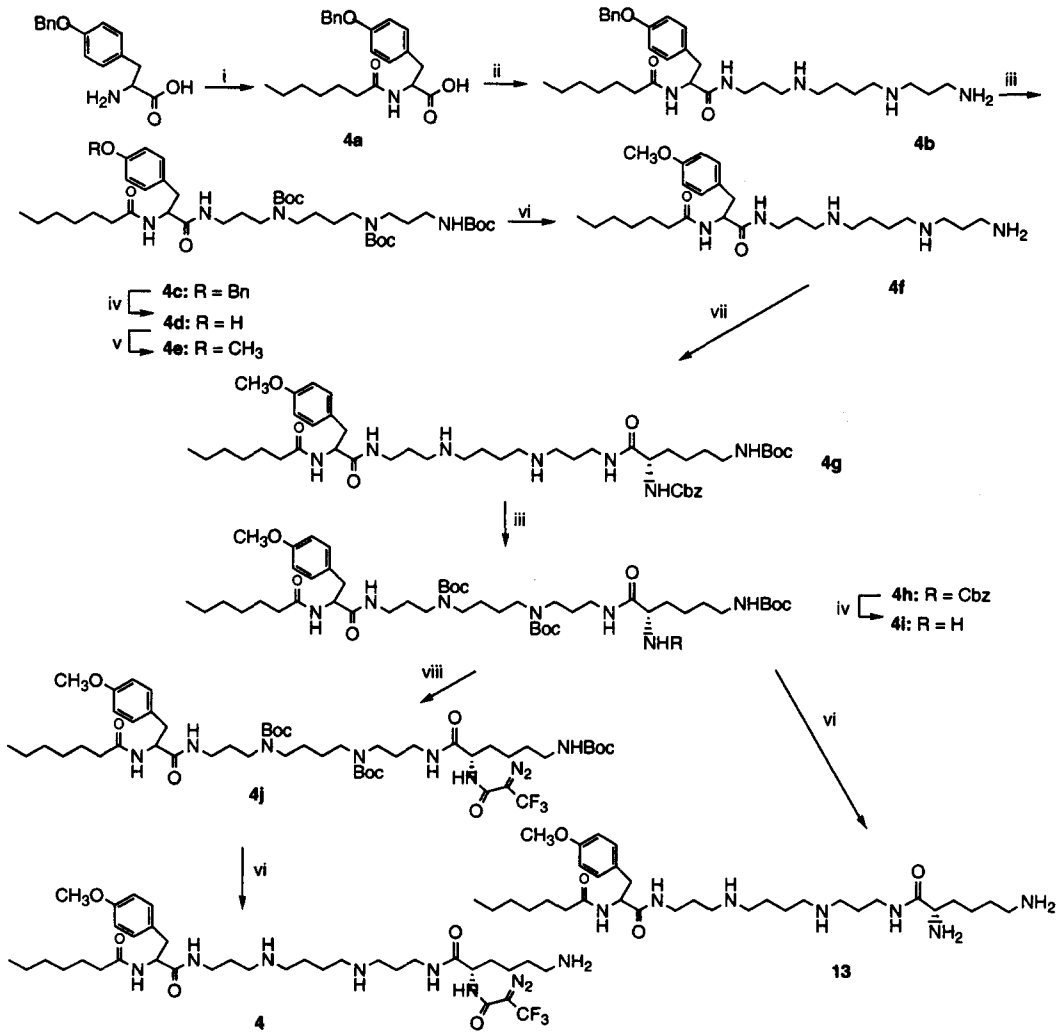


	Compound	IC_{50} (μM)	Relative potency	Remarks	Reference
19	R =	70 ± 1.10	0.9	These analogues presumably enter the channels from the synaptic side with the porphyrin group in the large hydrophilic domain, external to the transmembrane segments; polyamine extend into the channel	—*
20	R =	65 ± 1.95	0.9	Under assay conditions in buffer, the pyridines are not protonated. The slight enhancement in hydrophilicity does not affect binding (cf. **21**)	—*
21	R =	8 ± 0.14	6.3	Rotation of pyridine rings can bring the *meta*-Ns into closer contact of hydrophilic environments as compared to *para*-Ns (**20**)	—*
22	R =	3 ± 0.04	16.7	Quaternarization of pyridine N enhances binding due to the hydrophilicity of the external synaptic side	—*

*New compounds, synthesis described in this paper.

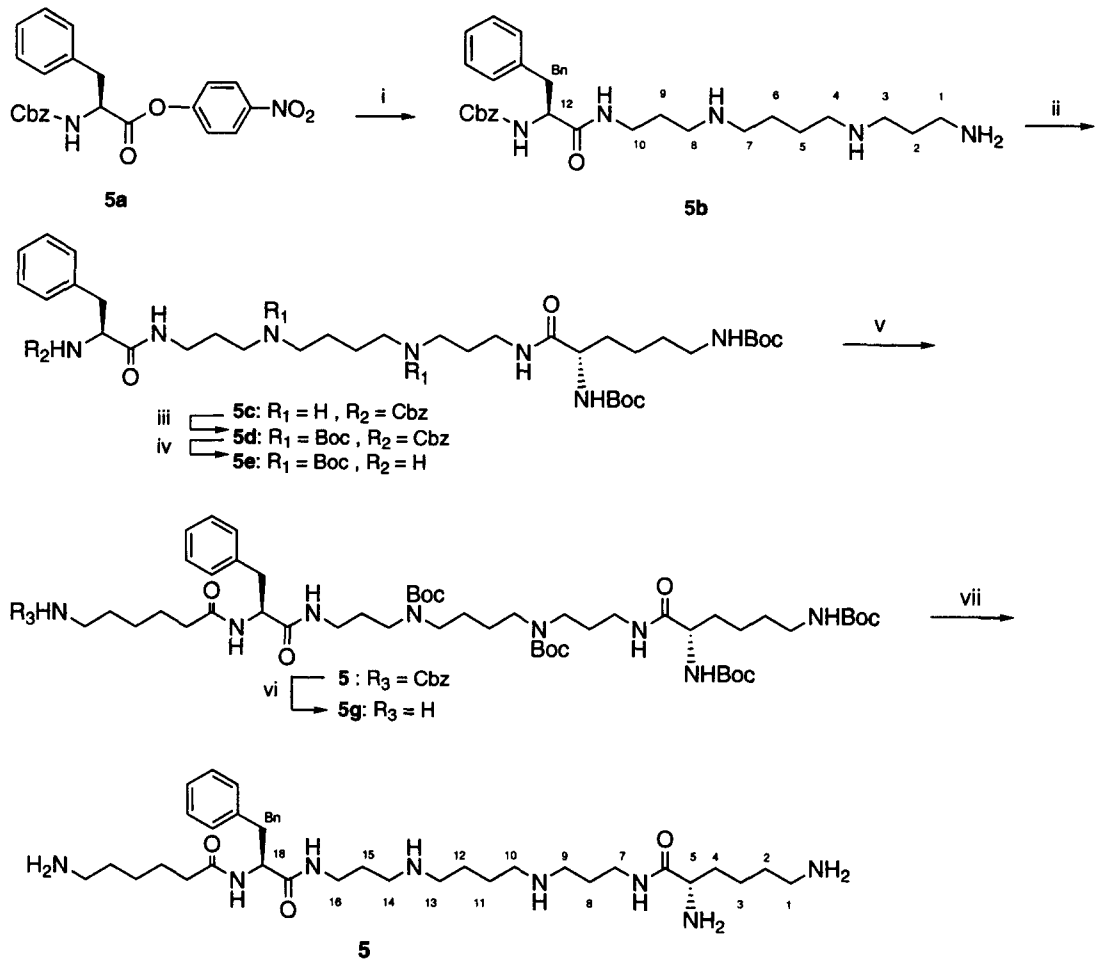
Table 5. BIG head PhTX analogues

	Compound	IC_{50} (μM)	Relative potency	Remarks	Reference
23		8 ± 0.19	6.3	Mode of entry of BIG analogues is probably same as with prophyrin analogues (cf. Table 4)	17
24		3 ± 0.08	16.7	The longer polyamine chain could extend deeper into the channel; stronger binding (cf. 23)	17
25		30 ± 0.60	1.7	Reason for the weaker binding of this analogue with longer polyamine chain is unclear	17



(i) heptanoyl chloride, aq NaOH/THF, rt, 3 h; (ii) CDI, spermine, Et_3N, THF; (iii) $(Boc)_2O$, Et_3N, MeOH; (iv) Pd/C, H_2, MeOH; (v) MeI, K_2CO_3, acetone; (vi) TFA, CH_2Cl_2; (vii) Et_3N, Nε-*t*-Boc-Nα-Cbz-L-Lysine *p*-nitrophenyl ester, DMF; (viii) 2-diazo- trifluoro-propionic acid *p*-nitrophenyl ester, Et_3N, DMF

Scheme 2.



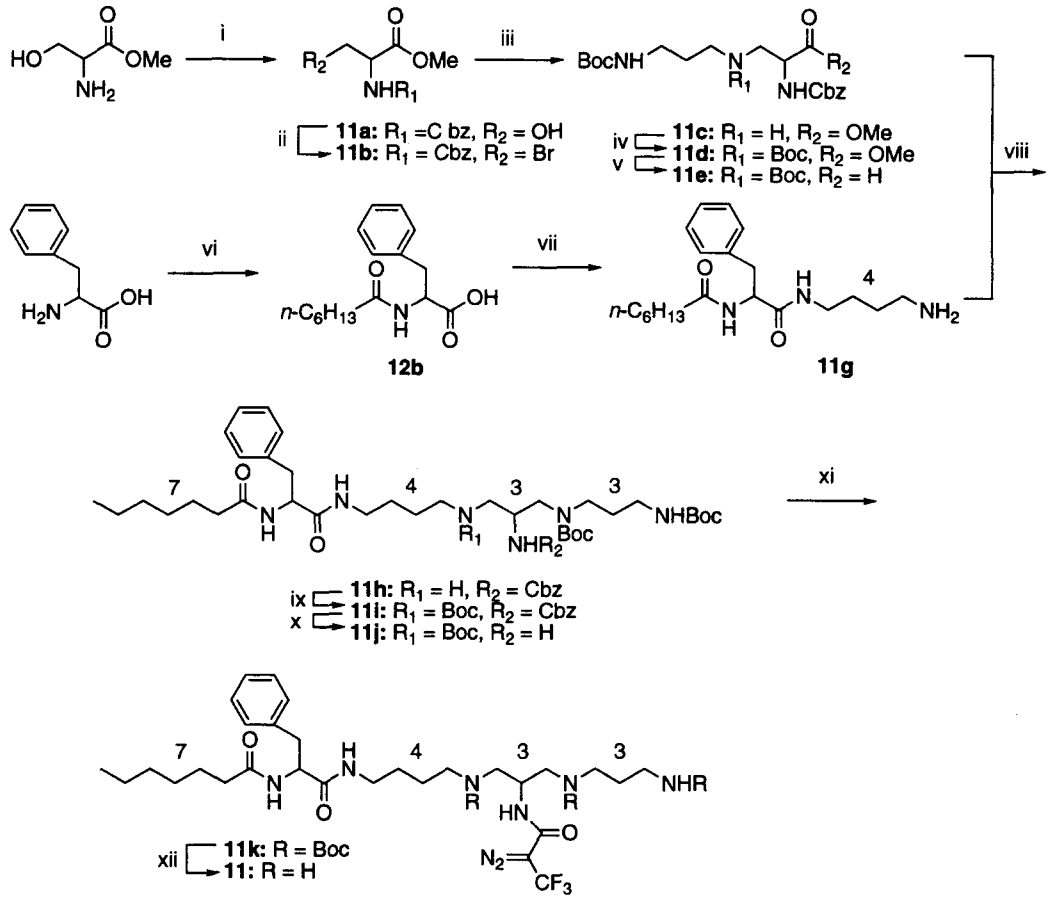
(i) spermine, THF, rt, 6 h , 72%; (ii) N,N di-*t*-Boc-L-lysine-N-hydroxysuccinimide ester, THF, Et_3N, rt, 5 h, 67%; (iii) $(Boc)_2O$, CH_2Cl_2, Et_3N, rt, 40 min, quant; (iv) H_2, Pd/C, rt, 6 h, 88%; (v) N-Cbz-6-amino hexanoic acid, EDC, DMAP, CH_2Cl_2, rt, 5 h , 88%; (vi) H_2, Pd/C, rt, 6 h, 87%; (vii) CH_2Cl_2, TFA, rt, overnight, quant

Scheme 3.

CH_2Cl_2, which was washed with 1 N HCl (40 mL × 3), H_2O (40 mL × 2), and brine (40 mL). The organic layer was dried over Na_2SO_4 and evaporated to dryness to afford a white solid (1.51 g, 99%). R_f (EtOAc:hexane, 1:1) 0.45; ^1H NMR (400 MHz, $CDCl_3$) δ 7.43–7.32 (m, 5H, $C_6\underline{H}_5$–CH_2–O), 7.10–7.06 (d, 2H, C–CH–C$\underline{H}$–C–OBn), 6.93–6.90 (d, 2H, C–CH–C$\underline{H}$–C–OBn), 5.45–5.41 (d, 1H, NH–CO–C$\underline{H}$–NH–COOH), 5.08–5.01 (s, 2H, C_6H_5-C$\underline{H}_2$–O), 4.85–4.80 (dd, 1H, C_6H_4–C$\underline{H}$–CH), 3.18–3.12 (dd, 2H, C_6H_4–C$\underline{H}$–CH), 2.39–2.35 (t, 2H, CH_2–C$\underline{H}_2$–CO), 2.10–1.25 (bm, 8H), 0.89–0.81 (t, 3H, C$\underline{H}_3$–CH_2); CI-MS ($C_{23}H_{29}NO_4$) 401 $(M + NH_3 + 1)^+$.

C_7-*O*-benzyl-tyrosine-343 (4b). To a solution of **4a** (0.77 g, 2 mmol) in 20 mL dry THF, was added 1,1′-carbonyldiimidazole (0.36 g, 2.2 mmol) and the mixture was stirred under argon for 30 min. Then the resulting solution was added to a solution of spermine (1.01 g, 5 mmol) and Et_3N (0.28 mL, 2 mmol) in dry THF (30 mL) dropwise by syringe. After stirred under argon for 5 h at rt, the solvent was removed under reduced pressure and the resulting mixture was pumped on a vacuum pump overnight. The product was submitted to next step without further purification.

C_7-*O*-benzyl-tyrosine-343-Boc (4c). To the mixture containing **4b** in MeOH (40 mL) were added di-*tert*-butyl-dicarbonate (2.18 g, 10 mmol) and Et_3N (0.85 mL, 6 mmol). The solvent was removed after stirring at rt overnight and the product was chromatographed on 200 g of silica with a step gradient system of CH_2Cl_2:MeOH from 99:1 to 95:5 which gave the desired product 1.1 g (63% in two steps). R_f (CH_2Cl_2:MeOH, 95:5) 0.3; ^1H NMR (400 MHz, $CDCl_3$) δ 7.43–7.32 (m, 5H, $C_6\underline{H}_5$–CH_2–O), 7.10–7.06 (d, 2H, C–CH–C$\underline{H}$–C–OBn), 6.93–6.90 (d, 2H, C–CH–C$\underline{H}$–C–OBn), 4.39–4.28 (t, 1H, NH–CO–C$\underline{H}$–NH–CO), 3.75–3.65 (s, 3H, O$\underline{CH}_3$), 3.28–2.92 (bm, 14H), 2.26–2.05 (m, 4H), 1.85–1.70 (bs, 4H), 1.61–1.51 (m, 2H), 1.47–1.02 (bm, 33H, including 27H from three Bocs), 0.89–0.81 (t, 3H, $CH_2C\underline{H}_3$); FABMS ($C_{48}H_{77}N_5O_9$) 869 $(M + 1)^+$.



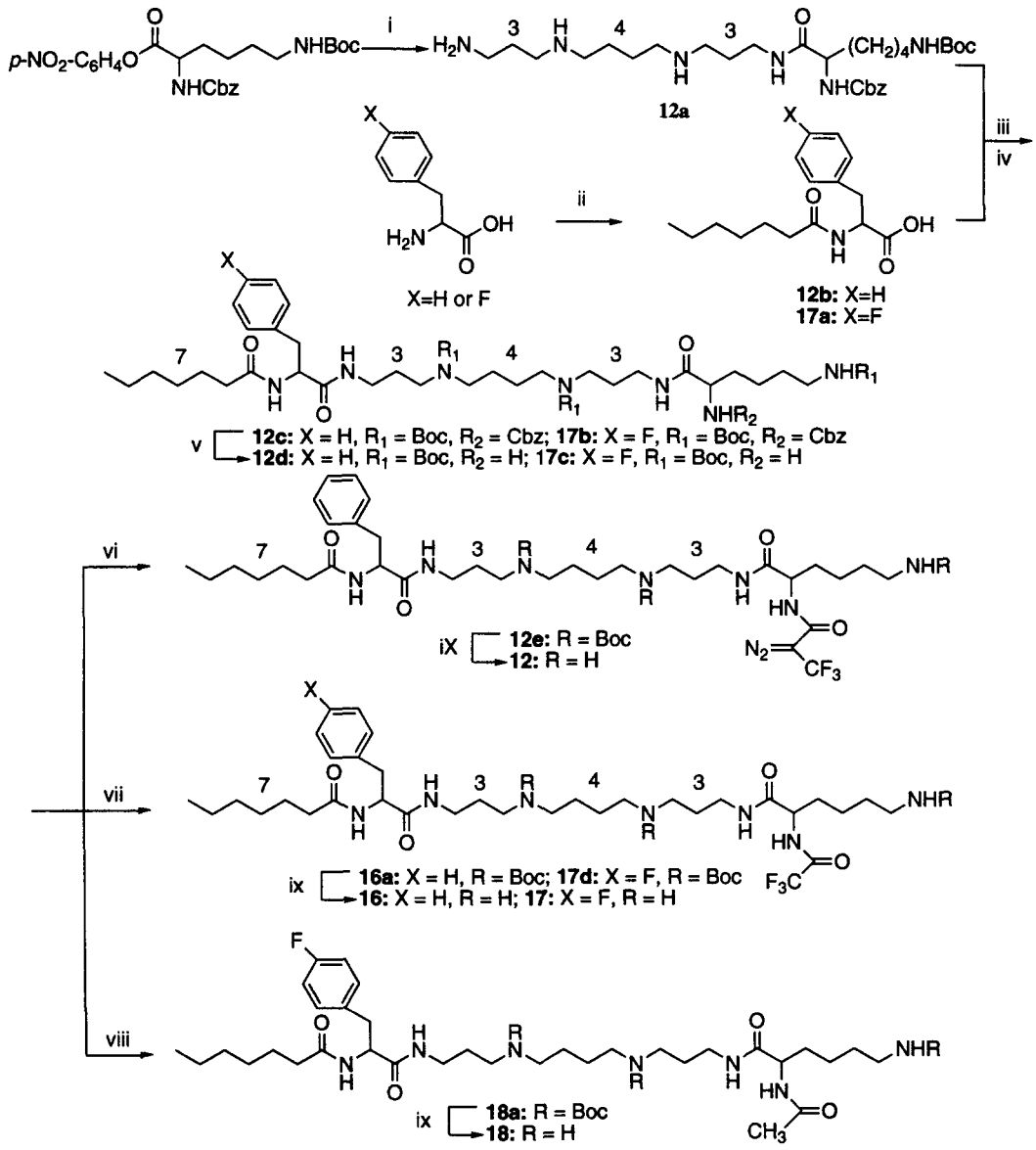
(i) CbzCl, Et_3N, CH_2Cl_2, rt, 5 h; (ii) NBS, PPh_3, DMF, 50 °C, 30 min; (iii) 1-N-Boc-propylamine, KF/Celite, CH_3CN, 10 h; (iv) $(Boc)_2O$, CH_2Cl_2, rt, 4 h; (v) DIBAL, CH_2Cl_2, -78 °C, 3 h; (vi) heptanoyl chloride, 1 N NaOH(H_2O)/THF, rt, 5 h; (vii) CDI, 1,4-diaminobutane, THF, rt, 7 h; (viii) Na_2SO_4, $NaBH_4$, EtOH, rt, 24 h; (ix) $(Boc)_2O$, CH_2Cl_2, rt, 3 h; (x) H_2, Pd/C, MeOH, rt, 6 h; (xi) 2-diazo-3,3,3-trifluoropropionic acid *p*-nitrophenyl ester, Et_3N, CH_2Cl_2, rt, overnight; (xii) TFA, CH_2Cl_2, rt, 2 h.

Scheme 4.

C_7-tyrosine-343-Boc (4d). To a MeOH (40 mL) solution of **4c** (0.869 g, 1 mmol) was added 10% Pd–C (0.5 g). This solution was purged several times with H_2 and stirred under H_2 atmosphere for 4 h. The reaction was terminated by filtration through celite and careful washing with copious volumes of MeOH. Removing of the solvent gave the raw product which was then chromatographed on 60 g of silica with CH_2Cl_2:MeOH (95:5) eluted **4d** 0.714 g (92%). R_f (CH_2Cl_2:MeOH, 90:10) 0.44; ^1H NMR (400 MHz, $CDCl_3$) δ 7.18–7.10 (d, 2H, C–CH–CH–C–OH), 6.96–6.91 (d, 2H, C–CH–CH–C–OH), 4.39–4.28 (t, 1H, NH–CO–CH–NH–CO), 3.75–3.65 (s, 3H, OCH_3), 3.28–2.92 (bm, 14H), 2.26–2.05 (m, 4H), 1.85–1.70 (bs, 4H), 1.61–1.51 (m, 2H), 1.47–1.02 (bm, 33H, including 27H from 3 Bocs), 0.89–0.81 (t, 3H, CH_2CH_3); FABMS ($C_{41}H_{71}N_5O_9$) 779 $(M + 1)^+$.

C_7-MeO-tyrosine-343-Boc (4e). Compound **4d** (0.5 g, 0.64 mmol), anhydrous K_2CO_3 (0.442 g, 3.2 mmol), and MeI (0.91 g, 6.4 mmol) in acetone (20 mL) was refluxed for 8 h, which was then filtered. Removing the solvent gave **4e** 0.492 g (97%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.10–7.02 (d, 2H, C–CH–CH–C–OCH_3), 6.75–6.65 (d, 2H, C–CH–CH–C–OCH_3), 4.39–4.28 (t, 1H, NH–CO–CH–NH–CO), 3.75–3.65 (s, 3H, OCH_3), 3.28–2.92 (bm, 14H), 2.26–2.05 (m, 4H), 1.85–1.70 (bs, 4H), 1.61–1.51 (m, 2H), 1.47–1.02 (bm, 33H, including 27H from three Bocs), 0.89–0.81 (t, 3H, CH_2CH_3); CI-MS ($C_{42}H_{73}N_5O_9$) 793$(M + 1)^+$.

C_7-MeO-tyrosine-343 (4f). The Boc deprotection of **4e** was achieved analogously to **2**. Compound **4f** (0.526 g, quantitative) was obtained in the form of yellowish solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.10–7.02 (d, 2H, C–CH–CH–C–OCH_3), 6.75–6.65 (d, 2H, C–CH–CH–C–OCH_3), 4.39–4.28 (t, 1H, NH–CO–CH–NH–CO), 3.75–3.65 (s, 3H, OCH_3), 3.28–2.85 (bm, 14H), 2.25–2.01 (m, 4H), 1.85–1.70 (bs, 4H), 1.61–1.51 (m, 2H), 1.10–1.41 (bm, 6H), 0.89–0.81 (t, 3H, CH_2CH_3); FABMS ($C_{27}H_{49}N_5O_3$ w/o TFA) 492 $(M + 1)^+$.



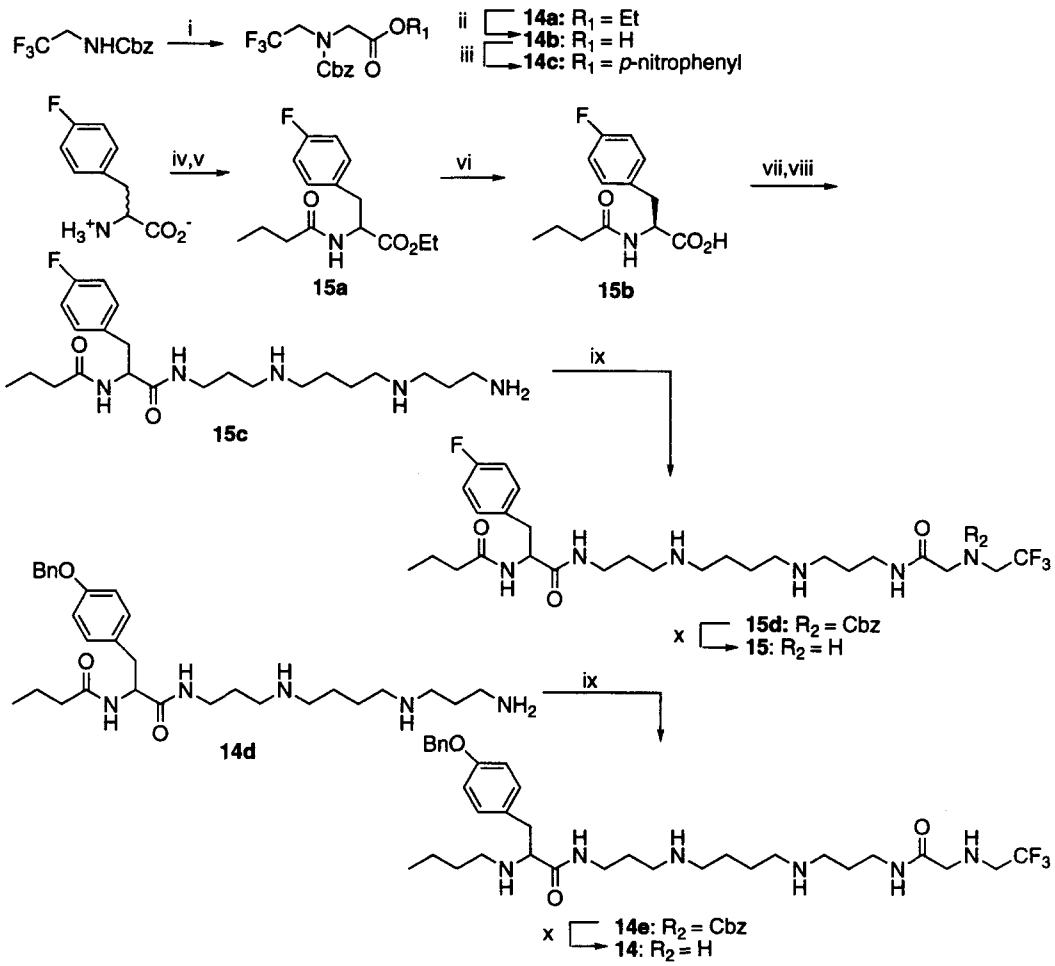
(i) spermine, Et_3N, THF, rt, 2.5 h; (ii) heptanoyl chloride, 2 N NaOH (in H_2O)/THF, rt, 5 h; (iii) CDI, Et_3N, THF, rt, 5 h; (iv) $(Boc)_2O$, CH_2Cl_2, rt, overnight; (v) H_2, Pd/C, MeOH, rt, onernight; (vi) 2-diazo-3,3,3-trifluoro propionic acid *p*-nitro phenyl ester, Et_3N, CH_2Cl_2, rt, 25 h; (vii) trifluoroacetic anhydride, Et_3N, DMAP (cat.), CH_2Cl_2, rt, 30 min; (viii) acetyl chloride, Et_3N, CH_2Cl_2, rt, 30 min; (ix) TFA, CH_2Cl_2, rt, quant.

Scheme 5.

C_7-MeO-tyrosine-343-lysine–Cbz (4g). To a MeOH (10 mL) solution of **4f** (0.769 g, 0.922 mmol) was added Et_3N (0.42 mL, 3 mmol), and the mixture was shaken well. After removing the volatile components, the yellowish residue was dissolved in DMF (12 mL), to which was added N_ε-*t*-Boc-N_α-Cbz-L-lysine *p*-nitrophenyl ester (0.463 g, 0.922 mmol). The reaction mixture was stirred overnight at rt. The product was submitted to next step without further purification.

C_7-MeO-tyrosine-343-lysine-Cbz-Boc (4h). The Boc protection of **4g** was achieved analogously to **4b** and the crude products were chromatographed on 60 g of silica with a step gradient system of CH_2Cl_2:MeOH from 99:1 to 97:3 yielded the desired product 0.338 g (35% in two steps). R_f (CH_2Cl_2:MeOH, 95:5) 0.19; ^1H NMR (400 MHz, $CDCl_3$) δ 7.28–7.12 (m, 5H, $C_6\underline{H}_5$–CH_2–O), 7.10–7.02 (d, 2H, C–CH–C$\underline{H}$–C–OCH_3), 6.75–6.65 (d, 2H, C–C$\underline{H}$–CH–C–OCH_3), 5.05–4.91 (s, 2H, C_6H_5–C$\underline{H}_2$–O), 4.39–4.28 (t, 1H, NH–CO–C$\underline{H}$–NH–CO), 3.75–3.65 (s, 3H, $OC\underline{H}_3$), 3.33–2.62 (bm, 14H), 2.15–2.01 (t, 2H, $C\underline{H}_2$–CO), 1.85–1.45 (m, 12H), 1.42–0.95 (bm, 39H, including 27H from three Bocs), 0.81–0.73 (t, 3H, $CH_2C\underline{H}_3$); FABMS ($C_{56}H_{91}N_7O_{12}$) 1055 $(M + 1)^+$.

C_7-MeO-tyrosine-343-lysine-Boc (4i). The Cbz deprotection of **4h** was achieved analogously to **4d**



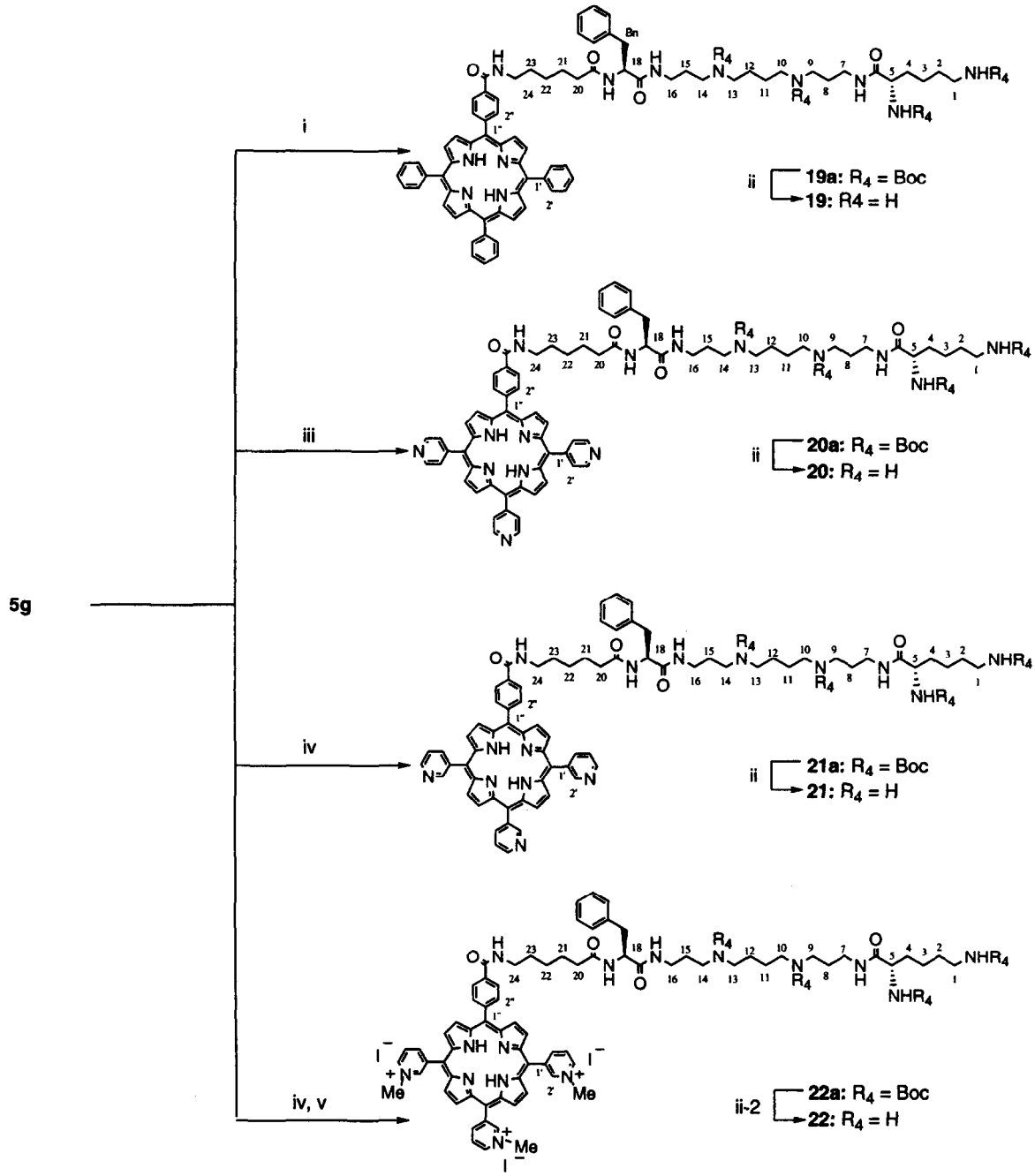
(i) *n*BuLi,THF,0 °C; then $BrCH_2CO_2Et$, 5 h; (ii) LiOH, MeOH-H_2O(2:1), overnight; (iii) *p*-nitrophenol, DCC, EtOAc; (iv) TsOH, EtOH, reflux; (v) butyl chloride,Et_3N,CH_2Cl_2, 0 °C; (vi) subtilisin carlsberg (0.05% w/w), 0.01 M KCl, 10-4 M K_2HPO_4, pH 8.0; (vii) *p*-nitrophenol, DCC, EtOAc; (viii) spermine, MeOH; (ix) **14c**, MeOH; (x) H_2,10% Pd/C, MeOH.

Scheme 6.

and the product was obtained in quantitative yield without purification. R_f (CH_2Cl_2:MeOH, 90:10) 0.2; ^{1}H NMR (400 MHz, CD_3OD) δ 7.08–6.99 (d, 2H, C–CH–CH–C–OCH_3), 6.75–6.65 (d, 2H, C–CH–CH–C–OCH_3), 4.42–4.35 (t, 1H, NH–CO–CH–NH–CO), 4.05–3.95 (t, 1H, CO–CH–NH_2), 3.66–3.58 (s, 3H, OCH_3), 3.24–2.41 (bm, 14H), 2.10–1.98 (t, 2H, CH_2–CO), 1.90–0.95 (bm, 51H, including 27H from three Bocs), 0.81–0.73 (t, 3H, CH_2CH_3); FABMS ($C_{48}H_{85}N_7O_{10}$) 921 $(M + 1)^+$.

C_7-MeO-tyrosine-343-lysine-diazo-Boc (4j). To an anhydrous DMF (3 mL) solution of **4i** (0.03 g, 0.0326 mmol), and 2-diazo-3,3,3-trifluoropropionic acid *p*-nitrophenyl ester (0.0099 g, 0.0359 mmol), was added Et_3N (5.04 mL, 0.0359 mmol) in dark. Then the reaction mixture was allowed to stir at rt in dark for 48 h. After removing the solvent at reduced pressure, the raw product was chromatographed on 3 g of silica gel with CH_2Cl_2:MeOH (90:10) under the red light, which yielded **4j** 0.015–0.027 g (43–63%). R_f (CH_2Cl_2:MeOH, 90:10) 0.14; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12–7.02 (d, 2H, C–CH–CH–C–OCH_3), 6.78–6.70 (d, 2H, C–CH–CH–C–OCH_3), 4.44–4.38 (t, 1H, NH–CO–CH–NH–CO), 3.71–3.62 (s, 3H, OCH_3), 3.25–2.68 (bm, 14H), 2.25–2.01 (m, 2H), 1.70–1.05 (bm, 51H, including 27H from three Bocs), 0.85–0.78 (t, 3H, CH_2CH_3); FABMS ($C_{51}H_{84}F_3N_9O_{11}$) 1057 $(M + 1)^+$.

C_7-MeO-tyrosine-PhTX-343-lysine-diazo (4). The Boc deprotection of **4j** was achieved analogously to **4f**, **4** was obtained in quantitative yield without purification. ^{1}H NMR (400 MHz, CD_3OD) δ 7.12–7.02 (d, 2H, C–CH–CH–C–OCH_3), 6.78–6.70 (d, 2H, C–CH–CH–C–OCH_3), 4.44–4.38 (t, 1H, NH–CO–CH–NH–CO), 3.71–3.62 (s, 3H, OCH_3), 3.25–3.25 (m, 2H), 3.19–2.65 (bm, 12H), 2.18–2.08 (m, 2H), 1.90–1.61 (bm, 12H), 1.58–1.42 (m, 4H), 1.45–1.10 (bm, 8H), 0.88–0.78 (t, 3H, CH_2CH_3); FABMS ($C_{36}H_{60}F_3N_9O_5$ w/o TFA) 757 $(M + 1)^+$.

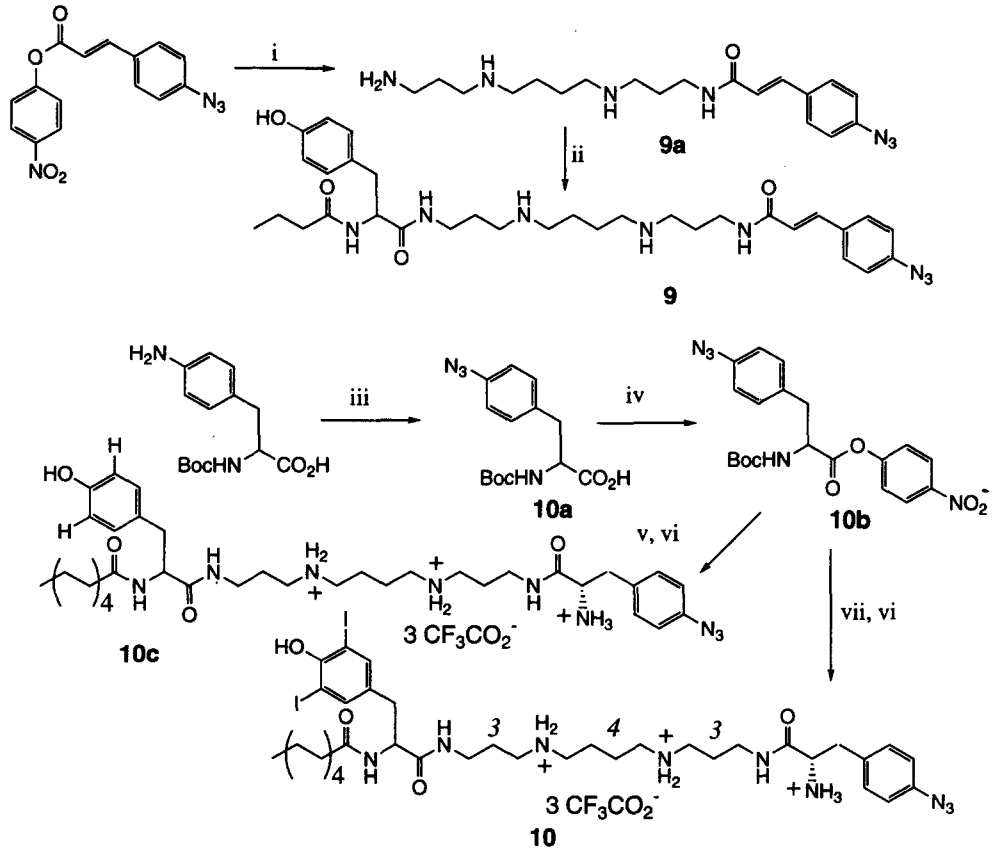


(i) TPP acid, EDC, DMAP, CH_2Cl_2, rt, 8 h, 96%; (ii) 1. TFA, CH_2Cl_2, rt, 1 h; 2. TFA, EtOH, rt, 8 h, quant; (iii) T4PyP acid, EDC, DMAP, CH_2Cl_2, rt, 8 h, 70%; (iv) T3PyP acid, EDC, DMAP, CH_2Cl_2, rt, 8 h, 82%; (v) CH_3I, rt, 8 h, quant.

Scheme 7.

L-Phenylalanine-(*N*-Cbz)-343 (5b). To a solution of spermine (1.08 g, 5.36 mmol) in dry THF was slowly added **5a** (1.06 g, 2.53 mmol) which resulted a bright yellow color in the solution. After completion of the addition, the solution was stirred at rt for 6 h. The solvent was removed under reduced pressure. **5b** (0.89 g, 73%) was obtained by flash chromatography. R_f (CH_2Cl_2:MeOH:*i*PrNH$_2$, 4:4:1) 0.26; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38–7.49 (br, m, 1H, NH), 7.18–7.29 (m, 10H, Cbz and Phe), 5.87–5.91 (m, 1H, NH), 5.04 (s, 2H, CH_2 of Cbz), 4.22–4.32 [m, 1H, H–C(12)], 3.19–3.39 (m, 2H, H-Bn), 2.99–3.07 [m, 2H, H–C(10)], 2.38–2.74 [m, 10H, H–C(1, 3, 4, 7, 8)], 1.42–1.68 [m, 8H, H–C(2, 5, 6, 9)]; CI-MS ($C_{27}H_{41}N_5O_3$) 484 $(M + 1)^+$.

L-Phenylalanine-(*N*-Cbz)-343-L-lysine-(di-Boc) (5c). To a solution of **5b** (0.89 g, 1.84 mmol) in dry THF was added *N*,*N*-di-*t*-Boc-L-lysine-*N*-hydroxysuccinimide ester (0.90 g, 2.02 mmol), followed by Et_3N (2



(i) spermine, MeOH, rt; (ii) N-butyryl-tyrosine *p*-nitrophenyl ester, MeOH; (iii) $NaNO_2$, AcOH-1.2 M HCl-H_2O, 4 °C; then, NaN_3, 4 °C; (iv) *p*-nitrophenol, DCC, EtOAc; (v) C_{10}-PhTX-343, DMF; (vi) TFA, CH_2Cl_2; (viii) C_{10}-I_2PhTX-343, DMF, rt

Scheme 8.

mL, 14.4 mmol). The reaction mixture was stirred at rt for 5 h. The solvent was removed by reduced pressure. Compound **5c** (0.99 g, 67%) was obtained by flash chromatography. R_f (CH_2Cl_2:MeOH:*i*PrNH$_2$, 85:15:1) 0.45; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54–7.65 (br, 1H, NH), 7.20–7.32 (m, 10H, Cbz and Phe), 5.92–5.96 (br, 1H, NH), 5.45–5.53 (br, 1H, NH), 4.95–5.05 (m, 2H, CH_2 of Cbz), 4.78–4.85 (br, 1H, NH), 4.40–4.44 [m, 1H, H–C(18)], 4.08–4.14 [m, 1H, H–C(5)], 3.18–3.38 [m, 4H, H–C(1), H-Bn], 2.98–3.12 [m, 4H, H–C(7, 16)], 2.66–2.85 [m, 8H, H–C(9, 10 ,13, 14)], 1.30–1.85 [m, 14H, H–C(2, 3, 4, 8, 11, 12, 15)], 1.42 (s, 9H, Boc), 1.39 (s, 9H, Boc); CI-MS ($C_{43}H_{69}N_7O_9$) 812 $(M + 1)^+$.

L-Phenylalanine-(*N*-Cbz)-343-(di-Boc)-L-lysine-(di-Boc) (5d). To the solution of **5c** (0.99 g, 1.22 mmol) in CH_2Cl_2 was added di-*t*-butyl dicarbonate (1.20 g, 5.45 mmol) followed by Et_3N (1.7 mL, 12.0 mmol). The reaction mixture was stirred at rt for 30 min. After extraction with satd $NaHCO_3$ solution (50 mL × 3), the combined organic layers were washed with brine (50 mL) once, dried over Na_2SO_4, and concentrated in vacuo. Compound **5d** (1.23 g, quat) was obtained by flash chromatography. R_f (CH_2Cl_2: MeOH, 20:1) 0.33; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18–7.32 (m, 10H, Cbz and Phe), 5.43–5.52 (br, 1H, NH), 5.19–5.32 (br, 1H, NH), 5.03–5.12 (m, 2H, CH_2 of Cbz), 4.62–4.73 (br, 1H, NH), 4.38–4.48 [br, 1H, H of C(18)], 3.98–4.13 [br, 1H, H of C(5)], 2.96–3.30 [m, 16H, H of Bn and C(1, 7, 9, 10, 13, 14, 16)], 1.22–1.90 [m, 14H, H of C(2, 3, 4, 8, 11, 12, 15)], 1.41–1.45 (m, 36H, Boc); CI-MS ($C_{53}H_{85}N_7O_{12}$) 1012 $(M + 1)^+$.

L-Phenylalanine-343-(di-Boc)-L-lysine-(di-Boc) (5e). Cbz group in **5d** was removed analogously to **4g**, which yielded **5e** (0.93 g, 88%). R_f (CH_2Cl_2: MeOH, 9:1) 0.57; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21–7.32 (m, 5H, Phe), 5.20–5.29 (br, m , 1H, NH), 4.64–4.73 (br, m, 1H, NH), 4.06–4.15 [m, 1H, H–C(5)], 3.57–3.66 [m, 1H, H–C(18)], 3.04–3.28 [m, 16H, H of Bn and C(1, 7, 9, 10, 13, 14, 16)], 1,33–1.88 [m, 14H, H–C(2, 3, 4, 8, 11, 12, 15)], 1.42–1.45 (m, 36H, Boc); FABMS ($C_{53}H_{85}N_7O_{12}$) 878 $(M + 1)^+$.

C_6-(*N*-Cbz)-L-phenylalanine-343-(di-Boc)-L-lysine-(di-Boc) (5f). To the mixture of **5e** (0.93 g, 1.06 mmol) and Cbz-6-aminohexanoic acid (0.31 g, 1.16 mmol) in CH_2Cl_2 (20 mL) was added EDC (0.26 g, 1.38 mmol) and DMAP (0.17 g, 1.38 mmol). The solution was

stirred at rt for 5 h. The solvent was removed under reduced pressure. Compound **5f** (1.05 g, 88%) was obtained by column chromatography. R_f (CH_2Cl_2:MeOH, 9:1) 0.60; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.35 (m, 10H, Cbz and Phe), 6.15–6.25 (m, 1H, NH), 5.19–5.32 (m, 1H, NH), 5.05–5.12 (s, 2H, CH_2 of Cbz), 4.63–4.74 [m, 1H, H–C(18)], 4.05–4.15 [m, 1H, H–C(5)], 2.98–3.30 [m, 18H, H of Bn and C(1, 7, 9, 10, 13, 14, 16, 24)], 2.15 [t, 2H, J = 6.4 Hz, H–C(20)], 1.21–1.88 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)], 1.41–1.44 (m, 36H, Boc); CI-MS ($C_{59}H_{96}N_8O_{13}$) 1142 (M+NH_3+ 1)$^+$, 1125 (M + 1)$^+$.

C_6-L-phenylalanine-343-(di-Boc)-L-lysine-(di-Boc) (5g). Cbz group in **5f** was removed analogously to **4g**, which yielded **5g** (0.80 g, 87%) as a colorless oil. R_f (CH_2Cl_2:MeOH:iPrNH$_2$, 85:15:1) 0.40; ^{1}H NMR (400 MHz, CD_3OD) δ 7.25–7.28 (m, 5H, Phe), 4.54–4.59 [m, 1H, H–C(18)], 3.88–3.95 [m, 1H, H–C–(5)], 3.02–3.24 (m, 16H, H of Bn and C [1, 7, 9, 10, 13, 14, 16)], 2.72 [t, 2H, J = 7.2 Hz, H–C(24)], 2.17-2.24 [m, 2H, H–C(20)], 1.32-1.80 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)], 1.42–1.45 (m, 36H, Boc); CI-MS ($C_{51}H_{90}N_8O_{11}$) 991 (M + 1)$^+$.

C_6-L-phenylalanine-343-L-lysine (5). Boc groups of **5g** were removed analogously to **2**, which yieldied **5** (5.9 mg, quat). ^{1}H NMR (400 MHz, CD_3OD) δ 7.20–7.31 (m, 5H, Phe), 4.45–4.55 [m, 1H, H–C(18)], 3.83–3.87 [m, 1H, H–C(5)], 3.39–3.49, 3.22–3.30, and 2.82–3.14 [m, 18H, H of Bn and C(1, 7, 9, 10, 13, 14, 16, 24)], 2.21 [t, J = 8.0 Hz, 2H, H–C(20)], 1.15–1.95 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)]; CI-MS ($C_{31}H_{58}N_8O_3$) 591 (M + 1)$^+$.

Spermine-Cinn-*p*-N_3 (9a). To a suspension of MeOH (10 mL) containing *p*-azidocinnamic acid *p*-nitrophenyl ester (1.01 g, 3.26 mmol) was added spermine (0.70 g, 3.44 mmol) in 10 mL MeOH. The mixture was stirred for 6 h, followed by evaporation of the solvent in vacuo. The residue obtained as an yellow oil was mixed with CH_2Cl_2:MeOH (1:1, 15 mL). The insoluble material was filtered through a pad of celite and washed with CH_2Cl_2:MeOH (1:1, 5 mL). The filtrate was concentrated and purified by flash column chromatography (silica) using a step gradient solvent system (CH_2Cl_2:MeOH, 9:1, CH_2Cl_2:MeOH:iPrNH$_2$, 15:5:1 and CH_2Cl_2:MeOH:iPrNH$_2$, 4:4:1). Product was obtained as a pale-orange oil in 48% yield (0.41 g). ^{1}H NMR (400 MHz, CD_3OD) δ 7.59–7.57 (d, 2H, J = 8.6 Hz), 7.51–7.47 (d, 1H, J = 15.6 Hz), 7.10–7.08 (d, 2H, J = 8.4 Hz), 6.57–6.53 (d, 1H, J = 15.8 Hz), 3.37–3.33 (t, 2H, J = 6.8 Hz), 2.69–2.56 (m, 10 H), 1.77–1.74 (q, 2H, J = 7.2 Hz), 1.68–1.64 (q, 2H, J = 7.2 Hz), 1.54–1.53 (bs, 4H).

PhTX-343-Cinn-*p*-N_3 (9). Compound **9a** (0.36 g, 0.96 mmol) and *N*-butyryl-L-tyrosine *p*-nitrophenyl ester (0.3 g) in 3 mL of MeOH were stirred at rt overnight. After evaporation of the reaction mixture, the orange residue was purified by silica gel flash column chromatography eluting with CH_2Cl_2:MeOH (9:1) and CH_2Cl_2:MeOH:iPrNH$_2$ (15:5:1). The desired product was obtained as a pale-yellow oil in 36% yield (176 mg). R_f (CH_2Cl_2:MeOH:iPrNH$_2$, 15:5:1) 0.27. ^{1}H NMR (400 MHz, CD_3OD) δ 7.58–7.56 (d, 2H, J = 8.4 Hz), 7.53–7.47 (d, 1H, J = 15.6 Hz), 7.08–7.04 (dd, 4H, J = 8.4 Hz), 6.72–6.69 (d, 2H, J = 8.4 Hz), 6.62–6.57 (d, 1H, J = 15.6 Hz), 4.50–4.45 (t, 1H, J = 7.2 Hz), 3.39–3.35 (t, 2HJ = 6.6 Hz), 3.22–3.15 (t, 2H, J = 6.2 Hz), 3.02–2.94 (dd, 1H, J = 13.5, 6.9 Hz), 2.84–2.77 (dd, 1H, J = 8.1, 13.5 Hz), 2.74–2.51 (m, 8H), 2.19–2.14 (t, 2H, J = 7.2 Hz), 1.83–1.79 (q, 2H, J = 6.9 Hz), 1.58–1.50 (m, 8H), 0.87–0.82 (t, 3H, J = 7.5 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 175.75, 173.99, 168.66, 157.41, 142.68, 140.57, 133.13, 131.25, 130.46, 128.92, 121.48, 120.48, 116.29, 115.73, 56.62, 49.80, 47.35, 47.11, 38.71, 38.30, 38.16, 37.87, 29.71, 29.29, 27.66, 27.58, 20.19, 13.95.

***N*-Boc-*p*-N_3-L-phenylalanine (10a)**. To a cold (4 °C) solution of *N*-Boc-*p*-amino-L-phenylalanine (1.28 g, 4.57 mmol) in 1.2 M AcOH:1.2 M HCl:water (1:1:1, 10 mL) was added $NaNO_2$ (0.316 g, 4.58 mmol) in H_2O (3 mL). After stirring for 30 min at 4 °C, NaN_3 (0.3 g) in H_2O (3 mL) was added to the diazotized solution, followed by stirring for 30 min at 4 °C until the generation of N_2 stopped. The aqueous solution was extracted with CH_2Cl_2 (100 mL), and the organic phase was dried over $MgSO_4$ before evaporation. The product was obtained as a pale-red oil: it was converted to solid by lyophilization of a frozen solution in MeCN:H_2O (1:1). The yield was 91% (1.33 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19–7.17 (t, 2H, J = 8.2 Hz), 6.98–6.96 (d, 2H, J = 8.2 Hz), 4.95–4.93 (bd, 1H, J = 7.2 Hz), 4.59–4.58 (q, 1H, J = 5.9 Hz), 3.19–3.16 (dd, 1H, J = 9.0, 13.8 Hz), 3.08–3.02 (dd, 1H, J = 5.9, 13.8 Hz), 1.43 (s, 9H).

***N*-Boc-*p*-N_3-L-phenylalanine *p*-nitrophenyl ester (10b)**. *N*-Boc-*p*-N_3-L-phenylalanine (1.33 g, 4.34 mmol) and *p*-nitrophenol (0.665 g, 4.78 mmol) in 25 mL EtOAc was cooled in ice bath. To this solution was added DCC (0.986 g, 4.78 mmol) while stirring at 4 °C. After 12 h, the precipitate was filtered through a pad of celite and washed with EtOAc (10 mL). The filtrate was evaporated in vacuo, and yielded a crude product that was purified with flash column chromatography (silica, Hexane:EtOAc, 2:1). The product was obtained as a pale-yellow solid in 85% yield (1.58 g). R_f (Hexane:EtOAc, 2:1) 0.79. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29–8.26 (d, 2H, J = 8.0 Hz), 7.24–7.19 (t, 4H, J = 8.0 Hz), 7.04–7.01 (d, 2H, J = 8.0 Hz), 5.04 (bd, 1H, J = 7.0 Hz), 4.79–4.77 (q, 1H, J = 6.7 Hz), 3.23–3.21 (m, 2H), 1.45 (s, 9H).

C_{10}-PhTX-343-phenylalanine-N_3-(N_α-Boc). To a solution of DMF (4 mL) containing **10b** (0.25 g, 0.58 mmol) was added C_{10}-PhTX-343 (0.286 g, 0.55 mmol) in DMF (1 mL). After stirring at rt for 24 h, solvent was removed. The resulting residue was purified with flash column chromatography (silica gel, CH_2Cl_2:MeOH:iPrNH$_2$, 15:5:1). The product was obtained as a pale-yellow solid in 66% yield (0.293 g). ^{1}H NMR

(400 MHz, $CDCl_3$ with 3 drops of CD_3OD) δ 7.23–7.21 (d, 2H, J = 8.0 Hz), 7.06–7.03 (d, 2H, J = 8.0 Hz), 6.96–6.94 (d, 2H, J = 8.0 Hz), 6.80–6.78 (d, 2H, J = 8.0 Hz), 4.53–4.39 (m, 2H), 3.51–3.19 (m, 6H), 2.91–2.22 (m, 8H), 2.21–2.18 (t, 2H, J = 7.6 Hz), 1.74–1.56 (bm, 8H), 1.37–1.25 (m, 23H), 0.89–0.86 (t, 3H, J = 6.4 Hz).

C_{10}-PhTX-343-phenylalanine-N_3 (10c). A solution of CH_2Cl_2 (2 mL) containing C_{10}-PhTX-343-phenylalanine-N_3-(N_α-Boc) (0.25 g) was mixed with TFA:CH_2Cl_2 (1:1, 4 mL). The mixture was stirred for 4 h at 4 °C. Volatiles were removed in vacuo, affording a pale-red oily residue. This crude product was solubilized in water, frozen, and lyophilized. ^1H NMR (400 MHz, D_2O) δ 7.25–7.23 (d, 2H, J = 8.4 Hz), 7.12–7.06 (m, 4H), 6.81–6.79 (d, 2H, J = 8.4 Hz), 4.40–4.36 (t, 1H, J = 7.2 Hz), 4.08–4.06 (t, 1H, J = 6.4 Hz), 3.26–2.70 (m, 16 H), 2.17–2.13 (m, 2H), 1.72–1.61 (bs, 6H), 1.41–1.31 (bs, 2H), 1.25–1.00 (m, 14 H), 0.82–0.80 (t, 3H, J = 6 Hz); FABMS ($C_{38}H_{61}N_9O_4$) 708 $(M + 1)^+$.

C_{10}-I_2PhTX-343-phenylalanine-N_3 (10). A DMF solution (2 mL) containing C_{10}-I_2PhTX-343 (18 mg, 0.023 mmol) and *N*-Boc-*p*-N_3-L-phenylalanine *p*-nitrophenyl ester (11 mg, 0.026 mmol) was stirred at rt for 24 h. After evaporation of DMF, the residue was purified with flash column chromatography (silica gel) elutied with CH_2Cl_2:MeOH (10:1) and CH_2Cl_2:MeOH:*i*$PrNH_2$ (15:5:1). The product C_{10}-I_2PhTX-343-phenylalanine-N_3-(N_α-Boc) was obtained in 69% yield (17 mg). The *N*-Boc group was removed by treating with TFA:CH_2Cl_2 (1:1, 2 mL) for 4 h at 4 °C. ^1H NMR (400 MHz, CD_3OD) δ 7.54 (s, 2H), 7.24–7.22 (d, 2H, J = 8.4 Hz), 6.99–6.97 (d, 2H, J = 8.4 Hz), 4.40–4.36 (t, 1H, J = 7.6 Hz), 3.78–3.75 (dd, 1H, J = 5.2, 7.2 Hz), 3.31–3.13 (m, 4H), 3.04–2.74 (m, 12H), 2.24–2.18 (m, 2H), 1.91–1.75 (m, 8H), 1.63–1.53 (m, 2H), 1.40–1.11 (m, 12H), 0.90–0.87 (t, 3H, J = 6.4 Hz); FABMS ($C_{38}H_{59}I_2N_9O_4$) 982 $(M + Na)^+$, 960 $(M + 1)^+$.

***N*-Cbz-serine methyl ester (11a).** To a solution of methyl serine HCl salt (5.00 g, 32.0 mmol) in CH_2Cl_2 (100 mL) were added Et_3N (11.2 mL, 80.3 mmol) and CbzCl (5.26 mL, 37.0 mmol), the mixture was stirred at rt for 5 h. The reaction was worked up by washing with H_2O (50 mL × 3), the organic layer was dried over Na_2SO_4, and concentrated to give the desired product (5.68 g, 70%) after purified by flash chromatography. R_f (EtOAc:Hexane, 1:1) 0.1; ^1H NMR (400 MHz, $CDCl_3$) δ 7.41–7.31 (m, 5H), 5.72–5.62 (br s, 1H), 5.14 (s, 2H), 4.78–3.83 (m, 1H), 3.82 (s, 3H), 3.77–3.72 (m, 2H); CI-MS ($C_{12}H_{15}NO_5$) 254 $(M + 1)^+$.

2-(*N*-Cbz-amino)-3-bromo-serine methyl ester (11b). NBS (325.3 mg, 1.827 mmol) was slowly added to a solution of **11a** (233.0 mg, 0.914 mmol) and PPh_3 (478.8 mg, 1.827 mmol) in DMF (15 mL). The mixture was stirred at 50 °C for 30 min, MeOH (1 mL) was added to destroy the excess reagent. After 5 min, ether (30 mL) was added, the organic layer was washed with H_2O (10 mL), sat $NaHCO_3$ (15 mL), brine (20 mL), and dried over Na_2SO_4. Purification by flash chromatography yielded the product (174.3 mg, 60%). R_f (EtOAc:hexane, 35:65) 0.5; ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.30 (m, 5H), 5.77–5.69 (br s, 1H), 5.12 (s, 2H), 4.48–4.38 (m, 2H), 3.75 (s, 3H), 2.30 (br s, 1H), 1.65 (br s, 1H); CI-MS ($C_{12}H_{14}BrNO_4$) 319 $(M + 1)^+$.

2-(*N*-Cbz-amino)-[1-(3-carbo-*tert*-butoxyamino)propyl]-1-aminopropanoic methyl ester (11c). To a solution of MeCN (20 mL) containing **11b** (174.3 mg, 0.548 mmol) and 270 mg of KF:celite was injected 3-*N*-Boc-diaminopropane (0.21 mL, 1.24 mmol) by syringe pump in MeCN (1 mL), and the mixture was refluxed for 10 h. The suspension then was filtered through celite, and the solvent was removed by evaporation. After column chromatography purification, product 219.7 mg (98%) was obtained. R_f (CH_2Cl_2:MeOH, 95:5) 0.25; ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.31 (m, 5H), 5.13 (s, 2H), 3.77 (s, 3H), 3.25–2.90 (m, 7H), 2.68–2.60 (m, 2H), 1.47 (s, 9H); CI-MS ($C_{20}H_{31}N_3O_6$) 410 $(M + 1)^+$.

2-(*N*-Cbz-amino)*N*-carbo-*tert*-*N*-[1-(3-carbo-*tert*-butoxyamino)propyl]-1-aminopropanoic methyl ester (11d). Boc protection was achieved analogously to **5d**. The yield was 60% (19.6 mg). R_f (EtOAc:hexane, 35:65) 0.3; ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.30 (m, 5H), 5.12 (s, 2H), 3.61 (s, 3H), 3.60–3.45 (m, 4H), 3.28–2.90 (m, 3H), 2.68–2.60 (m, 2H), 1.47 (s, 9H), 1.42 (s, 9H); CI-MS ($C_{25}H_{39}N_3O_8$) 510 $(M + 1)^+$.

2-(*N*-Cbz-amino)*N*-carbo-*tert*-*N*-[1-(3-carbo-*tert*-butoxyamino)propyl]-1-aminopropanal (11e). To a CH_2Cl_2 (10 mL) solution of ester **11d** (260.1 mg, 0.511 mmol) was injected 1.0 M DIBAL in hexane at −78 °C under argon protection. The reaction was stirred over 3 h until starting material disappeared by TLC analysis. The reaction was quenched by 1 N HCl (15 mL), and the aqueous solution was extracted with CH_2Cl_2. After the solvent was removed, the residue was applied to silica gel column to give the desired product (198.3 mg, 81%). R_f (EtOAc:hexane, 35:6) 0.5; ^1H NMR (400 MHz, $CDCl_3$) δ 9.61 (s, 1H), 7.33 (m, 5H), 5.02 (s, 2H), 3.67 (m, 2H), 3.45 (br, s , 2H), 3.28 (m, 3H), 1.68 (m, 2H), 1.47 (s, 9H), 1.42 (s, 9H); CI-MS ($C_{24}H_{37}N_3O_7$) 480 $(M + 1)^+$.

C_7-phenylalanine-43(2-*N*-Cbz)3 (11i). The mixture of **11g** (27.6 mg, 79.5 μmol) and **11e** (38.1 mg, 79.5 mol) in dry EtOH were stirred with Na_2SO_4 (113 mg, 0.795 mmol) as drying reagent under argon for 12 h. $NaBH_4$ (30.1 mg, 0.795 mmol) was added in portions at low temperature, and the solution was continuously stirred overnight. The reaction was terminated by quenching with H_2O (2 mL), extracted with CH_2Cl_2 (15 mL × 3), and the organic layer was dried over Na_2SO_4, then it was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (5 mL) and di-*tert*-

dicarbonate (17.3 mg, 79.5 mmol) was added. The mixture was stirred at rt for 3 h, and evaporated to afford a clear oil (51.5 mg, 80%) which was purified by prep. TLC (CH_2Cl_2:MeOH, 95:5); ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (m, 5H), 7.30–7.25 (m, 5H), 5.05 (s, 2H), 4.57–4.53 (t, 1H, J = 7.8 Hz), 4.09–4.00 (m, 2H), 3.20–2.87 (m, 12H), 2.20 (t, 2H, J = 7.8 Hz), 1.61–1.50 (m, 17H), 1.46 (s, 9H), 0.88 (t, 3H, J = 7.2 Hz); CI-MS ($C_{50}H_{80}N_6O_{10}$) 926 $(M + 1)^+$.

C_7-phenylalanine-43(2-amine)3 (11j). The Cbz group was removed analogously to **4g**, yield was 91% (14.2 mg). ^1H NMR (400 MHz, $CDCl_3$) δ 7.30–7.19 (m, 5H), 4.45 (t, 1H, J = 7.8 Hz), 4.09–4.04 (m, 2H), 3.20–2.87 (m, 12H), 2.20 (t, 2H, J = 7.8 Hz), 1.61–1.50 (m, 17H), 1.46 (s, 9H), 0.88 (t, 3H, J = 7.2 Hz); CI-MS ($C_{42}H_{74}N_6O_8$) 792 $(M + 1)^+$.

C_7-phenylalanine-tri-Boc-43(2-N_2CF_3)3 (11k). This compound was synthesized analogously to **4j**, with 33% yield (2.2 mg). ^1H NMR (400 MHz, $CDCl_3$) δ 7.30–7.19 (m, 5H), 4.45 (t, 1H, J = 7.8 Hz), 4.09–4.04 (m, 2H), 3.20–2.87 (m, 12H), 2.20 (t, 2H, J = 7.8 Hz), 1.61–1.50 (m, 17H), 1.46 (s, 9H), 0.88 (t, 3H, J = 7.2 Hz); CI-MS ($C_{44}H_{71}F_3N_8O_9$) 914 $(M + 1)^+$.

C_7-Phe 43(2-N_2CF_3)3 (11). The Boc groups were removed analogously to **2**. The crude was applied to RP-HPLC purification [gradient of H_2O (0.1% TFA)MeCN from 95:5 to 5:95 over 30 min], retention time: 17–18.2 min. ^1H NMR (400 MHz, CD_3OD) δ 7.30–7.19 (m, 5H), 4.45 (t, 1H, J = 7.8 Hz), 4.09–4.04 (m, 2H), 3.79–3.75 (m, 1H), 3.20–2.87 (m, 12H), 2.20 (t, 2H, J = 7.8 Hz), 1.61–1.40 (m, 17H), 0.88 (t, 3H, J = 7.2 Hz); CI-MS ($C_{29}H_{47}F_3N_8O_3$) 614 $(M + 1)^+$.

343-Lysine-(N_ε-*t*-Boc-N_α-Cbz) (12a). To a solution of spermine (606 mg, 3.0 mmol) and Et_3N (0.42 mL, 3.0 mmol) in 20 mL of THF, N_ε-*t*-Boc-N_α-Cbz-L-lysine *p*-nitrophenyl ester (501 mg, 1.0 mmol) in 10 mL of THF was added dropwise while stirring. The reaction was stirred at rt for 2.5 h which was then terminated by removing the solvent. The crude was purified with silica gel flash column chromatography (CH_2Cl_2:MeOH:*i*PrNH$_2$, 80:20:10), which gave the desired product in 90% yield (507 mg). R_f (CH_2Cl_2:MeOH:*i*PrNH$_2$, 4:4:1) 0.3; ^1H NMR (400 MHz, CD_3OD) δ 7.37–7.31 (m, 5H), 5.08 (s, 2H), 4.02–3.93 (m, 1H), 3.27–3.18 (m, 2H), 3.03–2.95 (m, 2H), 2.72–2.54 (m, 10H), 1.74–1.37 (m, 23H).

C_7-phenylalanine (12b). To a solution of phenylalanine (1.155 g, 7.0 mmol) in 20 mL of 2 N NaOH:THF (1:1) was added heptanoyl chloride (2.2 mL, 14 mmol). The mixture was stirred at rt for 5 h, which pH was then adjusted to 5 by 1 N HCl, extracted by CH_2Cl_2 (50 mL × 3). The organic layer was dried with brine and $MgSO_4$, and concentrated to give the desired product in 96% yield (1.867 g). R_f (CH_2Cl_2:MeOH, 9:1) 0.5.

C_7-Phenylalanine-343(di-Boc)-lysine(N_ε-*t*-Boc-N_α-Cbz) (12c). To a solution of **12b** (249 mg, 899 μmol) in 6 mL of THF was added 1,1′-carbonyldiimidazole (175 mg, 1.079 mmol). The mixture was stirred at rt for 1.5 h, which was then slowly added to the solution of **12a** (507 mg, 899 μmol) and Et_3N (0.4 mL, 2.697 mmol) in 10 mL of THF while stirring. The reaction was stirred for 3.5 h and terminated by removing the solvent. The crude was purified by silica gel flash chromatography which gave 561 mg of the product (76%). R_f (CH_2Cl_2:MeOH:*i*PrNH$_2$, 90:10:2) 0.3.

The Boc protection of the above product (561 mg, 682 mol) was achieved analogously to **5d**, which gave the desired product in 95% yield (659 mg). R_f (CH_2Cl_2:MeOH, 9:1) 0.3; ^1H NMR (400 MHz, CD_3OD) δ 7.37–7.17 (m, 5H), 5.06 (s, 2H), 4.59–4.50 (m, 1H), 4.04–3.95 (m, 1H), 3.23–2.94 (m, 15H), 2.89–2.78 (m, 1H), 2.20–2.10 (m, 2H), 1.80–1.10 (m, 49H), 0.88 (t, 3H, J = 7.2 Hz); CI-MS ($C_{55}H_{89}N_7O_{11}$) 1025 $(M + 1)^+$.

C_7-phenylalanine-343(di-Boc)-lysine(N_ε-*t*-Boc) (12d). Cbz group of **12c** was removed analogously to **4g**, which gave the desired product in 84% yield (543 mg). R_f (CH_2Cl_2:MeOH, 9:1) 0.5; ^1H NMR (400 MHz, CD_3OD) δ 7.25–7.11 (m, 5H), 4.55–4.45 (m, 1H), 3.28–2.90 (m, 16H), 2.87–2.74 (m, 1H), 2.18–2.05 (m, 2H), 1.75–1.05 (m, 49H), 0.88 (t, 3H, J = 7.2 Hz).

C_7-phenylalanine-343(di-Boc)-lysine(N_ε-*t*-Boc-N_α-$COCF_3$) (12e). To a solution of **12d** (54 mg, 61 μmol), Et_3N (0.025 mL, 188 μmol) and DMAP (cat. amount) in 3 mL of dry CH_2Cl_2 was added trifluoroacetic anhydride, the mixture was then stirred at rt for 30 min. The reaction was terminated by removing the solvent. The crude product was purified by silica gel flash chromatography, which gave the desired product (43.54 mg, 92%). R_f (CH_2Cl_2:MeOH, 9:1) 0.6; ^1H NMR (400 MHz, CD_3OD) δ 7.35–7.15 (m, 5H), 4.65–4.50 (m, 1H), 4.35–4.25 (m, 1H), 3.30–2.97 (m, 15H), 2.98–2.80 (m, 1H), 2.28–2.12 (br s , 2H), 1.92–1.12 (m, 49H), 0.88 (t, 3H, J = 7.2 Hz).

C_7-phenylalanine-343-lysine-(N_α-$COCF_3$) (12). The Boc groups were removed analogously to **2**. The crude was applied to RP-HPLC [gradient of H_2O (0.1% TFA) MeCN from 85:15 to 0:100 over 30 min], the retention time was 8.5 min. The product was obtained in 97% yield (36.5 mg). R_f (CH_2Cl_2:MeOH:*i*PrNH$_2$, 85:10:5) 0.1; ^1H NMR (400 MHz, CD_3OD) δ 7.27–7.15 (m, 5H), 4.46–4.39 (m, 1H), 4.28–4.02 (m, 1H), 3.48–3.33 (m, 1H), 3.27–2.82 (m, 15H), 2.18–2.08 (m, 2H), 2.00–1.60 (m, 12H), 1.53–1.37 (m, 4H), 1.30–1.10 (m, 6H), 0.85 (t, 3H, J = 7.2 Hz); FABMS ($C_{34}H_{58}F_3N_7O_4$) 686 $(M + 1)^+$.

C_7-MeO-tyrosine-343-lysine (13). The Boc deprotection of **4i** was achieved analogously to **4f** and **13** was obtained in quantitative yield. No further purification was necessary. R_f (CH_2Cl_2:MeOH:*i*PrNH$_2$, 4:4:1) 0.6; ^1H NMR (400 MHz, CD_3OD) δ 7.04–6.98 (d, 2H, C–CH–C<u>H</u>–C–OCH_3), 6.72–6.67 (d, 2H, C–C<u>H</u>–CH–C–

OCH_3), 4.30–4.24 (t, 1H, NH–CO–CH–NH–CO), 3.75–3.68 (t, 1H, CO–CH–NH_2), 3.62–3.59 (s, 3H, OCH_3), 3.36–3.25 (m, 2H), 3.20–2.65 (bm, 12H), 2.10–1.98 (t, 2H, CH_2–CO), 1.90–1.25 (bm, 16H), 1.20–1.02 (m, 8H), 0.81–0.72 (t, 3H, CH_2CH_3); FABMS ($C_{33}H_{61}N_7O_4$ w/o TFA) 621 $(M + 1)^+$.

N_α-CH_2CF_3-N_α-Cbz-glycine ethyl ester (14a). To a solution of CF_3CH_2NH-Cbz (1.5 g, 6.38 mmol) in 30 mL THF at 0 °C was added *n*-BuLi (3.5 mL, 2.0 M solution in cyclohexane). After stirring for 10 min, 1-bromomethyl acetate (1.17 g, 7.00 mmol) was injected. The mixture was stirred for 5 h and then allowed to warm to rt, to which 10 mL H_2O was added. The aqueous layer was extracted with EtOAc. The organic layer was dried over $MgSO_4$ and applied to silica gel column after evaporation of solvent (hexane:EtOAc, 4:1); 1.85 g of product was obtained (91%). R_f (hexane:EtOAc, 2:1) 0.60; ^{1}H NMR (200 MHz,$CDCl_3$) δ 7.34–7.31 (m, 5H, $C_6H_5CH_2$), 5.18–5.15 (d, 2H, $C_6H_5CH_2$), 4.24–4.07 (two q, 2H, COO–CH_2CH_3), 4.13–4.07 (d, 2H, NCH_2COOEt), 4.03–3.90 (q, 2H, CF_3CH_2–N), 1.29–1.14 (two t, 3H, $COOCH_2CH_3$); CI-MS ($C_{14}H_{16}F_3NO_4$) 337 $(M+NH_3+ 1)^+$.

N_α-CH_2CF_3-N_α-Cbz-glycine (14b). To a solution of **14a** (1.45 g,4.52 mmol) in 30 mL MeOH was added a solution of LiOH monohydrate (776 mg, 18 mmol) in 5 mL H_2O. The mixture was then stirred at rt overnight; 1.19 g was obtained (90%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.42 (br, s, 1H, COOH), 7.28–7.18 (m, 5H, $C_6H_5CH_2$), 5.12–5.11 (d, 2H, $C_6H_5CH_2$), 4.12–4.08 (d, 2H, NCH_2COOEt), 4.05–3.80 (two q, 2H, CF_3CH_2–N); CI-MS ($C_{12}H_{12}F_3NO_4$) 309 $(M + NH_3+ 1)^+$.

For the synthesis of **14d**, see ref 21.

C_4-*p*-F-phenylalanine-343-glycine-N_α-CH_2CF_3(14). ^{1}H NMR (300 MHz, HCl salt, CD_3OD) δ 7.09–7.06 (d, 2H, C–CH–CH–C–OH), 6.74–6.71 (d, 2H, C–CH–CH–C–OH), 4.46–4.41 (t, 1H, CH_2CHCO), 4.16–4.06 (q, 2H, NCH_2CF_3), 4.04 (s, 2H, $COCH_2N$;), 3.40–3.23 (m, 4H), 3.10–2.96 (m, 6H), 2.84–2.81 (m, 4H), 2.23–2.18 (t, 2H, CH_3CH_2–CH_2CO), 1.99–1.77 (br, m, 8H), 1.60–1.51 (m, 2H, CH_3CH_2–CH_2CO), 0.87–0.82 (t, 3H, $CH_3CH_2CH_2$–CO); ^{13}C NMR (75.43 MHz, HCl salt, CD_3OD) δ 176.27 ($CONH(CH_2)_3$–NH), 174.67 (CO–NH–CH), 166.16 (N–CO–CH_2N), 157.34, 131.31, 128.95, 116.33, 56.95, 49.35, 48.22, 46.59, 46.33, 38.60, 38.04, 37.40, 36.92, 27.29, 27.23, 24.30, 20.23, 13.93. ^{19}F NMR (373 MHz, ^{1}H decoupled) δ −66.2 (CF_3; HCl salt, solid-state), −70.7 (CF_3; HCl salt, solution); DCI-MS ($C_{27}H_{45}F_3N_6O_4$) 575 $(M + 1)^+$.

C4-*p*-F-phenylalanine-343 (15c). ^{1}H NMR (300 MHz, TFA salt, CD_3OD) δ 7.29–7.24 (dd, 2H, C–CH–CH–C–F), 7.04–6.98 (dd, 2H, C–CH–CH–C–F), 4.51–4.46 (dd, 1H, NH–CH–CO), 3.33–2.88 (m, 14H), 2.20–2.16 (t, 2H, CH_2CH_2CO), 2.20–2.09 (q, 2H, $CONHCH_2CH_2CH_2NH$), 1.88–1.81 (br, m, 6H), 1.58–1.50 (m, 2H, $CH_3CH_2CH_2CO$), 0.86–0.81 (t, 3H, $CH_3CH_2CH_2CO$); FABMS ($C_{23}H_{40}FN_5O_2$) 438 $(M + 1)^+$.

C_4-*p*-F-phenylalanine-343-glycine-N_α-Cbz-N_α-CH_2CF_3 (15d). The coupling was achieved analogously to **2b** with the yield of 86%. R_f (CH_2Cl_2:MeOH:*i*$PrNH_2$, 15:5:1) 0.64; ^{1}H NMR (400 MHz, HCl salt, CD_3OD) δ 7.33–7.27 (m, 5H, $C_6H_5CH_2$), 7.24–7.20 (dd, 2H, C–CH–CH–C–F), 6.98–6.94 (dd, 2H, C–CH–CH–C–F), 5.15 (s, 2H, $C_6H_5CH_2$), 4.46–4.43 (dd, 1H, CH_2CHCO), 4.11–4.06 (q, 1H), 4.05–4.01 (s, 2H, $COCH_2N$), 3.37–3.14 (m, 5H), 3.08–3.03 (dd, 1H, CH_2CH–CO), 2.98–2.80 (m, 8H), 2.14–2.10 (t, 2H, $CH_3CH_2CH_2CO$), 1.92–1.75 (m, 4H), 1.50–1.46 (m, 2H, $CH_3CH_2CH_2CO$), 0.79–0.75 (t, 3H, CH_3CH_2–CH_2CO); ^{13}C NMR (75.43 MHz, CD_3OD) δ 176.13 [$CONH$–$(CH_2)_3$], 174.60 (CO–NH–CH), 171.58 (N–CO–CH_2N), 164.82, 161.60 (C–CH–CH–C–F), 137.33, 134.48, 134.43 (C–CH–CH–C–F), 132.04, 131.93 (C–CH–CH–C–F), 129.59, 129.31, 128.83, 116.18, 115.89(C–CH–CH–C–F), 69.23, 56.69, 52.41, 51.65, 48.15, 46.29, 45.06, 38.66, 37.91, 36.99, 27.51, 27.39, 21.48, 20.82, 20.42, 13.88; CI-MS ($C_{25}H_{50}F_4N_6O_5$) 711 $(M + 1)^+$.

C_4-*p*-F-phenylalanine-343-glycine-N_α-CH_2CF_3 (15). The Cbz group was removed analogously to **4g** with the yield of 91%. R_f (CH_2Cl_2:MeOH:*i*$PrNH_2$, 15:5:1) 0.50; ^{1}H NMR (400 MHz, HCl salt, CD_3OD) δ 7.27–7.24 (dd, 2H, C–CH–CH–C–F), 7.03–6.99 (dd, 2H, C–CH–CH–C–F), 4.51–4.47 (dd, 1H, CH_2CH–CO), 3.30–3.14 (m, 8H), 3.10–3.04 (dd, 1H, CH_2–CHCO), 2.90–2.85 (dd, 1H, CH_2CH–CO), 2.76–2.63 (m, 8H), 2.17–2.14 (t, 2H, CH_3–CH_2CH_2CO), 1.80–1.60 (m, 8H), 1.60–1.50 (m, 2H, $CH_3CH_2CH_2CO$), 0.85–0.81 (t, 3H, $CH_3CH_2CH_2CO$); ^{13}C NMR (75.43 MHz, HCl salt, CD_3OD) δ 176.09 [$CONH$–$(CH_2)_3NH$], 174.49 (CO–NH–CH), 166.82 (N–CO–CH_2N), 164.77, 161.54 (d, *C*–CH–CH–C–F), 134.55, 134.51 (d, C–CH–CH–C–F), 132.04, 131.93 (d, *C*–CH–CH–C–F), 116.13, 115.85 (d, *C*–CH–CH–C–F), 56.51, 49.85, 48.19, 46.63, 46.54, 46.37, 45.33, 45.24, 45.15, 38.72, 37.90, 37.45, 37.33, 37.11, 37.00, 27.25, 24.25, 20.84; ^{19}F NMR (373 MHz, ^{1}H decoupled) δ −71.8 (CF_3; HCl salt, solid-state), −116.6 (F; HCl salt, solid-state); FABMS ($C_{27}H_{44}F_4N_6O_3$) 577 $(M + 1)^+$.

C_7-phenylalanine-343-lysine-(N_ε-*t*-Boc-N_α-N_2CF_3) (16a). This compound was synthesized analogously to **4j**, which gave the desired product (70 mg, 90%). R_f (CH_2Cl_2:MeOH, 9:1) 0.4; FABMS ($C_{50}H_{82}F_3N_9O_{10}$) 1027 $(M + 1)^+$.

C_7-phenylalanine-343-lysine-(N_α-N_2CF_3) (16). The Boc groups were removed analogously to **2**. The crude was applied to RP-HPLC [gradient of H_2O (0.1% TFA) MeCN from 85:15 to 0:100 over 30 min], and the retention time was 8.2 min. ^{1}H NMR (400 MHz, CD_3OD) δ 7.31–7.17 (m, 5H), 4.50–4.40 (m, 1H), 3.50–3.37 (m, 1H), 3.25–2.70 (m, 16H), 2.37–

2.03 (m, 4H), 1.95–1.55 (m, 10H), 1.55–1.40 (m, 2H), 1.39–1.10 (m, 8H), 0.86 (t, 3H, J = 7.2 Hz); FABMS ($C_{35}H_{58}F_3N_9O_4$) 727 $(M + 1)^+$.

C_7-*p*-F-phenylalanine (17a). Compound **17a** was synthesized analogously to **4a,** the yield was 54%. R_f (CH_2Cl_2: MeOH:HOAc, 9:1:0.1) 0.43; ^{1}H NMR (400 MHz, CD_3OD) δ 7.23–7.20 (dd, 2H, C–CH–CH–C–F), 6.98–6.93 (dd, 2H, C–CH–CH–C–F), 4.59–4.54 (dd, 1H, CONHCHCOOH), 3.21–3.15 (dd, 1H, *p*-F-Phe–CH_2–CH), 2.93–2.87 (dd, 1H, *p*-F-Phe–CH_2CH), 2.15–2.11 (t, 2H, CH_2CH_2–CONH), 1.49–1.45 (t, 2H), 1.29–1.17 (m, 6H), 0.89–0.85 (t, 3H, CH_3CH_2).

C_7-*p*-F-phenylalanine-343-(di-Boc)-lysine-(N_α-Cbz-N_ε-Boc) (17b). Coupling between **17a** and **12a** was achieved analogously to **4b**. Boc groups were added analogously to **5d**. Overall yield was 83%. R_f (CH_2Cl_2:MeOH, 9:1) 0.65; ^{1}H NMR (400 MHz, CD_3OD) δ 7.39–7.27 (m, 5H, $C_6H_5CH_2O$), 7.27–7.24 (dd, 2H, C–CH–CH–C–F), 7.02–6.98 (dd, 2H, C–CH–CH–C–F), 5.10 (s, 2H, $C_6H_5CH_2O$), 4.57–4.53 (m, 1H, *p*-F-Phe–CH_2CH), 4.02–3.98 (m, 1H, Cbz–NHCH), 3.20–2.98 (br, m, 17H), 2.88–2.83 (dd, 1H), 2.18–2.14 [t, 2H, $CH_3(CH_2)_4$–CH_2CONH], 1.75–1.55(m, 12H), 1.50–1.40 (two s, 27H, CH_3 of Boc), 1.30–1.15 (m, 6H), 0.88–0.85 [t, 3H, $CH_3(CH_2)_5$CONH]; FABMS ($C_{55}H_{88}FN_7O_{11}$) 1042$(M + 1)^+$.

C_7-*p*-F-phenylalanine-343-(di-Boc)-lysine-(N_ε-Boc) (17c). Cbz group was removed analogously to **4g**. Yield was 84%; R_f (CH_2Cl_2:MeOH, 9:1) 0.45; ^{1}H NMR (400 MHz, CD_3OD) δ 7.28–7.24 (dd, 2H, C–CH–CH–C–F), 7.02–6.98 (dd, 2H, C–CH–CH–C–F), 4.55–4.51 (m, 1H, *p*-F-Phe-CH_2–CH), 3.23–3.02 (br, m, 17H), 2.19–2.17 (t, 2H, CH_2CH_2CO–NH), 1.78–1.60 (m, 12H), 1.50–1.45 (two s, 27H, CH_3 of Boc), 1.39–1.17 (m, 6H), 0.91–0.89 [t, 3H, $CH_3(CH_2)_5$CONH]; FABMS ($C_{47}H_{82}FN_7O_9$) 908 $(M + 1)^+$.

C_7-*p*-F-phenylalanine-343-lysine-(N_α-$COCF_3$) (17). Trifluoroacetylation was achieved analogously to **16a**. Yield was 74%; R_f (CH_2Cl_2:MeOH, 10:1) 0.78. The Boc groups were removed analogously to **2.** The crude product was then purified by RP-HPLC [gradient of H_2O (0.05% HCl) MeCN from 85:15 to 0:100 over 30 min], retention time was 19 min. UV–vis (MeOH) 218, 265, 272 nm. ^{1}H NMR (400 MHz, CD_3OD) δ 7.28–7.24 (dd, 2H, C–CH–CH–C–F), 7.04–6.99 (dd, 2H, C–CH–CH–C–F), 4.45–4.40 (m, 1H, *p*-F-Phe–CH_2–CH), 4.32–4.27 (m, 1H, COCHNH–$COCF_3$), 3.48–3.43 (m, 2H), 3.25–2.89 (m, 20H), 2.18–2.15 (t, 2H, CH_2CH_2CO–NH), 1.92–1.78 (m, 12H), 1.73–1.65 (m, 2H), 1.49–1.44 (m, 4H), 1.30–1.19 (m, 6H), 0.89-0.86 [t, 3H, $CH_3(CH_2)_5$–CONH]; FABMS ($C_{34}H_{57}F_4N_7O_4$) 704 $(M + 1)^+$.

C_7-*p*-F-phenylalanine-343-lysine-(N_α-$COCH_3$) (18). Compound **17c** (30 mg, 33 μmol) in 10 mL dry CH_2Cl_2 and 14 μL Et_3N was purged with argon. To this solution was carefully injected acetyl chloride (2.8 μL, 40 μmol) in 2 mL of dry CH_2Cl_2 under stirring. The mixture was stirred for 15 min at rt, which was then evaporated and applied to silica gel column eluted with CH_2Cl_2:MeOH (20:1); 26 mg of the desired product was obtained (83%). R_f (CH_2Cl_2:MeOH, 10:1) 0.80. The Boc groups were removed analogously to **2**. The crude product was then purified by RP-HPLC [gradient of H_2O(0.05% HCl)MeCN from 85:15 to 0:100 over 30 min], retention time was 21 min. UV–vis (MeOH) 215, 264, 271 nm. ^{1}H NMR (400 MHz, CD_3OD) δ 7.28–7.25 (dd, 2H, C–CH–CH–C–F), 7.04–7.00 (dd, 2H, C–CH–CH–C–F), 4.46–4.43 (m, 1H, *p*-F-Phe–CH_2–CH), 4.18–4.15 (m, 1H, CO–CH–$NHCOCH_3$), 3.22–2.87 (m, 20H), 2.19–2.16 (t, 2H, CH_2–CH_2CONH), 2.02 (s, 3H, NHCOCH), 1.91–1.79 (m, 12H), 1.71–1.66 (m, 2H), 1.48–1.44 (m, 4H), 1.28–1.18 (m, 6H), 0.89–0.86 [t, 3H, $CH_3(CH_2)_5$CONH].

5-(4′-Carboxyphenyl)-10,15,20-triphenyl porphyrin (TPP), 5-(4′-carboxyphenyl)-10,15,20-tri-(3′-pyridine) porphyrin (T3PyP) and 5-(4′-carboxyphenyl)-10,15,20-tri-(4′-pyridine) porphyrin (T4PyP). These compounds were synthesized according to the procedures described in ref 16.

TPP-C_6-L-phenylalanine-343-(di-Boc)-lysine-(di-Boc) (19a). To a solution of **5g** (26 mg, 26.3 μmol), EDC (25.2 mg, 132 μmol) and DMAP (9.6 mg, 79 μmol) in 3 mL dry CH_2Cl_2 was added **TPP** (26 mg, 40 μmol). The mixture was stirred at rt for 8 h, then CH_2Cl_2 (20 mL) was added. The solution was extracted with sat aqueous NH_4Cl solution (20 mL × 3), washed with brine (20 mL), dried (Na_2SO_4), and evaporated under reduced pressure to give a purple crude product. Purification by column chromatography afforded **19a** (41.2 mg, 96%) as a purple solid. R_f (CH_2Cl_2:MeOH, 9:1) 0.64; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82–8.84 (m, 8H, pyrrole), 8.79 [d, 2H, J = 8.0 Hz, H–C(2″, 6″)], 8.28 [d, 2H, J = 8.0 Hz, H–C(3″, 5″)], 8.18–8.21 [m, 6H, H–C(2′, 6′)], 7.70–7.82 [m, 9H, H–C(3′-5′)], 7.18–7.30 (m, 5H, Phe), 6.84–6.90 (m, 1H, NH), 4.68–4.80 [m, 1H, H–C(18)], 4.10–4.18 [m, 1H, H–C(5)], 3.56–3.64 [m, 2H, H–C(24)], 2.98–3.30 [m, 16H, H of Bn and C (1, 7, 9, 10, 13, 14, 16)], 2.26–2.40 [m, 2H, H–C(20)], 1.22–1.88 [m, 20H, H–C (2, 3, 4, 8, 11, 12, 15, 21, 22, 23)], 1.41–1.46 (m, 36H, Boc); FABMS ($C_{96}H_{118}N_{12}O_{12}$) 1632 $(M + 1)^+$.

TPP-C_6-L-phenylalanine-343-L-lysine (19). To a solution of **19a** (41.2 mg, 25.3 μmol) in dry CH_2Cl_2 was added ca. 30 equivalence of TFA. The resulting green solution was stirred at rt until it became colorless and a green solid precipitated (1 h). The solvent was removed, and the green solid was dissolved in a solution of TFA (ca. 30 eqivalence) in dry EtOH (2 mL), and stirred for 8 h at rt. Evaporation of the solvent afforded **19** (42.6 mg, quat.) as a green solid. ^{1}H NMR (400 MHz, CD_3OD) 8.88–8.92 (m, 8H, pyrrole), 8.74 [d, 2H, J = 8.0 Hz, H–C(2″, 6″)], 8.60–8.70 [m, 6H, H–C(2′, 6′)], 8.51 [d,

2H, J = 8.0 Hz, H–C(3″, 5″)], 8.02–8.14 [m, 9H, H–C(3′-5′)], 7.20–7.28 (m, 5H, Phe), 4.48–4.58 [m, 1H, H–C(18)], 3.82–3.90 [m, 1H, H–C(5)], 3.50–3.58 [m, 2H, H–C(24)], 2.86–3.28 [m, 16H, Bn and C (1, 7, 9, 10, 13, 14, 16)], 2.30–2.38 [m, 2H, H–C(20)], 1.26–1.96 [m, 20H, H–C (2, 3, 4, 8, 11, 12, 15, 21, 22, 23)]; FAB-HRMS ($C_{76}H_{87}N_{12}O_4$) calcd 1233.7130 $(M + 1)^+$, found 1233.7110 $(M + 1)^+$.

T4PyP-C_6-L-phenylalanine-343-(di-Boc)-L-lysine-(di-Boc) (20a). Compound **20a** (9.2 mg, 70%) was synthesized according to the procedures of **19a** except that T4PyP was used instead of TPP. R_f (CH_2Cl_2:MeOH, 20:1) 0.37; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.00–9.08 (m, 8H, pyrrole), 8.83–8.90 [m, 6H, H-(C2′, C6′)], 8.80 [d, 2H, J = 4.4 Hz, H-C(2″, 6″)], 8.19 (d, 2H, J = 4.4 Hz, H–C(3′, 5′), 8.08–8.18 (m, 6H, H–C (3′, 5″)], 7.18–7.28 (m, 5H, phe), 6.80–6.90 (m, 1H, NH), 5.20–5.28 (m, 1H, NH), 4.63–4.78 [m, 1H, H –C(18)], 4.05–4.15 [m, 1H, H–C(5)], 3.57–3.64 [m, 2H, H–C(24)], 2.98–3.26 [m, 16H, H of Bn and C (1, 7, 9, 10, 13, 14, 16)], 2.28–2.36 [m, 2H, H–C(20)], 1.22–1.86 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)], 1.42–1.47 (m, 36H, Boc), −2.78 (m, 2H, pyrrole NH); FABMS ($C_{93}H_{115}N_{15}O_{12}$) 1635 $(M + 1)^+$.

T4PyP-C_6-L-phenylalanine-343-L-lysine (20). Boc groups of **20a** were removed according to the procedures of **19** yielded **20** (5.8 mg, quat) as a green solid. ^{1}H NMR (400 MHz, CD_3OD) δ 9.22–9.28 [m, 6H, H–C(2″, 6″)], 8.86–9.20 [m, 8H, pyrrole], 8.78–8.82 [m, 6H, H–C(3″, 5″)], 8.34 [d, 2H, J = 8.4 Hz, H–C(2′, 6′)], 8.29 [d, 2H, J = 8.4 Hz, H–C(3″,5″)], 7.22–7.38 [m, 5H, Phe], 4.52–4.58 [m, 1H, H–C(18)], 3.82–3.90 [m, 1H, H–C(5)], 3.50–3.58 [m, 2H, H–C(24)], 2.82–3.16 [m, 16H, H of Bn and C(1, 7, 9, 10, 13, 14, 16)], 2.28–2.36 [m, 2H, H–C(20)], 1.24–1.95 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)]; FAB-HRMS ($C_{73}H_{83}N_{15}O_4$) calcd 1234.6830 $(M + 1)^+$, found 1234.6830 $(M + 1)^+$.

T3PyP-C_6-L-phenylalanine-343-(di-Boc)-L-lysine-(di-Boc) (21a). Compound **21a** (26.9 mg, 82%) was synthesized according to the procedures of **19a** except that T3PyP was used. R_f (CH_2Cl_2:MeOH, 9:1) 0.57; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.38–9.42 [m, 3H, H–C(2′)], 9.03–9.08 [m, 3H, H–C(4′)], 8.80–8.86 (m, 8H, pyrrole), 8.76–8.80 [m, 3H, H–C(6′)], 8.46–8.53 [m, 3H, H–C(5′)], 8.17–8.26 [m, 4H, H–C(2″, 3″, 4″, 5″)], 7.14–7.26 (m, 5H, Phe), 4.62–4.76 [m, 1H, H–C(18)], 4.05–4.15 [m, 1H, H–C(5)[, 3.56–3.62 [m, 2H, H–C(24)], 2.96–3.24 [m, 16H, H of Bn and C(1, 7, 9, 10, 13, 14, 16)], 2.24–2.32 [m, 2H, H–C(20)], 1.24–1.82 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)], 1.42–1.49 (m, 36H, Boc), −2.86 (s, 2H, pyrrole NH); FABMS ($C_{93}H_{115}N_{15}O_{12}$) 1635 $(M + 1)^+$.

T3PyP-C_6-L-phenylalanine-343-L-lysine (21). Compound **21** (15.0 mg, quat) was obtained according to the procedures of **19** as a green solid. ^{1}H NMR (400 MHz, CD_3OD) δ 9.55–9.59 [m, 3H, H–C(2′)], 9.18–9.24 [m, 3H, H–C(4′)], 9.04–9.10 [m, 3H, H–C(6′)], 8.84–9.04 (m, 8H, pyrrole), 8.30–8.35 [m, 3H, H–C(5′)], 8.22–8.29 [m, 4H, H–C(2″, 3″, 4″, 5″)], 7.20–7.32 (m, 5H, Phe), 4.50–4.56 [m, 1H, H–C(18)], 3.85–3.92 [m, 1H, H–C(5)], 3.48–3.56 [m, 2H, H–C(24)], 2.85–3.19 [m, 16H, H of Bn and C(1, 7, 9, 10, 13, 14, 16)], 2.28–2.34 [m, 2H, H–C(20)], 1.25–1.96 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)]; FAB-HRMS ($C_{73}H_{83}N_{15}O_4$) calcd 1234.6830 $(M + 1)^+$; found 1234.6830 $(M + 1)^+$.

T3MePyP-C_6-L-phenylalanine-343-(di-Boc)-L-lysine-(di-Boc) (22a). A solution of **21a** (12.4 mg, 7.6 μmol) in 2 mL CH_3I was stirred overnight. Evaporation of the solvent gave **22a** (15.4 mg, 98%). ^{1}H NMR (400 MHz, CD_3OD) δ 9.90–9.98 [m, 3H, H–C(2′)], 9.40–9.48 [m, 3H, H–C(4′)], 9.32–9.40 [m, 3H, H–C(6′)], 8.84–9.24 (m, 8H, pyrrole), 8.54–8.62 [m, 3H, H–C(5′)], 8.24–8.34 [m, 4H, H–C(2″, 3″, 4″, 5″)], 7.16–7.36 (m, 5H, Phe), 6.50–6.62 (br, m, 1H, NH), 4.75–4.80 (s, 9H, N–CH_3), 4.50–4.56 [m, 1H, H–C(18)], 3.85–3.92 [m, 1H, H–C(5)], 3.48–3.56 [m, 2H, H–C(24)], 2.82–3.30 [m, 16H, H of Bn and C (1, 7, 9, 10, 13, 14, 16)], 2.25–2.34 [m, 2H, H–C(20)], 1.20–1.80 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)], 1.41–1.47 [m, 36H, Boc]; FABMS ($C_{96}H_{124}N_{15}O_{12}I_3$) 1680 $(M + 1)^+$.

T3MePyP-C_6-L-phenylalanine-343-L-lysine (22). To a solution of **22a** (15.4 mg, 9.2 μmol) in 3 mL EtOH, was added ca. 30 equivalence of TFA. The resulting green solution was stirred for 8 h at rt. Evaporation of the solvent afforded **22** (16.3 mg, quat) as a green solid. ^{1}H NMR (400 MHz, CD_3OD) δ 9.92–9.96 [m, 3H, H–C(2′)], 9.42–9.48 [m, 3H, H–C(4′)], 9.36–9.42 [m, 3H, H–C(6′)], 8.92–9.32 (m, 8H, pyrrole), 8.54–8.60 [m, 3H, H–C(5′)], 8.27–8.36 [m, 4H, H–C(2″, 3″, 4″, 5″)], 7.22–7.32 [m, 5H, Phe], 4.75–4.80 [s, 9H, N–CH_3], 4.50–4.56 [m, 1H, H–C(18)], 3.85–3.92 [m, 1H, H–C(5)], 3.48–3.55 [m, 2H, H–C(24)], 2.88–3.30 [m, 16H, H of Bn and C(1, 7, 9, 10, 13, 14, 16)], 2.26–2.32 [m, 2H, H–C(20)], 1.22–1.96 [m, 20H, H–C(2, 3, 4, 8, 11, 12, 15, 21, 22, 23)].

Receptor preparation and binding assay

A nicotinic AChR (nAChR) enriched membrane preparation was obtained from frozen *Torpedo nobiliana* electric organs as described previously.[18] In summary, the tissue kept at −80 °C was thawed, diced, then homogenized in equal volume of buffer (154 mM NaCl, 50 mM Tris–HCl, pH 7.4 containing 0.02% NaN_3 and 1 mM EDTA). The homogenate was centrifuged at 1000*g*, for 10 min and the supernatant fraction recentrifuged at 30,000*g* for 60 min. The pellets were resuspended in the same buffer at a final concentration of 1 mg/mL.

[^{3}H]H_{12}-HTX (specific activity = 10 Ci/mmole) was used to label allosteric site on the nAChR using thin-layer liquid scintillation.[19,20] A 96-well Skatron cell harvester was used to transfer incubation mixtures from

96-well microtiter plates onto a GF/B filtermat. Binding reactions were initiated by adding 25 μg of Torpedo nAChR-enriched membranes into a final volume of 250 μL buffer containing 5.6 nM [^{3}H]-H_{12}-HTX and 100 μM carbamylcholine. Incubation time was 5 min at 23 °C. The binding reaction was terminated by vacuum filtration over filter mats presoaked in 0.05% polyethyleneimine. The wash protocol allowed for each sample site to receive 2.5 mL of wash buffer. After drying the filtermats in a ventilated oven at 60 °C, 50 μL of Betaplate Scint (LKB Scintillation Products) were pipetted on each sample site to form a thin layer of scintillant. The filtermat was then sealed in a plastic bag, placed in a cassette holder and radioactivity of the 96 sample sites on the filtermat counted in a Betaplate 1205 Scintillation Counter (Wallac, Gaithersburg, MD).

The PhTXs were dissolved in the assay buffer except for the insoluble PhTX, which were dissolved first in DMSO then diluted in the assay buffer. All PhTXs were assayed at concentration ranging from 0.01 to 100 μM. All assays were performed in triplicates and the results were calculated as the means of triplicate measurements ± standard errors of the means. Amantadine (5 mM) was used to identify the specific binding of [^{3}H]-H_{12}-HTX. Each assay included two triplicates of total (i.e., no amantadine) and two triplicates for nonspecific (i.e., plus amantadine) binding to calculate the control level of specific [^{3}H]-H_{12}-HTX binding. Mean binding values in presence of PhTXs were calculated as percent of control and plotted against the logarithm of the concentration. The concentrations of PhTXs that caused 50% inhibition of [^{3}H]-H_{12}-HTX binding (IC_{50}) were obtained from linear transformations of the semilogarithmic plots.

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