

Structure–Activity Relationships of Methoctramine-Related Polyamines as Muscular Nicotinic Receptor Noncompetitive Antagonists. 2.¹ Role of Polymethylene Chain Lengths Separating Amine Functions and of Substituents on the Terminal Nitrogen Atoms

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Received October 19, 2001

Polymethylene tetraamine methoctramine (**1**) is a prototypical antimuscarinic ligand endowed with a significant affinity for muscular nAChRs. Thus, according to the universal template approach, structural modifications were performed on **1** in order to improve affinity and selectivity for the muscle-type nAChR. The polyamine derivatives synthesized were tested at both frog rectus and *Torpedo* nAChRs and at guinea pig left atria (M₂) and ileum longitudinal muscle (M₃) mAChRs. All of the compounds, like prototype **1**, were noncompetitive antagonists of nicotinic receptors while being competitive antagonists at M₂ and M₃ mAChRs. The biological profile of polyamines **4–7** revealed that increasing the number of amine functions and the chain length separating these nitrogen atoms led to a significant improvement in potency at nAChRs. Moreover, the role of the number and type of amine functions in the interaction with nAChRs was further investigated through the synthesis of compounds **9** and **10**. Tetraamines **8** and **11**, bearing a rather rigid spacer between the nitrogen atoms instead of the very flexible polymethylene chain, displayed a profile similar to that of **1** at nAChRs, whereas a significant decrease in potency was observed at mAChRs. Tetraamine **12**, bearing a 2-methoxyphenethyl group, was less potent than **1**, whereas tetraamine **13**, carrying a diphenylethyl moiety, was more potent than **1**, confirming that an increase in size of the hydrophobic group on the terminal nitrogen atoms increases significantly the binding affinity for nAChRs. Tetraamines **14–17** were significantly more potent than prototype **2** at both frog rectus and *Torpedo* nAChRs, confirming that an increase in the distance between the amine functions results in a parallel increase in the affinity for nAChRs. To gain insight into the mode of interaction of polymethylene tetraamines with nAChRs, photolabile (**19** and **20**) and fluorescent (**21** and **22**) compounds were synthesized. A most intriguing finding was the observation that **19**, which bears two identical azido groups on the terminal nitrogen atoms, was found to bind the *Torpedo* nAChR with a 1:1 stoichiometry, suggesting a U-shaped conformation in the receptor interaction. Moreover, the high potency shown by fluorescent compounds **21** and **22** appears promising for a further characterization of the polymethylene tetraamines binding site with the muscle-type nAChR.

Introduction

Recently, we have modified the structure of methoctramine (**1**, Figure 1), the prototype polymethylene tetraamine for antagonism of muscarinic acetylcholine receptors (mAChRs),² to produce polyamines that have high affinity and selectivity for the muscle-type nicotinic acetylcholine receptor (nAChR).^{1,3,4} The rationale for these structural modifications was based on the observation that **1**, at micromolar concentrations, antagonizes nAChRs, hence providing the prospect of applying the universal template approach⁵ to the design of polyamines as nAChR ligands.¹

The nAChR, a prototypical member of the superfamily of ligand-gated ion channels, is a heteropentameric transmembrane protein with the subunit stoichiometry $\alpha_2\beta\gamma\delta$.^{6–8} The five receptor subunits are arranged around a central pore, which is permeable for cations upon agonist binding. Each subunit contains four transmembrane sequences M1–M4. The M2 sequences of all subunits contribute structurally to the formation of the ion pore, thereby facing the lumen of the channel.^{9,10} The selectivity filter for cations is formed by several rings of negatively charged amino acid side chains protruding into the lumen of the pore.^{11–13} Multiple ligand binding domains have been characterized on the nAChR. Two binding sites for agonists and competitive antagonists are located in the extracellular region, mainly on each of the two α -subunits at the α – δ and α – γ interfaces,^{14,15} whereas several binding sites for noncompetitive antagonists, such as triphenylmeth-

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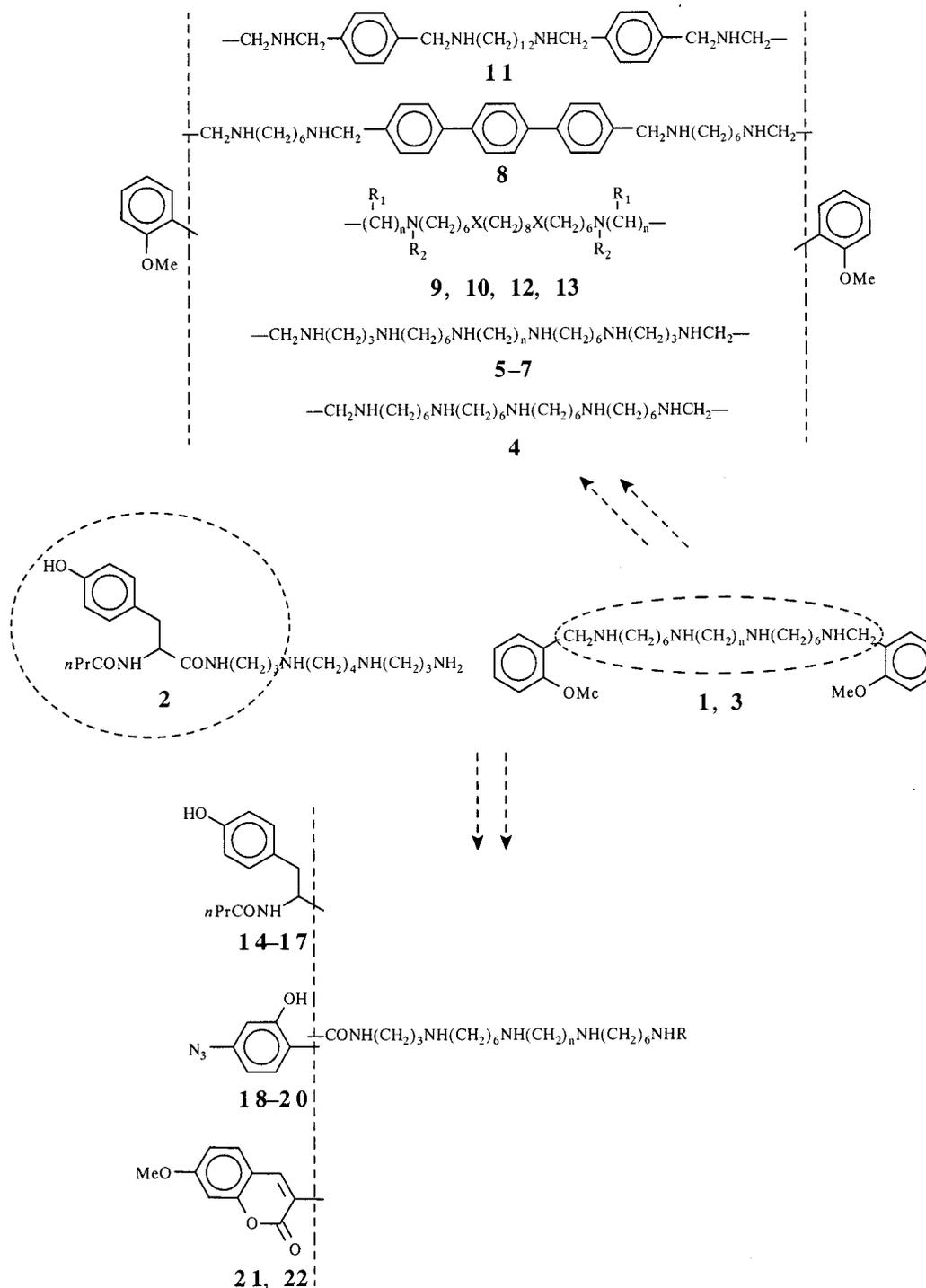


Figure 1. Design strategy for the synthesis of **4–8**, **11**, **14–17**, and **19–22** by modifying the polymethylene tetraamine backbone of methoctramine (**1**) and by inserting selected aryl substituents of the structural features of philanthotoxin PhTX-343 (**2**) on the terminal nitrogen atoms of **1**-related compounds.

ylphosphonium bromide (TPMP⁺), have been found within the ion channel close to the M2 transmembrane domain.^{16,17} Luminal noncompetitive antagonists are assumed to enter the open channel and to bind deep in the ion channel close to the negatively charged selectivity filter, thereby inhibiting the ion flow.¹⁸ Therefore, it may well be that the positively charged backbone of tetraamines is able to interact with the negatively charged amino acid residues located at the constriction of the channel and on the extracellular side of this constriction as well, provided that the distance separat-

ing the amine functions of the ligand fits the distance between the carboxylate residues of the receptor.

Increasing the number of methylenes separating the inner nitrogen atoms of **1** resulted in a significant improvement in potency at the nAChR while decreasing the affinity for the M₂ mAChR. Furthermore, the tetraamine backbone of **1** was modified by replacing the terminal 2-methoxybenzyl groups by other pharmacophores with the aim of achieving selectivity for the muscle-type nAChR.¹ To this end, we choose the butyryltyrosyl moiety of philanthotoxin-343 (PhTX-343) (**2**,

Figure 1), a synthetic analogue of a wasp (*Philanthus triangulum*) toxin PhTX-433,¹⁹ since it is known that an aromatic moiety at one end and a primary amine function at the other end of the molecule are important for philanthotoxin binding at the *Torpedo* nAChR.^{20,21} By combining the structural features of tetraamine **1** and philanthotoxin **2**, we designed novel inhibitors of the muscle-type nAChR. These novel polyamines were potent noncompetitive antagonists of the nAChR while being competitive mAChR antagonists. The promising results obtained following these structural modifications prompted us to modify further the structure of **1** to achieve additional information on the structural requirements that allow differentiation between muscle-type nAChRs and mAChRs.

The finding that the higher homologue **3** of **1** was 12-fold and 250-fold more potent than prototypes **1** and **2**, respectively, suggests clearly that the length of the spacer between the inner nitrogen atoms is important for potency at both nAChRs and mAChRs.¹ To improve hopefully the selectivity, we have designed polyamines **4** and **5**, in which the total number of methylenes has been preserved with respect to **3** while inserting additional amine functions, and hexaamines **6** and **7**, in which additional nitrogen atoms have been included at the extremities of the polyamine backbone of prototypes **1** and **3**, respectively. These structural modifications might give an indication of the number of protonable nitrogen atoms for optimal interaction with the nAChR ion channel. To investigate further the role of the number and type of amine functions for potency at nAChRs, dioxadiazine **9**²² and *N,N,N',N''*-tetramethyltetraamine **10**²² have been included in this study. Tetraamines **8**, which carries a (4''-aminomethyl-[1,1':4',1'']terphenyl-4-yl)methylamine fragment instead of a 1,12-dodecanediamine residue, and **11**, which bears a *p*-xylene moiety instead of a hexamethylene chain between the inner and outer nitrogen atoms of **3**, may contribute in determining whether flexibility is an important determinant of potency with respect to nAChR antagonism. Tetraamines **12**³⁵ and **13**³⁵ were also investigated to verify whether the terminal 2-methoxybenzyl groups in symmetrically substituted methoctramine-related tetraamines are relevant for potency at the nAChR.

Unsymmetrical hybrid polyamines **14**–**17**, bearing the structural features of prototypes **1** and **2**, were designed to define whether the chain length between the nitrogen atoms plays a similar role in asymmetrically and symmetrically substituted polyamines. Interestingly, using fluorescent titration and photo-cross-linking, we have shown recently that the asymmetrically substituted photolabile tetraamine **18** binds in the vestibule, that is, in the wide water-filled entrance of the pore, located above the narrow part of the channel and of the selectivity filter and the gating mechanism of the nAChR.^{3,4} The terminal aromatic moiety that carries the photolabile azido group was found to photolabel the hydrophobic sequence $\alpha 186$ – $\alpha 189$ of the α -subunit. This sequence is located in the upper part of the ion channel close to the agonist binding site. Furthermore, the long, positively charged polyamine tail of **18** overlaps in the narrow part of the nAChR ion channel with the high-affinity noncompetitive inhibitor

site. In direct binding studies, a binding stoichiometry of two molecules of **18** per nAChR monomer was determined, suggesting that each of the aromatic moieties of the two **18** molecules interact with one of the two α -subunits.⁴ Thus, the symmetrically substituted photolabile tetraamines **19** and **20** were designed to verify whether these compounds interact with the receptor's α -subunit with only one of the two hydrophobic moieties or with both of them. To this end, compound **19** was selected for iodination with ¹²⁵I at both aromatic rings, generating ¹²⁵I₂-**19**, since this compound was slightly more potent in binding assays than its higher homologue **20**. Finally, to gain insight into the mode of interaction of polymethylene tetraamines with the nAChR, we have designed the fluorescent analogues **21** and **22**.

All of the compounds synthesized in this study were tested on muscle-type nAChRs, and most of them were tested as well on peripheral M₂ and M₃ mAChRs.

Chemistry

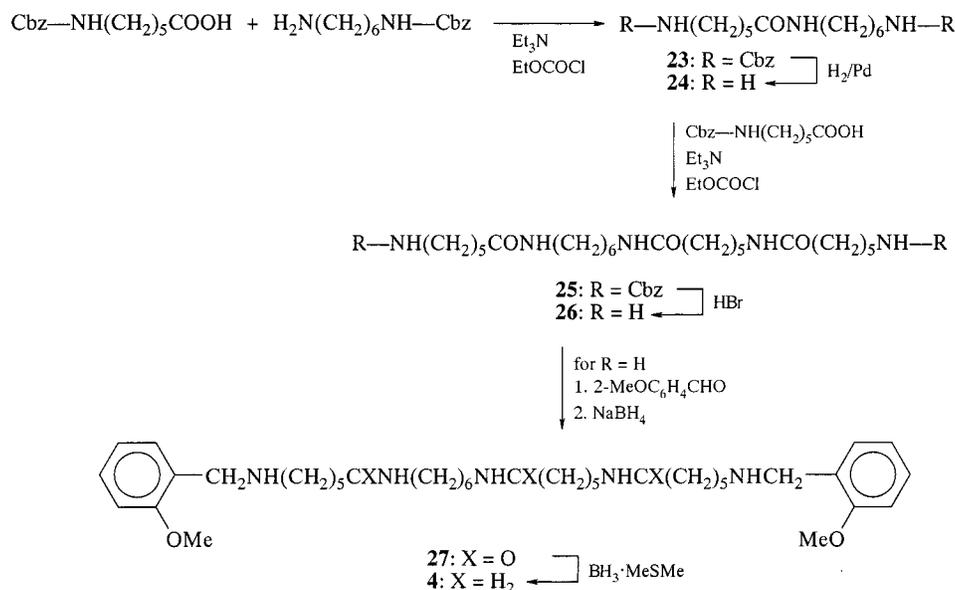
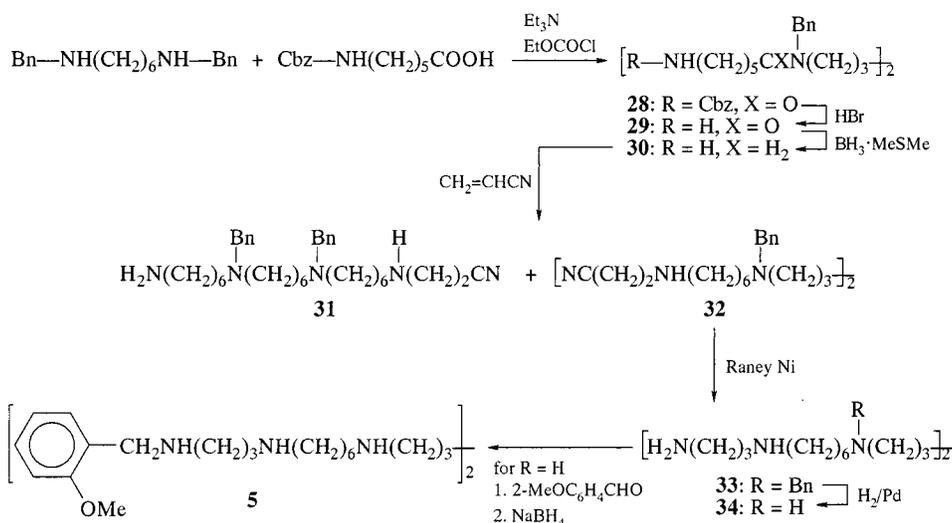
The design strategy for our compounds is shown schematically in Figure 1.

All the compounds were synthesized according to standard procedures (Schemes 1–10) and were characterized by IR, ¹H NMR, mass spectra, and elemental analysis.

N-[(Benzyloxy)carbonyl]-6-aminocaproic acid was amidated with *N*-[(benzyloxy)carbonyl]-1,6-hexanediamine¹ to give **23**. Removal of the *N*-(benzyloxy)carbonyl group was achieved by catalytic hydrogenation over 10% palladium on charcoal. Thus, hydrogenolysis of **23** gave **24**,²³ which was reacted with *N*-[(benzyloxy)carbonyl]-6-aminocaproic acid to afford **25**. Removal of the *N*-(benzyloxy)carbonyl group of **25** by hydrolysis with HBr gave **26**, which was treated with 2-methoxybenzaldehyde followed by reduction with NaBH₄ of the formed Schiff base to diaminetriamide **27**, which in turn was reduced with borane to **4** (Scheme 1).

N-[(Benzyloxy)carbonyl]-6-aminocaproic acid was amidated with *N,N*-dibenzylhexane-1,6-diamine²⁴ to give **28**. Removal of the *N*-(benzyloxy)carbonyl group of **28** by hydrolysis with HBr gave diaminediamide **29**, which was reduced with borane to tetraamine **30**. The addition of acrylonitrile to **30** gave a mixture of **31** and **32** that were separated by chromatography. Dinitrile **32** was reduced with Raney Ni into the corresponding amine **33**. Hydrogenolysis of **33** gave **34**, which was treated with 2-methoxybenzaldehyde followed by reduction with NaBH₄ of the formed Schiff base to hexaamine **5** (Scheme 2).

Selective protection of amine functions of **35**² and **36**² was achieved by following an adapted procedure described for spermine.²⁵ Thus, tetraamines **35** and **36** were treated first with ethyl trifluoroacetate and then with di-*tert*-butyl dicarbonate followed by basic hydrolysis to give **37** and **39**, and **38** and **40**, respectively. The addition of acrylonitrile to **39** and **40** gave **41** and **42**, respectively, which were transformed into the corresponding **43** and **44**. These nitriles were reduced with Raney Ni into **45** and **46**, which were treated with 2-methoxybenzaldehyde followed by reduction with NaBH₄ of the formed Schiff base followed by acidic hydrolysis to give hexaamines **6** and **7** (Scheme 3).

Scheme 1^a^a Cbz = C₆H₅CH₂OCO-.Scheme 2^a^a Cbz = C₆H₅CH₂OCO-; Bn = C₆H₅CH₂-.

Tetraamine **8** was obtained through the condensation of dialdehyde **47**²⁶ with diamine **48**²⁷ followed by reduction with NaBH₄ of the intermediate Schiff base (Scheme 4). It was obtained in poor yields because of its very low solubility.

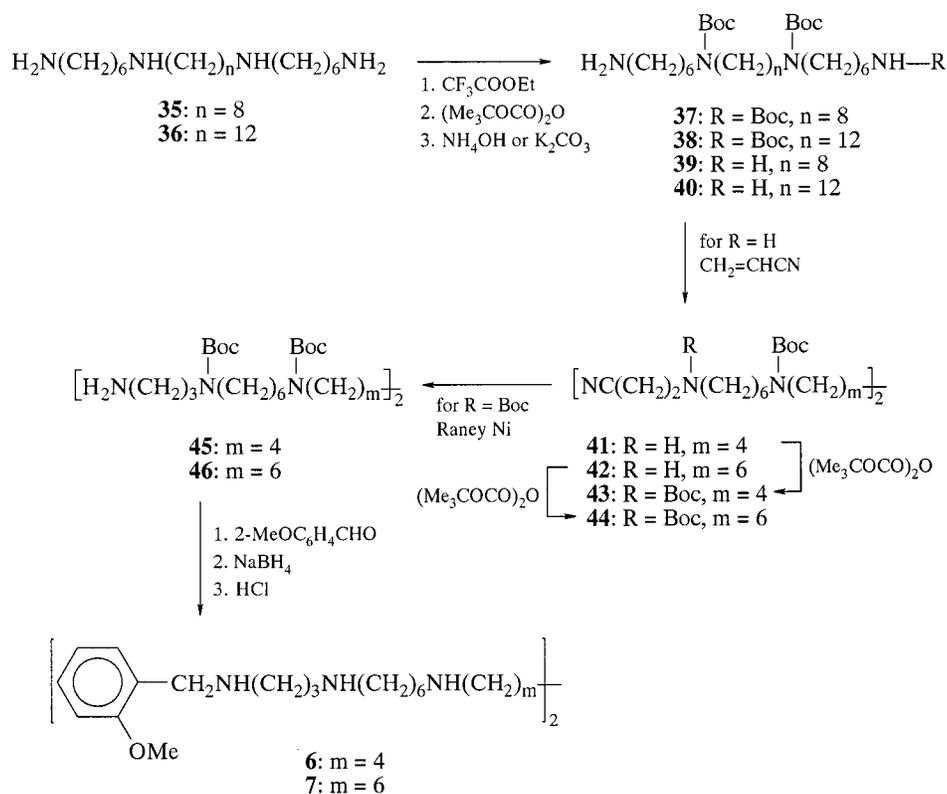
1,12-Dodecanedioic acid was amidated with amine **49**¹ to give **50**, which in turn was treated with HBr to afford diaminediamide **51**. Condensation of **51** with 2-methoxybenzaldehyde in the presence of NaBH₄ gave **52**, which was reduced with borane to **11** (Scheme 5).

Amine functions of nitrile **31** were protected with di-*tert*-butyl dicarbonate to give **53**, which was reduced with Raney Ni to **54**. Coupling **54** with the activated ester **55**²⁸ afforded **56**. Removal of the *N*-(*tert*-butoxy)-carbonyl group of **56** by acidic hydrolysis gave **57**, which, upon catalytic hydrogenation, afforded hybrid polyamineamide **14**. The introduction of a 2-methoxybenzyl moiety on the terminal amine function of **14** gave **15** (Scheme 6). The higher homologues **16** and **17** were synthesized by a different synthetic pathway. The

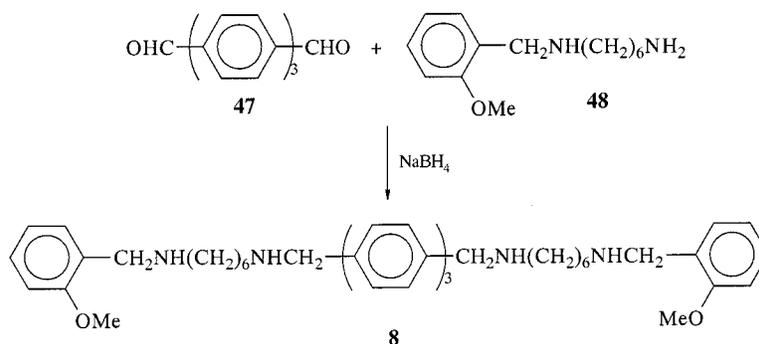
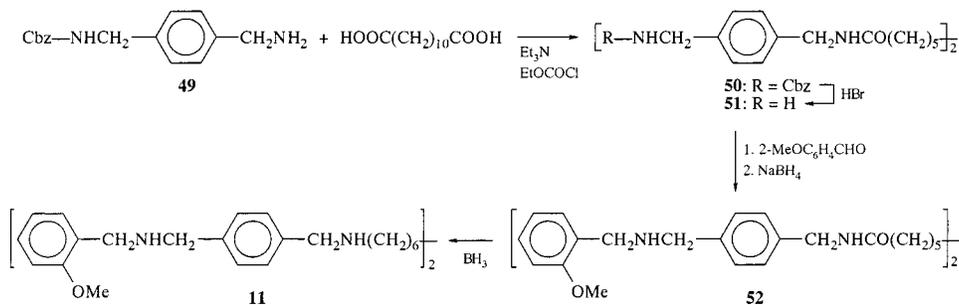
addition of acrylonitrile to **38** gave **58**, which in turn was transformed into **59**. Reduction of **59** with Raney Ni afforded **60**, which in turn was coupled to **55**²⁸ yielding **61**. Removal of the *N*-(*tert*-butoxy)carbonyl groups of **61** by hydrolysis with CF₃COOH gave **62**, which, upon catalytic hydrogenation, afforded **16**. The 2-methoxybenzyl group on the terminal nitrogen of **16** was introduced as for **15**, giving **17** (Scheme 7).

Symmetrically substituted analogues **19** and **20** of the photolabile tetraamine **18**¹ were synthesized as shown in Scheme 8. Thus, azidosalicilic acid *N*-hydroxysuccinimide ester²⁹ was amidated with **45** and **46** to give **63** or **64**, which, upon treatment with CF₃COOH to remove the protecting groups, afforded the photolabile compounds **19** and **20**, respectively.

Nitrile **65**¹ was treated with di-*tert*-butyl dicarbonate to give **66**, which, upon reduction with Raney Ni, afforded **67**. Amidation of 2,5-dioxotetrahydro-1*H*-pyrrolyl-7-methoxy-2-oxo-2*H*-3-chromene carboxylate with

Scheme 3^a^a Boc = Me₃COCO-.

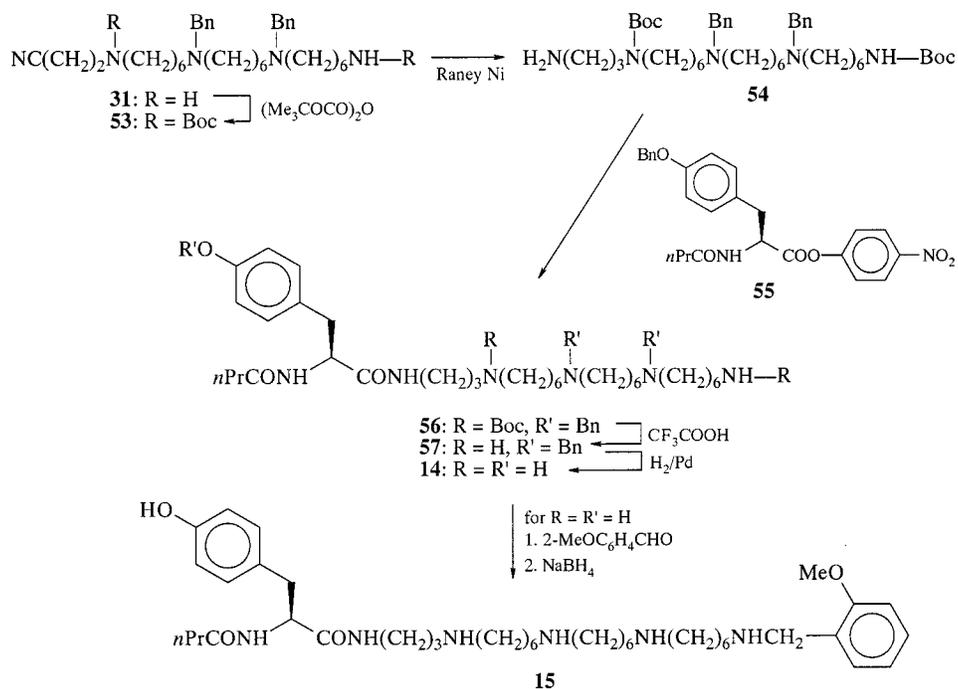
Scheme 4

Scheme 5^a^a Cbz = C₆H₅CH₂OCO-.

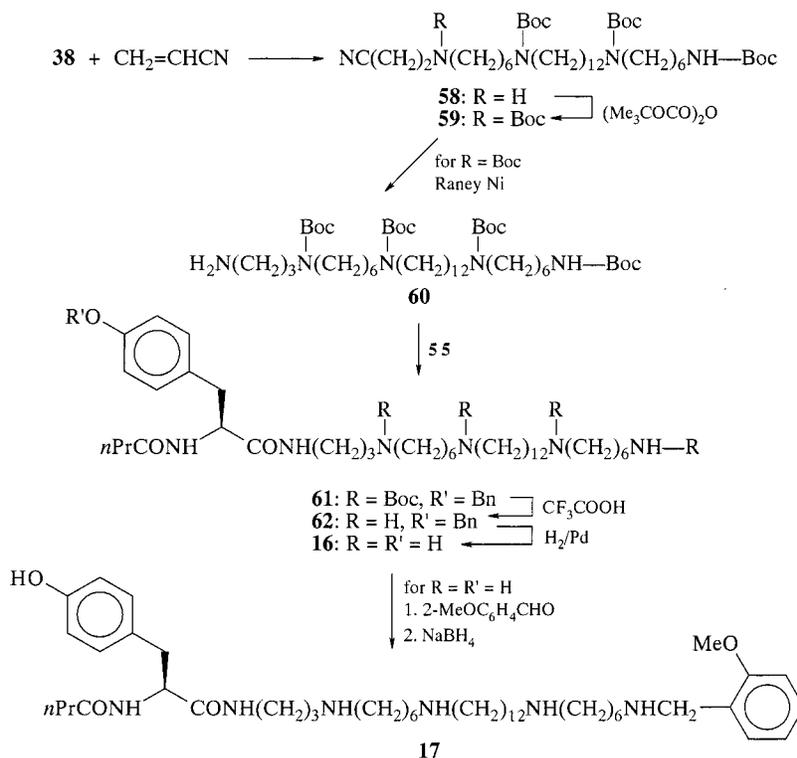
67 gave **68**, which, following acidic hydrolysis with CF₃-COOH, afforded the fluorescent compound **21** (Scheme 9).

A different route was used to synthesize the iodinated analogue **22** of **21**. Addition of acrylonitrile to **37** gave **69**, which was reduced with Raney Ni to **70**. Treatment with ethyl trifluoroacetate afforded the protection of the

primary amine function of **70**, giving **71**. Subsequent acidic hydrolysis with CF₃COOH of **71** gave the mono-protected pentaamine **72**, which could be selectively alkylated on the other free terminal amine function through condensation with 2-methoxy-5-iodo-benzaldehyde³⁰ and reduction with NaBH₄ to afford **73**. Treatment of **73** with di-*tert*-butyl dicarbonate gave **74**, which,

Scheme 6^a

^a Boc = Me₃COCO-; Bn = C₆H₅CH₂-.

Scheme 7^a

^a Boc = Me₃COCO-; Bn = C₆H₅CH₂-.

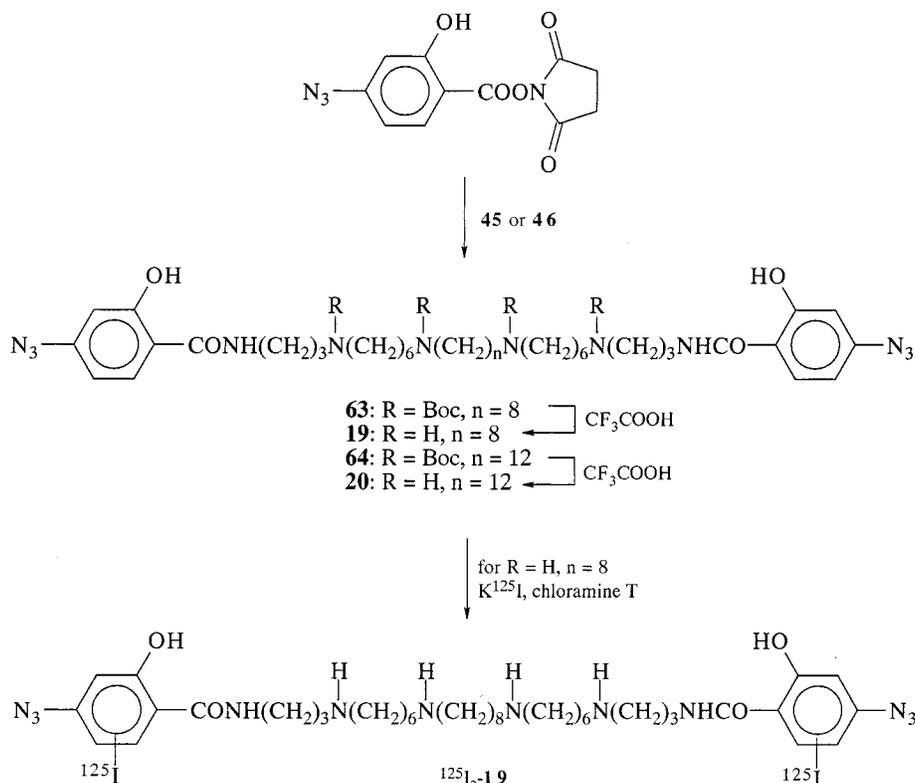
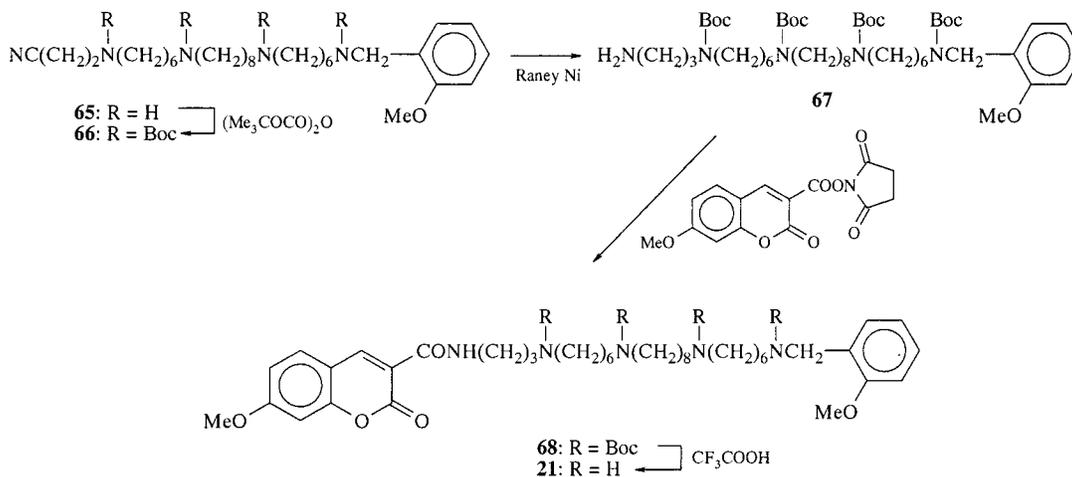
upon basic hydrolysis with aqueous ammonia, afforded **75**, whose free terminal amine function was allowed to react with 2,5-dioxotetrahydro-1*H*-pyrrolyl-7-methoxy-2-oxo-2*H*-3-chromene carboxylate, giving **76**, which, following acidic hydrolysis with CF₃COOH to remove the protecting groups, yielded the unsymmetrically substituted tetraamine **22** (Scheme 10).

Photolabile compound **19** was radioactively iodinated with ¹²⁵I to afford ¹²⁵I₂-**19**, using the chloramine T

method (Scheme 8).³¹ However, it was not ascertained whether the iodine atom entered at position 3 or 5 of each terminal phenyl ring.

Biology

The effects of compounds **1–22** on M₂ mAChRs were determined using guinea pig left atria stimulated electrically at 1 Hz. The guinea pig ileum longitudinal muscle was used to study their effects on M₃ mAChRs.¹

Scheme 8^a^a Boc = Me₃COCO-.Scheme 9^a^a Boc = Me₃COCO-.

In both cases the agonist was arecaidine propargyl ester (APE). The biological data are expressed as the negative logarithm of the apparent dissociation constants (pK_B) according to Furchgott.³²

The effects of compounds **1–22** on muscle-type nAChRs were studied using the frog rectus abdominis muscle and carbachol-induced contractions as the measured parameter.³³ The results are expressed as pIC_{50} values, i.e., the negative logarithm of the concentrations required to inhibit the maximal response to carbachol by 50%.

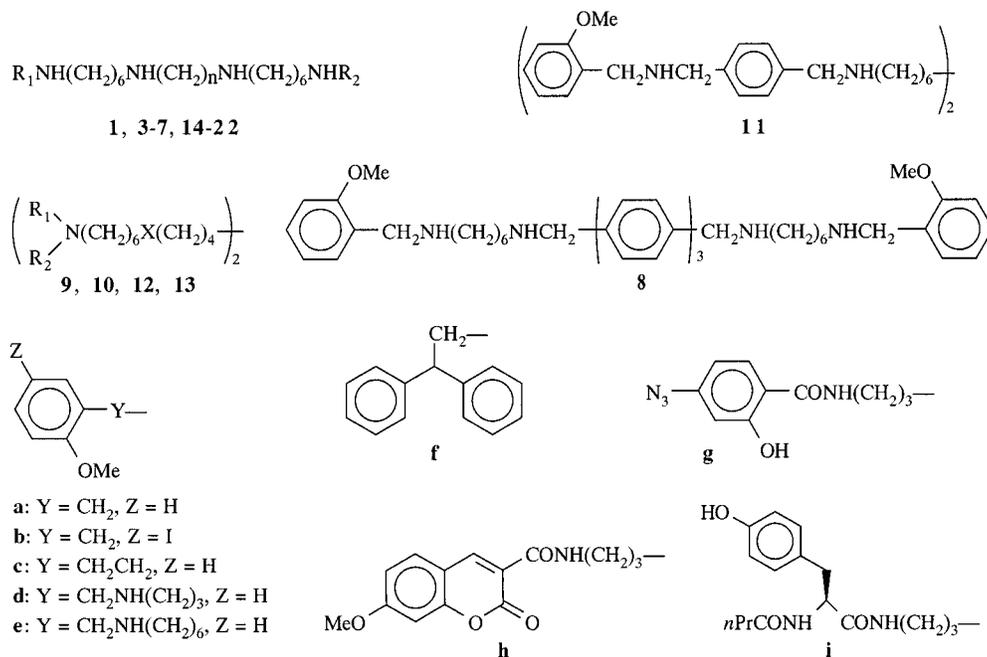
Apparent binding affinities (pK_{app}) of compounds **1–3**, **5–8**, **11**, and **14–22** at the noncompetitive binding site of *Torpedo* nAChRs were determined by using the fluorescent noncompetitive inhibitor ethidium in a

displacement assay.³ Binding of the radioactively labeled polyamine ¹²⁵I-**19** to the *Torpedo* nAChR was also investigated.⁴

Polyamines **1** and **2** and the noncompetitive nAChR antagonist TPMP⁺ were used as standards, and their pIC_{50} , pK_{app} , or pK_B values were within the error of previous determinations.

Results and Discussion

Summaries of the results are presented in Table 1 and Figures 2 and 3. Over the concentration range investigated, all of the newly synthesized compounds were noncompetitive antagonists of the muscle-type nAChR. The maximum response to carbachol was reduced, and the magnitude of this reduction was

Table 1. Antagonist Affinities, Expressed as pIC₅₀ or pK_B Values, at Nicotinic Acetylcholine Receptors (nAChRs) of the Isolated Frog Rectus Abdominis Muscle (FRA) and *Torpedo* and at Muscarinic Acetylcholine Receptors (mAChRs) of Guinea Pig Left Atrium (M₂) and Longitudinal Ileum (M₃)

| no. ^a | R ₁ | R ₂ | X | n | nAChR | | mAChR | |
|--------------------------------|----------------|-----------------|------------------|----|--------------------------|--------------------------------------|-----------------------------------|-----------------------------------|
| | | | | | pIC ₅₀ (FRA) | pK _{app} (<i>Torpedo</i>) | pK _B (M ₂) | pK _B (M ₃) |
| 1 | a | a | | 8 | 5.93 ± 0.03 ^b | 6.21 ± 0.10 | 7.91 ± 0.03 ^b | 6.14 ± 0.06 ^b |
| 2 | a | a | | 12 | 4.62 ± 0.03 ^b | 3.94 ^c | <5 ^b | <5 ^b |
| 3 | a | a | | 6 | 7.02 ± 0.04 ^b | 5.66 ± 0.05 | 7.35 ± 0.09 ^b | 5.98 ± 0.07 ^b |
| 4 | a | e | | 6 | 6.68 ± 0.06 | | 6.98 ± 0.05 ^b | 5.30 ± 0.11 ^b |
| 5 | d | d | | 6 | 6.42 ± 0.04 | 6.40 ± 0.09 | 6.15 ± 0.01 | <5 |
| 6 | d | d | | 8 | 6.44 ± 0.09 | 6.66 ± 0.11 | <6 | <6 |
| 7 | d | d | | 12 | 6.66 ± 0.02 | 7.04 ± 0.09 | 6.50 ± 0.27 | <5 |
| 8 | | | | | 6.30 ± 0.02 | 6.35 ± 0.06 | <5 | <5 |
| 9 | a | H | O | | 5.14 ± 0.04 | | <5 ^d | <5 ^d |
| 10 | a | CH ₃ | NCH ₃ | | 5.88 ± 0.08 | | 6.79 ± 0.11 ^e | <5 ^e |
| 11 | | | | | 6.43 ± 0.02 | 6.19 ± 0.07 | <5 | <5 |
| 12 | c | H | NH | | 5.36 ± 0.06 | | 6.64 ± 0.10 ^f | 5.32 ± 0.06 ^f |
| 13 | f | H | NH | | 6.31 ± 0.11 | | 5.98 ± 0.15 ^f | 5.40 ± 0.20 ^f |
| 14 | i | H | | 6 | 5.62 ± 0.07 | 4.63 ± 0.08 | <5 | 5.16 ± 0.08 |
| 15 | i | a | | 6 | 5.48 ± 0.11 | 6.12 ± 0.03 | 5.87 ± 0.08 | 5.08 ± 0.03 |
| 16 | i | H | | 12 | 6.30 ± 0.02 | 5.28 ± 0.06 | | |
| 17 | i | a | | 12 | 6.27 ± 0.01 | 5.89 ± 0.07 | | |
| 18 | g | H | | 8 | 6.39 ± 0.01 ^b | 5.46 ± 0.06 | 7.01 ± 0.09 ^b | 7.35 ± 0.03 ^b |
| 19 | g | g | | 8 | 5.80 ± 0.02 | 5.86 ± 0.06 | | |
| 20 | g | g | | 12 | 6.17 ± 0.07 | 5.72 ± 0.02 | | |
| 21 | h | a | | 8 | 6.21 ± 0.04 | 6.41 ± 0.05 | 7.77 ± 0.11 | 6.19 ± 0.08 |
| 22 | h | b | | 8 | 7.11 ± 0.05 | 6.80 ± 0.07 | 6.88 ± 0.13 | 5.68 ± 0.23 |
| TPMP ⁺ ^g | | | | | 6.05 ± 0.09 | 5.70 ± 0.12 | | |

^a 1, 3, 8, 10–13, 15, 17, tetrahydrochlorides; 2, trihydrochloride; 4, pentahydrochloride; 5–7, hexahydrochlorides; 9, dihydrochloride; 14, 16, 18–22, tetratrifluoroacetates. ^b Data from ref 1. ^c Data from ref 3. ^d Data from ref 22. ^e Data from ref 27. ^f Data from ref 35. ^g Triphenylmethylphosphonium bromide.

phobic headgroup of 2-related compounds increased significantly the binding affinity for nAChRs.³

Very recently,¹ we have demonstrated that increasing the number of methylenes separating the inner nitrogen atoms of 1 resulted in a significant increase of potency at nAChRs, the most potent homologue being 3. However, when the fluorescent noncompetitive antagonist ethidium was used in a displacement assay, 3 was found to be 23-fold less potent than in functional assays (see below). Keeping constant the two 2-methoxybenzyl groups on the terminal amine functions and the total number of methylenes of 3, we investigated 4 and 5, bearing one and two additional nitrogen atoms, respectively. It turned out that pentaamine 4 and hexamine

5 retained most of the potency at nAChRs, being only 2-fold and 4-fold less potent than prototype 3, respectively. This finding clearly suggests that additional protonable nitrogen atoms did not alter significantly the binding with the receptor and might well interact with negatively charged or aromatic amino acid residues by way of formation of cation–anion or cation– π interactions. It derives that an appropriate modification of the chain length separating the nitrogen atoms might improve the potency for nAChRs. To this end, we have studied hexamines 6 and 7, bearing 8 and 12 methylenes between the inner nitrogen atoms. Again, when the length of the spacer between the inner nitrogen atoms was increased, there was an increase in potency

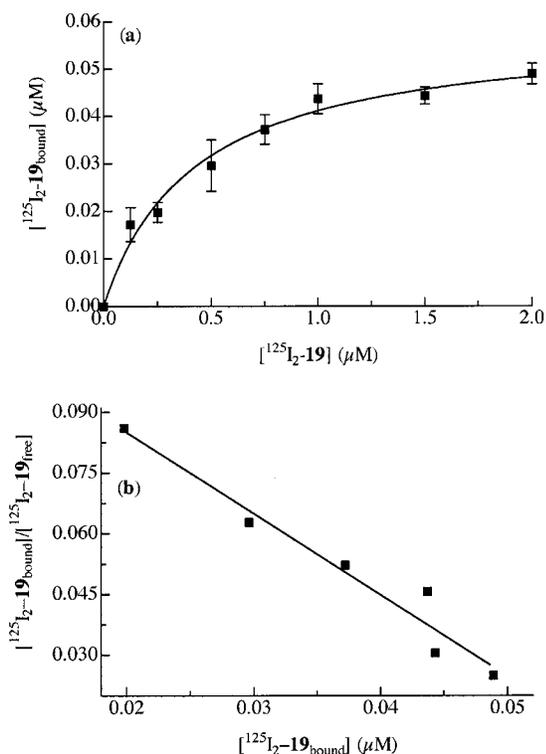


Figure 2. (a) Binding of $^{125}\text{I}_2\text{-19}$ to the *Torpedo* nicotinic receptors. Nicotinic receptor-rich membranes (0.3 mg/mL protein) were incubated with increasing concentrations of $^{125}\text{I}_2\text{-19}$. The specific binding of $^{125}\text{I}_2\text{-19}$ was determined by subtracting the binding in the presence of a 100-fold molar excess of $\text{I}_2\text{-19}$ from the total binding. Each value is the mean \pm SD of three separate experiments. (b) Scatchard plot of $^{125}\text{I}_2\text{-19}$ binding to *Torpedo* nicotinic receptors. The K_{app} value was $1.0 \pm 0.2 \mu\text{M}$. When the α -BTX binding assay was used to determine the number of receptor monomers per microgram of protein, the binding stoichiometry of $^{125}\text{I}_2\text{-19}$ was calculated to be 1 mole of $^{125}\text{I}_2\text{-19}$ per mole of nicotinic receptor monomer.

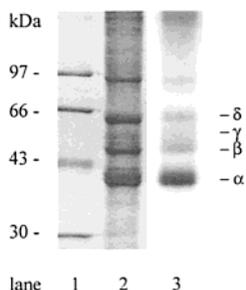


Figure 3. Photoaffinity labeling of nicotinic receptors with $^{125}\text{I}_2\text{-19}$. Nicotinic receptor-rich membranes (25 μg) were photolabeled with 10 μM $^{125}\text{I}_2\text{-19}$, and labeled receptor subunits were separated on a 10% SDS polyacrylamide gel. The gel was stained with Coomassie (lane 2), and radioactive protein bands were visualized by autoradiography (lanes 3). Lane 1 shows the molecular mass markers in kDa: phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). Exposure time was 1 day.

at both frog rectus and *Torpedo* nAChRs. Interestingly, hexamine **7** was 4-fold more potent than the lower homologue **5** and 24-fold more potent than the prototype **3** at the *Torpedo* nAChR. Furthermore, unlike **3**, it was more potent at the *Torpedo* nAChR than at the frog rectus nAChR.

Previously, we showed that the reduction in the flexibility of the hexamethylene chain between the inner

and outer nitrogen atoms and of the octamethylene chain between the inner nitrogen atoms of prototype **1** did not influence activity at the nAChR and the M_3 mAChR, while decreasing markedly the potency at the M_2 mAChR.¹ Tetraamine **8** carries a (4''-aminomethyl-[1,1';4',1'']terphenyl-4-yl)methylamine function instead of the 1,12-dodecanediamine fragment of **3**, whereas tetraamine **11** contains a *p*-xylene moiety instead of a hexamethylene spacer between the inner and outer nitrogen atoms of **3**. Unlike **3**, compound **8** showed the same potency at both frog rectus and *Torpedo* nAChRs and was 5-fold less potent than **3** at the frog rectus nAChR and 5-fold more potent than **3** at the *Torpedo* nAChR. Furthermore, **8**, unlike **3**, was devoid of activity at both M_2 and M_3 mAChRs. This finding may have relevance because the replacement of the polymethylene chain between the inner nitrogen atoms of tetraamines with a rigid spacer is highly detrimental for the interaction with mAChRs while not affecting the affinity for nAChRs. Moreover, in **8**, the inner nitrogen atoms are unlikely to be less than 15 Å apart because, owing to the rigidity of the terphenyl moiety, the only possibility of altering the distance between the two inner amine functions is restricted to the rotation along the axis of the two bonds between the inner nitrogen atoms and the 4'',4'-carbon atoms. It follows that this distance is important for the interaction with two anionic sites of the channel located most likely at a distance comparable with that between the inner nitrogen atoms of **8**.

Interestingly, compound **11** displayed a biological profile at nAChRs and mAChRs similar to that observed for **8**. This finding clearly suggests that also an appropriate rigid spacer between the inner and outer nitrogen atoms may allow significant differentiation between muscle-type nAChRs and mAChRs.

The results obtained for hybrid tetraamines **14**–**17** deserve comment. All of the compounds were significantly more potent than prototype **2** at both frog rectus and *Torpedo* nAChRs, confirming that an increase in the distance between the amine functions results in a parallel increase in the affinity for nAChRs. However, the higher homologues **14** and **16** were (like **2** but to a greater extent) more potent at frog rectus versus *Torpedo* nAChRs. The insertion of a 2-methoxybenzyl group on the terminal primary amine function of **14** and **16**, affording **15** and **17**, respectively, caused a slight decrease in potency for the frog rectus nAChR and a significant increase in affinity for the *Torpedo* nAChR. The increase was more pronounced for **15**, which, contrary to the higher homologue **17**, was more potent (4-fold) at the *Torpedo* nAChR relative to the frog rectus nAChR. This finding and the results obtained for **3** clearly suggest that there are likely subtle differences in the mode of interaction of polyamines with the ion channel of frog rectus and *Torpedo* nAChRs. We do not know at present whether the different potencies displayed by **14** and **16** and by **15** and **17** as well as by **3** at the frog rectus relative to the *Torpedo* nAChR are the result of structural differences in the ion channel or simply a different bioavailability of the compounds at the receptor site.

The asymmetrically substituted photolabile tetraamine **18**¹ behaved like **14** and **16**, since it was almost 10-fold more potent at frog rectus nAChRs than at

Torpedo nAChRs, confirming that a terminal primary amine function may interact differently with the nAChR of different species. Again, the inclusion of an aromatic group on the terminal amine function of **18**, affording **19**, caused an increase in potency for the *Torpedo* nAChR. The higher homologue **20** retained high affinity for both frog rectus and *Torpedo* nAChRs, but potency was higher for the frog rectus nAChR.

In a reversible binding assay, the radioactively labeled $^{125}\text{I}_2$ -**19** was shown to bind with an affinity of $pK_{\text{app}} = 6.0 \pm 0.1$. The non-iodinated **19** binds with $pK_{\text{app}} = 5.86 \pm 0.06$. The introduction of two iodine atoms in **19** results in a moderate increase in the binding affinity of **19**, an effect that was previously observed when iodinating the asymmetrical **18**.⁴ The straight line obtained in the Scatchard plot indicates that $^{125}\text{I}_2$ -**19** binds to a single class of binding sites (Figure 2). When ^{125}I -labeled α -BTX in a binding assay was used to determine the number of receptor monomers per microgram of protein,³⁶ the binding stoichiometry of $^{125}\text{I}_2$ -**19** was calculated to be one molecule per receptor monomer. As shown in Figure 3, $^{125}\text{I}_2$ -**19** was found to photolabel exclusively the nAChR α -subunits. No radioactivity was incorporated into the other receptor subunits at detectable levels. Considering the biological profile of ^{125}I -**18** (see Introduction),⁴ our present results clearly demonstrate that symmetrically substituted tetraamines interact, at least with the *Torpedo* nAChR, differently from monosubstituted tetraamines bearing a terminal primary amine function. It was found that two molecules of ^{125}I -**18** label one receptor monomer with a 2:1 stoichiometry whereas $^{125}\text{I}_2$ -**19** interacted with the receptor with a 1:1 stoichiometry. It is derived that most likely the positively charged terminal primary amine function of **18** and the second *N*-aryl-substituted moiety of **19** bind at different sites within the receptor lumen. We have advanced that the positively charged tetraamine tail of **18** interacts deep in the lumen of the nAChR ion channel with the high-affinity noncompetitive inhibitor site. This binding site is close to the negatively charged amino acids that are part of the selectivity filter, suggesting that the positively charged tetraamine tail interacts with these negative amino acid residues, which is in agreement with previous models for polyamines binding,^{12,13} while the aryl moiety of this compound binds to a hydrophobic region in the upper part of the ion channel. As demonstrated in ^{125}I -**18** in photoaffinity labeling studies, this site lies within the hydrophobic sequence HWVY (residues $\alpha 186$ – $\alpha 189$) containing three aromatic residues and is located close to the agonist binding site.⁴ The presence of noncompetitive antagonists prevent ^{125}I -**18** photo-cross-linking; thus, the binding site must be accessible from the water-filled lumen of the channel.⁴ Since the photolabile azido group of **18** is linked to the aromatic headgroup, the site of ^{125}I -**18** cross-linking identified the region to which the aryl moiety of the molecule binds. Since two molecules of ^{125}I -**18** bind within the nAChR channel, each of the aromatic moieties of the two ^{125}I -**18** interacts most likely with one of the two α -subunits of the nAChR monomer. It is derived that $^{125}\text{I}_2$ -**19**, which bears two identical azido groups at both ends of its polyamine tail and interacts with the receptor molecule with a 1:1 stoichiometry, apparently shows a conformation in which $^{125}\text{I}_2$ -

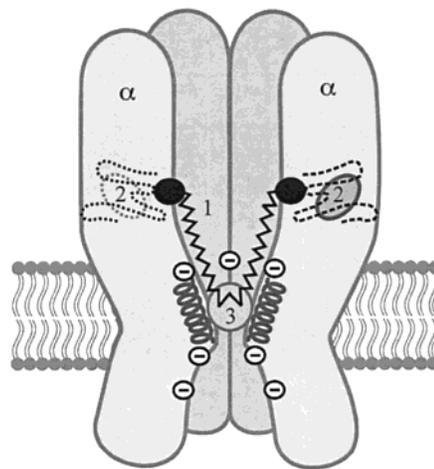


Figure 4. Model of $^{125}\text{I}_2$ -**19** binding in the *Torpedo* nAChR ion channel. Each of the two aromatic moieties of $^{125}\text{I}_2$ -**19** most likely interacts with one of the two α -subunits of the nAChR monomer. The long positively charged polyamine chain, which overlaps with the noncompetitive inhibitor site, reaches further down into the ion channel and is located close to the negatively charged selectivity filter. (1) $^{125}\text{I}_2$ -**19**; (2) agonist binding site; (3) high-affinity noncompetitive inhibitor site.

19 is in contact with both α -subunits of the nAChR (Figure 4). Consequently, to achieve cross-linking with both receptor α -subunits, **19** might assume a folded U-shaped conformation with the long, positively charged polyamine tail reaching down the ion channel. An attractive working hypothesis may be that a U-shaped conformation of **19** recognizes the hydrophobic sequence HWVY of each receptor α -subunit by way of the two terminal aryl moieties while interacting with two negatively charged rings of the receptor channel, thanks to the positive charges of the polymethylene tetraamine backbone. This model is in line with the observation that $^{125}\text{I}_2$ -**19** exclusively photolabels the α -subunit (Figure 3). Because of the generally low cross-linking yield of 1–2% per azido group in photoaffinity-labeling experiments, it was not possible to detect α -subunit dimers cross-linked together by $^{125}\text{I}_2$ -**19**, since the probability that both azido groups of $^{125}\text{I}_2$ -**19** react with an α -subunit is only 0.04–0.01%. This view satisfactorily rationalizes the high affinity of hexaamines **5**–**7** toward the nAChR by admitting that in a U-shaped conformation these compounds may interact in an improved manner with the negative charges of the selectivity filter of the ion channel lumen, thanks to the six positively charged nitrogen atoms that are lined in a manner such that they are able to face the negative charges of the receptor.

To gain insight into the interaction mode of polyamines bearing aryl substituents on the terminal nitrogen atoms, we have investigated asymmetrically disubstituted tetraamines **21** and **22**. These compounds bear a fluorescent moiety on one terminus and a substituted benzyl group on the other terminus. Iodine was introduced into the aryl moiety of **21**, affording **22**, because it is known that this element may determine quenching of the fluorescence when facing the fluorophore group. Consequently, in a conformation in which the two aromatic moieties of compound **22** would be in proximity, i.e., if the two aromatic moieties of **22** would refold to generate a polyamine ring structure with the two

aromatic moieties of **22** interacting by stacking forces, the fluorescence intensity of **22** should be lower than that of **21**, which lacks iodine. Preliminary results have shown that the presence of iodine in **22** did not affect the intensity of fluorescence relative to **21** (Bixel et al., unpublished results). This observation would not support the hypothesis that symmetrical polyamine binds in a U-shaped conformation as shown in Figure 4. However, it still may be possible that the distance between the fluorophor and the iodine may not be suitable for quenching in at least two reasonable aspects. First, a spacer of five atoms and one atom separates the fluorophor and the iodine-bearing aryl group of **22**, respectively, from the basic nitrogen atoms to which they are linked. Assuming that these nitrogen atoms interact with two anionic sites and that each of them is located in a similar position on both α -subunits, it is derived that the fluorophor and the aryl moiety can hardly be facing each other, thus preventing the quenching of the fluorescence. Second, the distance between the sites on the α -subunits where the fluorophor and the aryl moieties bind might be large enough to prevent quenching of the fluorescence. Besides this intriguing aspect, the results obtained for **21** and **22** appear promising. Tetraamine **21** displayed a biological profile similar to that of prototype **1** at all the receptors tested. Interestingly enough, the introduction of an iodine atom in **21**, affording **22**, resulted in a significant increase in potency at nAChRs while giving a decrease in potency at mAChRs. As a matter of fact, compound **22** resulted in the most potent noncompetitive antagonist at the frog rectus nAChR while being only slightly less potent than hexaamine **7**, which resulted the most potent at the *Torpedo* nAChR. It is derived that the insertion of appropriate substituents on the benzene ring of **1** may be important for making relevant the structure–activity relationship studies for improving affinity and selectivity for muscle-type nAChRs.

Experimental Section

Chemistry. Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR, MALDI-TOF-MS, and NMR spectra were recorded on Perkin-Elmer 297, Bruker Biflex III, and Varian VXR 300 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Although IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. The elemental compositions of the compounds agreed to within $\pm 0.4\%$ of the calculated value. When the elemental analysis is not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck), medium pressure (Biotage, KP-SIL 60, 0.032–0.063 mm), or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F₂₅₄) that were visualized in an iodine chamber. The term “dried” refers to the use of anhydrous sodium sulfate.

[6-(6-Benzoyloxycarbonylaminohexanoylamino)hexyl]-carbamic Acid Benzyl Ester (23). Ethyl chlorocarbonate (0.72 mL, 7.6 mmol) in dry dioxane (5 mL) was added dropwise to a stirred and cooled (5 °C) solution of *N*-[(benzyloxy)-

carbonyl]-6-aminocaproic acid (2.0 g, 7.5 mmol) and triethylamine (1.05 mL, 7.5 mmol) in dioxane (50 mL), followed after standing for 30 min by the addition of *N*-[(benzyloxy)carbonyl]-1,6-hexanediamine¹ (1.88 g, 7.5 mmol) in dioxane (15 mL). After being stirred at room temperature overnight, the mixture was evaporated, affording a residue that was suspended in water (400 mL). The precipitate was filtered and washed with 2 N KHSO₄, aqueous NaHCO₃ saturated solution, and water to give **23**: 78% yield; mp 115–118 °C; ¹H NMR (CDCl₃) δ 1.23–1.69 (complex m, 14), 2.17 (t, 2), 3.16–3.22 (m, 6), 4.81 (br s, 2, exchangeable with D₂O), 5.03 (s, 4), 5.58 (br s, 1, exchangeable with D₂O), 7.30–7.39 (m, 10).

6-Aminoheptanoic Acid (6-Aminoheptyl)amide (24). A solution of **23** (2.93 g, 5.88 mmol) in MeOH (150 mL) was hydrogenated over 10% Pd on charcoal (wet, Degussa type E101 NE/W) (290 mg) for 2 h. Following catalyst removal, the solvent was evaporated, affording **24** in a quantitative yield: ¹H NMR (CDCl₃) δ 1.29–1.72 (complex m, 14 + 4 exchangeable with D₂O), 2.18 (t, 2), 2.68 (t, 4), 3.22 (q, 2), 5.70 (br s, 1, exchangeable with D₂O).

(5-{6-[6-(6-Benzoyloxycarbonylaminohexanoylamino)hexanoylamino]hexyl}carbonyl)pentyl)carbamic Acid Benzyl Ester (25). It was obtained as a white solid from **24** (1.35 g, 5.88 mmol) and *N*-[(benzyloxy)carbonyl]-6-aminocaproic acid (3.11 g, 11.7 mmol) following the procedure described for **23**: 70% yield; mp 132–134 °C; ¹H NMR (DMSO-*d*₆) δ 1.12–1.51 (complex m, 26), 2.01 (t, 6), 2.91–3.06 (m, 10), 5.00 (s, 4), 7.21 (br s, 2, exchangeable with D₂O), 7.31–7.35 (m, 10), 7.73 (br s, 3, exchangeable with D₂O).

6-Aminoheptanoic Acid {6-[6-(6-Aminoheptanoylamino)hexanoylamino]hexyl}amide (26). A solution of 30% HBr in acetic acid (20 mL) was added to a solution of **25** (2.98 g, 4.06 mmol) in acetic acid and CF₃COOH (1:1; 60 mL), and the resulting mixture was stirred for 2 h. Ether (100 mL) was then added, yielding a solid that was dissolved in water. The solution was made basic with NaOH pellets and extracted with CHCl₃ (3 \times 50 mL). Removal of the dried solvent gave in a quantitative yield **26**: ¹H NMR (CD₃OD) δ 1.27–1.70 (complex m, 26), 2.12–2.25 (m, 6), 2.70 (t, 4), 3.16 (t, 6).

6-(2-Methoxybenzylamino)hexanoic Acid (6-{6-[6-(2-Methoxybenzylamino)hexanoylamino]hexanoylamino}-hexyl)amide (27). A mixture of **26** (1.89 g, 4.06 mmol) and 2-methoxybenzaldehyde (1.21 g, 8.95 mmol) in refluxing MeOH (100 mL) was stirred for 2 h. After the mixture was cooled, NaBH₄ (0.34 g, 8.95 mmol) was added and stirring was continued at room temperature for 5 h. The mixture was then cautiously made acidic with 3 N HCl, filtered, and evaporated. The residue was dissolved in water, and the resulting solution was washed with ether, made basic with 2 N NaOH, and extracted with CHCl₃. Removal of the dried solvent afforded **27** as a foam solid: 78% yield; ¹H NMR (CDCl₃) δ 1.20–1.82 (complex m, 26 + 2 exchangeable with D₂O), 2.18 (t, 6), 2.54 (t, 4), 3.11–3.26 (m, 6), 3.70–3.83 (m, 10), 5.95 (br s, 3, exchangeable with D₂O), 6.79–6.93 (m, 4), 7.14–7.28 (m, 4).

***N*-(2-Methoxybenzyl)-*N*-(6-{6-[6-(2-methoxybenzylamino)hexylamino]hexylamino}hexyl)hexane-1,6-diamine Pentahydrochloride (4).** A solution of 10 M BH₃·MeSMe (2.2 mL) in dry diglyme (2 mL) was added dropwise at room temperature to a solution of **27** (2.2 g, 3.16 mmol) in dry diglyme (100 mL) with stirring under a stream of dry nitrogen. When the addition was completed, the reaction mixture was heated at 120 °C for 10 h. After the mixture was cooled at 0 °C, excess borane was destroyed by cautious dropwise addition of MeOH (10 mL). The resulting mixture was left to stand for 4 h at room temperature, cooled at 0 °C, treated with HCl gas for 15 min, and then heated at 120 °C for 4 h. After the mixture was cooled, ether was added and the resulting mixture was stirred overnight at room temperature, yielding a solid that was filtered and dissolved in water. The solution was made basic with 2 N NaOH and extracted with CHCl₃, affording the free base that was purified by flash chromatography. Elution with MeOH/aqueous 28% ammonia (8.5:1.7) afforded crude **4** that was converted into the pentahydrochloride salt: 25% yield; mp 233–235 °C (from EtOH/MeOH); ¹H NMR (free base)

(CDCl₃) δ 1.21–1.58 (complex m, 32 + 5 exchangeable with D₂O), 2.56 (t, 16), 3.76 (s, 4), 3.86 (s, 6), 6.80–6.94 (m, 4), 7.14–7.28 (m, 4). Anal. (C₄₀H₇₆Cl₅N₅O₂) C, H, N.

[5-(Benzyl-{6-[benzyl-(6-benzoyloxycarbonylamino-hexanoyl)amino]hexyl}carbamoyl)pentyl]carbamic Acid Benzyl Ester (28). It was synthesized from *N,N*-dibenzylhexane-1,6-diamine²⁴ (5.73 g, 19.3 mmol) and *N*-[(benzyloxy)carbonyl]-6-aminocaproic acid (10.5 g, 39.6 mmol) following the procedure reported for **23** and purified by flash chromatography. Elution with a step gradient system of EtOAc/cyclohexane (6:4 to 7:3) afforded **28** as a foam solid: 70% yield; ¹H NMR (CDCl₃) δ 1.23–1.75 (complex m, 20), 2.22–2.38 (m, 4), 3.09–3.35 (m, 8), 4.52 (d, 4), 4.83 (br s, 2, exchangeable with D₂O), 5.09 (s, 4), 7.11–7.39 (m, 20).

6-Amino-hexanoic Acid {6-[(6-Amino-hexanoyl)benzyl-amino]hexyl}benzylamide (29). It was synthesized from **28** (8.83 g, 11.2 mmol) and 30% HBr in acetic acid (65 mL) following the procedure described for **26**: 90% yield; mp 204–205 °C; ¹H NMR (CDCl₃) δ 1.13–1.69 (complex m, 20 + 4, exchangeable with D₂O), 2.21–2.35 (m, 4), 3.56–3.68 (m, 4), 3.09 (t, 2), 3.27 (t, 2), 4.48 (d, 4), 7.07–7.28 (m, 10).

N¹-{6-[(6-Amino-hexyl)benzylamino]hexyl}-N⁴-benzyl-hexane-1,6-diamine (30). This compound was obtained by reduction of **29** (5.3 g, 10.14 mmol) with 10 M BH₃·MeSMe (5 mL) following the procedure described for **4**, and the free base was purified by medium-pressure chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (8.5:1.5:0.15) gave **30** as a foam solid: 40% yield; ¹H NMR (CDCl₃) δ 1.24–1.44 (complex m, 24 + 4, exchangeable with D₂O), 2.37 (t, 8), 2.65 (t, 4), 3.52 (s, 4), 7.18–7.30 (m, 10).

3-[6-{(6-[(6-Amino-hexyl)benzylamino]hexyl}benzyl-amino)hexylamino]propionitrile (31) and 3-[6-[Benzyl-(6-{benzyl-[6-(2-cyanoethylamino)hexyl]amino}hexyl)-amino]hexylamino]propionitrile (32). A solution of acrylonitrile (0.14 mL, 2.06 mmol) in MeOH (3 mL) was added dropwise to a cooled (0 °C) and stirred solution of **30** (1.02 g, 2.06 mmol) in MeOH (20 mL). Stirring and cooling were continued for 3 h, then removal of the solvent gave a mixture of compounds that were separated by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (9.5:0.5:0.05) gave **31** and **32** as yellow oils.

31: 35% yield; *R_f* = 0.52 [eluent system, CHCl₃/MeOH/aqueous 28% ammonia (9.5:1.5:0.15)]; ¹H NMR (CDCl₃) δ 1.18–1.58 (complex m, 24 + 3, exchangeable with D₂O), 2.31–2.39 (m, 8), 2.44 (t, 2), 2.58 (t, 2), 2.63 (t, 2), 2.88 (t, 2), 3.52 (s, 4), 7.21–7.33 (m, 10).

32: 30% yield; *R_f* = 0.95 [eluent system, CHCl₃/MeOH/aqueous 28% ammonia (9.5:1.5:0.15)]; ¹H NMR (CDCl₃) δ 1.18–1.62 (complex m, 24 + 2, exchangeable with D₂O), 2.31–2.38 (m, 8), 2.42–2.61 (m, 8), 2.87 (t, 4), 3.51 (s, 4), 7.19–7.31 (m, 10).

N-(3-Aminopropyl)-N'-(6-{[6-(3-aminopropylamino)-hexyl]benzylamino}hexyl)-N'-benzylhexane-1,6-diamine (33). A suspension of **32** (0.37 g, 0.62 mmol), aqueous 40% NaOH (0.4 mL), and Raney Ni (nickel sponge; suspension in water) (0.25 g) in EtOH (8 mL) was hydrogenated for 6 h at room temperature and a pressure of 15 psi. Following catalyst removal, the solvent was evaporated, yielding a residue that was dissolved in water (50 mL) and extracted with CHCl₃ (3 × 20 mL). Removal of dried solvents gave a residue that was purified by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (5:4.5:1) afforded **33** as an oil: 90% yield; ¹H NMR (CDCl₃) δ 1.24–1.44 (complex m, 24), 1.51–1.68 (m, 4), 1.90 (br s, 6, exchangeable with D₂O), 2.31–2.38 (m, 8), 2.52–2.76 (m, 12), 3.51 (s, 4), 7.19–7.38 (m, 10).

N-(3-Aminopropyl)-N'-(6-[6-(3-aminopropylamino)-hexylamino]hexyl)hexane-1,6-diamine (34). A solution of **33** (0.34 g, 0.55 mmol) and CF₃COOH (0.5 mL) in EtOH (15 mL) was hydrogenated over 10% Pd on charcoal (wet, Degussa type E101 NE/W) (34 mg) for 2 h. Following catalyst removal the solvent was evaporated, affording **34** as a white solid: quantitative yield; mp 143–145 °C; ¹H NMR (D₂O) δ 1.12–1.55 (complex, 24), 1.81–2.00 (m, 4), 2.78–3.02 (m, 20).

N-[3-(2-Methoxybenzylamino)propyl]-N'-(6-{6-[3-(2-methoxybenzylamino)propylamino]hexylamino}hexyl)-hexane-1,6-diamine Hexahydrochloride (5). A mixture of **34** (free base, 0.24 g, 0.55 mmol), molecular sieves (3 Å), and 2-methoxybenzaldehyde (167 mg, 1.23 mmol) in EtOH (20 mL) was stirred for 30 min at room temperature, then NaBH₄ (47 mg, 1.23 mmol) was added, and the stirring was continued overnight. Following removal of molecular sieves, the solution was made acidic with 6 N HCl (3 mL). Removal of the solvent gave a residue that was dissolved in water (20 mL). The solution was washed with ether (3 × 20 mL) to remove nonbasic materials, then was made basic with 2 N NaOH, and finally was extracted with CHCl₃ (3 × 20 mL). Removal of washed (brine) and dried solvents gave **5** that was transformed into the hexahydrochloride salt: 40% yield; mp 270 °C (dec) (from MeOH); ¹H NMR (free base) (CDCl₃) δ 1.18–1.44 (complex m, 24), 1.58–1.68 (m, 4 + 6 exchangeable with D₂O), 2.42–2.61 (m, 20), 3.68 (s, 4), 3.73 (s, 6), 6.76–6.88 (m, 4), 7.16–7.20 (m, 4). Anal. (C₄₀H₇₈Cl₆N₆O₂) C, H, N.

{8-[(6-Amino-hexyl)-tert-butoxycarbonylamino]octyl}-(6-tert-butoxycarbonylamino-hexyl)carbamic Acid tert-Butyl Ester (37) and (6-Amino-hexyl)-{8-[(6-amino-hexyl)-tert-butoxycarbonylamino]octyl}carbamic Acid tert-Butyl Ester (39). Ethyl trifluoroacetate (0.35 mL, 2.9 mmol) was added to a stirred and cooled (–80 °C) solution of **35**² (1.0 g, 2.9 mmol) in MeOH (65 mL). Following stirring at –80 °C for 1 h and then to 0 °C over 1 h, di-tert-butyl dicarbonate (2.55 g, 11.7 mmol) was added to the solution. After 1 h of stirring at room temperature, the pH was increased to 11 with aqueous 28% ammonia and stirring was vigorously continued for 3 days. Removal of the solvent gave a residue that was dissolved in water (30 mL), and the resulting solution was extracted with CHCl₃ (3 × 20 mL). Evaporation of the dried solvent afforded a mixture of compounds that were separated by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (9:1:0.13) gave **37** and **39** as yellow oils.

37: 35% yield; *R_f* = 0.50 [eluent system, CHCl₃/MeOH/aqueous 28% ammonia (9:1:0.1)]; ¹H NMR (CDCl₃) δ 1.16–1.55 (complex m, 55 + 2 exchangeable with D₂O), 2.71–2.84 (m, 4), 3.06–3.19 (m, 8), 4.62 (br s, 1, exchangeable with D₂O).

39: 30% yield; *R_f* = 0.25 [eluent system, CHCl₃/MeOH/aqueous 28% ammonia (9:1:0.1)]; ¹H NMR (CDCl₃) δ 1.29–1.51 (complex m, 46 + 4 exchangeable with D₂O), 2.69 (t, 4), 3.11–3.22 (m, 8).

{12-[(6-Amino-hexyl)-tert-butoxycarbonylamino]dodecyl}-(6-tert-butoxycarbonylamino-hexyl)carbamic Acid tert-Butyl Ester (38) and (6-Amino-hexyl)-{12-[(6-amino-hexyl)-tert-butoxycarbonylamino]dodecyl}carbamic Acid tert-Butyl Ester (40). Ethyl trifluoroacetate (0.15 mL, 1.25 mmol) was added to a stirred and cooled (–80 °C) solution of **36**² (0.50 g, 1.25 mmol) in MeOH (30 mL). Stirring was continued at –80 °C for 1 h and then to 0 °C for an additional 1 h. Di-tert-butyl dicarbonate (1.09 g, 5 mmol) was added to the mixture that was stirred at room temperature for 1 h. Removal of the solvent gave a residue that was taken up in MeOH (40 mL) and water (2 mL). The resulting solution was treated with K₂CO₃ (6 g, 0.05 mol) and heated under reflux for 2 h. The residue was treated with 40% NaOH and extracted with CHCl₃ (3 × 20 mL). Removal of the dried solvent gave a mixture of compounds that were purified by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (9:1:0.05) gave **38** and **40** as yellow oils:

40: yield 25%; *R_f* = 0.45 [eluent system, CHCl₃/MeOH/aqueous 28% ammonia (8.5:1.5:0.2)]; ¹H NMR (CDCl₃) δ 1.12–1.55 (complex m, 54), 1.72 (br s, 4, exchangeable with D₂O), 2.63 (t, 4), 3.05–3.18 (m, 8).

38: yield 45%; *R_f* = 0.70 [eluent system, CHCl₃/MeOH/aqueous 28% ammonia (8.5:1.5:0.2)]; ¹H NMR (CDCl₃) δ 1.05–1.40 (complex m, 63 + 2 exchangeable with D₂O), 2.50–2.63 (m, 2), 2.82–3.10 (m, 10), 4.89 (br s, 1, exchangeable with D₂O).

(8-{tert-Butoxycarbonyl-[6-(2-cyanoethylamino)hexyl]-amino}octyl)-[6-(2-cyanoethylamino)hexyl]carbamic Acid tert-Butyl Ester (41). A solution of acrylonitrile (0.13 mL, 2.02 mmol) in MeOH (4 mL) was added dropwise to a solution

of **39** (0.55 g, 1.01 mmol) in MeOH (40 mL). After being stirred at room temperature overnight, the mixture was evaporated, affording a residue that was purified by flash chromatography. Elution with petroleum ether/CH₂Cl₂/EtOH/aqueous 28% ammonia (6:3:1:0.02) gave **41** as an oil: 95% yield; ¹H NMR (CDCl₃) δ 1.15–1.41 (complex m, 46 + 2 exchangeable with D₂O), 2.41 (t, 4), 2.52 (t, 4), 2.82 (t, 4), 2.98–3.08 (m, 8).

(12-{tert-Butoxycarbonyl-[6-(2-cyanoethylamino)hexyl]amino}dodecyl)-[6-(2-cyanoethylamino)hexyl]carbamic Acid tert-Butyl Ester (42). This compound was obtained from **40** (0.55 g, 0.918 mmol) and acrylonitrile (0.18 mL, 0.669 mmol) as described for **41**. Eluting with petroleum ether/CH₂Cl₂/EtOH/aqueous 28% ammonia (6:3:1:0.01) gave **42** as an oil: 80% yield; ¹H NMR (CDCl₃) δ 1.15–1.55 (complex m, 54 + 2 exchangeable with D₂O), 2.45–2.65 (m, 8), 3.05–3.20 (m, 8), 3.91 (t, 4).

(6-{tert-Butoxycarbonyl-[8-(tert-butoxycarbonyl-{6-[tert-butoxycarbonyl-(2-cyanoethyl)amino]hexyl}amino)octyl]amino}hexyl)-(2-cyanoethyl)carbamic Acid tert-Butyl Ester (43). A solution of **41** (0.63 g, 0.97 mmol) and di-*tert*-butyl dicarbonate (0.45 g, 2.08 mmol) in dry CHCl₃ was stirred overnight and then was washed with aqueous saturated NaHCO₃ solution (2 × 30 mL) and brine (2 × 30 mL). Removal of the dried solvent gave **43** in a quantitative yield: ¹H NMR (CDCl₃) δ 1.09–1.45 (complex m, 64), 2.36–2.53 (m, 4), 2.95–3.18 (m, 12), 3.37 (t, 4).

(6-{tert-Butoxycarbonyl-[12-(tert-butoxycarbonyl-{6-[tert-butoxycarbonyl-(2-cyanoethyl)amino]hexyl}amino)dodecyl]amino}hexyl)-(2-cyanoethyl)carbamic Acid tert-Butyl Ester (44). It was obtained in quantitative yield from **42** (0.50 g, 0.728 mmol) and di-*tert*-butyl dicarbonate (0.35 g, 1.6 mmol) following the procedure described for **43**: ¹H NMR (CDCl₃) δ 1.15–1.55 (complex m, 72), 2.45–2.62 (m, 4), 3.03–3.12 (m, 8), 3.20 (t, 4), 3.40 (t, 4).

(3-Aminopropyl)-(6-{[8-({6-[(3-aminopropyl)-*tert*-butoxycarbonylamino]hexyl)-*tert*-butoxycarbonylamino]octyl}-*tert*-butoxycarbonylamino}hexyl)carbamic Acid tert-Butyl Ester (45). This compound was obtained by reduction of **43** (0.81 g, 0.95 mmol) with Raney Ni (nickel sponge; suspension in water) (0.30 g) as described for **33**. Removal of the dried solvent gave a residue that was purified by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (9:1:0.05) gave **45** as an oil: 50% yield; ¹H NMR (CDCl₃) δ 1.14–1.50 (complex m, 64), 1.55–1.63 (m, 4), 1.95 (br s, 4, exchangeable with D₂O), 2.62 (t, 4), 3.01–3.22 (m, 16).

(3-Aminopropyl)-(6-{[12-({6-[(3-aminopropyl)-*tert*-butoxycarbonylamino]hexyl)-*tert*-butoxycarbonylamino]dodecyl}-*tert*-butoxycarbonylamino}hexyl)carbamic Acid tert-Butyl Ester (46). It was obtained by reduction of **44** (0.76 g, 0.38 mmol) with Raney Ni as described for **33**: 78% yield; ¹H NMR (CDCl₃) δ 1.18–1.55 (complex m, 72 + 4 exchangeable with D₂O), 1.58–1.66 (m, 4), 2.65 (t, 4), 3.05–3.20 (m, 16).

***N,N*-Bis-[6-[3-(2-methoxybenzylamino)propylamino]hexyl]octane-1,8-diamine Hexahydrochloride (6)**. It was synthesized from **45** (0.19 g, 0.22 mmol) and 2-methoxybenzaldehyde (0.066 g, 0.49 mmol) following a slightly modified procedure described for **5**. Following removal of molecular sieves, the solvent was evaporated to give a residue that was taken up in 3 N HCl (10 mL). The resulting mixture was stirred for 3 h at room temperature, was washed with ether to remove nonbasic materials, then was made basic with 2 N NaOH, and finally was extracted with CHCl₃ (3 × 20 mL). Removal of the dried solvent gave **6** as free base that was transformed into the hexahydrochloride salt: 30% yield; mp 295 °C (dec); ¹H NMR (free base) (CDCl₃) δ 1.14–1.40 (m, 18), 1.45–1.65 (m, 10), 1.75–1.85 (m, 4), 2.26 (br s, 6, exchangeable with D₂O), 2.52–2.80 (m, 20), 3.78 (s, 6), 3.88 (s, 4), 6.85–6.98 (m, 4), 7.20–7.32 (m, 4). Anal. (C₄₂H₈₂Cl₆N₆O₂) C, H, N.

***N,N*-Bis-[6-[3-(2-methoxybenzylamino)propylamino]hexyl]dodecane-1,12-diamine Hexahydrochloride (7)**. It was synthesized from **46** (0.30 g, 0.33 mmol) and 2-methoxybenzaldehyde (0.098 g, 0.72 mmol) following the procedure described for **6**. The free base was transformed into the

hexahydrochloride salt: 50% yield; mp 283–285 °C (from EtOH/Et₂O); ¹H NMR (free base) (CDCl₃) δ 1.18–1.60 (complex m, 36), 1.65–1.80 (m, 4), 1.97 (br s, 6, exchangeable with D₂O), 2.50–2.78 (complex m, 20), 3.78 (s, 4), 3.82 (s, 6), 6.82–6.98 (m, 4), 7.20–7.35 (m, 4). Anal. (C₄₆H₉₀Cl₆N₆O₂) C, H, N.

***N*-(2-Methoxybenzyl)-*N'*-(4'-[6-(2-methoxybenzylamino)hexylamino]methyl)-[1,1',4',1''terphenyl-4-ylmethyl]-hexane-1,6-diamine Tetrahydrochloride (8)**. A solution of **47**²⁶ (0.17 g, 0.59 mmol) and **48**²⁷ (0.37 g, 1.19 mmol) in EtOH (20 mL) was stirred at 60 °C for 2 h, then NaBH₄ (0.05 g, 1.32 mmol) was added, and the stirring was continued overnight at room temperature. Removal of the solvent gave a residue that was purified by flash chromatography. Elution with CH₂Cl₂/MeOH/aqueous 28% ammonia (9:1:0.1) gave **8** as free base that was transformed into the tetrahydrochloride salt: 14% yield; mp >300 °C (from EtOH/Et₂O); ¹H NMR (free base) (CDCl₃) δ 1.26–1.73 (complex m, 16 + 4 exchangeable with D₂O), 2.50–2.60 (m, 8), 3.78 (s, 4), 3.83 (s, 10), 6.83–6.95 (m, 4), 7.20–7.26 (m, 4), 7.39–7.43 (m, 4), 7.59–7.67 (m, 8); MALDI-MS calcd for C₄₈H₆₃N₄O₂ 727.49 [M + H⁺], found 727.44. Anal. (C₄₈H₆₆Cl₄N₄O₂) C, H, N.

[4-({11-[4-(Benzyloxycarbonylamino)methyl]benzyl]carbamic Acid Benzyl Ester (50). It was obtained as a white solid from 1,12-dodecanedioic acid (0.35 g, 1.5 mmol) and **49**¹ (0.83 g, 3.0 mmol) following the procedure described for **23**: 80% yield; mp 209 °C; ¹H NMR (DMSO-*d*₆) δ 1.18–1.31 (m, 12), 1.40–1.52 (m, 4), 2.03–2.21 (m, 4), 4.16–4.25 (m, 8), 5.04 (s, 4), 7.12–7.38 (m, 18), 7.81 (t, 2, exchangeable with D₂O), 8.30 (t, 2, exchangeable with D₂O).

Dodecanedioic Acid Bis(4-aminomethylbenzylamide) (51). It was obtained in a quantitative yield by hydrolysis with HBr of **50** (0.9 g, 1.2 mmol) following the procedure described for **26**: mp 195–200 °C; ¹H NMR (D₂O) δ 1.15–1.39 (m, 12), 1.51–1.79 (m, 4), 2.25–2.50 (m, 4), 4.12–4.45 (m, 8), 7.31–7.57 (m, 8).

Dodecanedioic Acid Bis[4-[(2-methoxybenzylamino)methyl]benzylamide] (52). It was obtained in low yield (15%) because of its insolubility from **51** (0.65 g, 1.4 mmol) and 2-methoxybenzaldehyde (0.39 g, 2.9 mmol) following the procedure described for **27**: mp 120–125 °C; ¹H NMR (CDCl₃) δ 1.09–1.39 (m, 12), 1.53–1.71 (m, 4), 1.82 (br s, 2, exchangeable with D₂O), 2.18 (t, 4), 3.74 (s, 4), 3.78 (s, 4), 3.82 (s, 6), 4.39 (d, 4), 5.82 (br t, 2, exchangeable with D₂O), 6.85–6.96 (m, 4), 7.20–7.31 (m, 12).

***N,N*-Bis[4-[(2-methoxybenzylamino)methyl]benzyl]-dodecane-1,12-diamine Tetrahydrochloride (11)**. It was obtained as a tetrahydrochloride salt by reduction with BH₃·MeSMe (0.1 mL) of **52** (0.15 g, 0.21 mmol) following the procedure described for **4**: mp >300 °C (from EtOH/Et₂O); ¹H NMR (D₂O) δ 1.03–1.22 (m, 16), 1.42–1.61 (m, 4), 2.85–2.93 (m, 4), 3.70 (s, 6), 4.07 (s, 4), 4.11 (s, 4), 4.14 (s, 4), 6.83–6.94 (m, 4), 7.14–7.41 (m, 12). Anal. (C₄₄H₆₆Cl₄N₄O₂) C, H, N.

[6-(Benzyl-[6-[benzyl-(6-*tert*-butoxycarbonylamino)hexyl]amino]hexyl)-(2-cyanoethyl)carbamic Acid tert-Butyl Ester (53). It was obtained in a quantitative yield from **31** (0.39 g, 0.71 mmol) and di-*tert*-butyl dicarbonate (0.34 g, 1.47 mmol) following the procedure described for **43**: oil; ¹H NMR (CDCl₃) δ 1.18–1.56 (complex m, 42), 2.32–2.38 (m, 8), 2.50–2.61 (m, 2), 3.01–3.12 (m, 2), 3.18–3.23 (m, 2), 3.39–3.46 (m, 2), 3.48–3.55 (m, 4), 4.48 (br s, 1, exchangeable with D₂O), 7.19–7.38 (m, 10).

(3-Aminopropyl)-[6-(benzyl-[6-(benzyl-(6-*tert*-butoxycarbonylamino)hexyl]amino)hexyl]amino]hexyl]carbamic Acid tert-Butyl Ester (54). It was obtained by reduction of **53** (0.52 g, 0.72 mmol) with Raney Ni (nickel sponge; suspension in water) (0.20 g) as described for **33**. Removal of dried solvents gave a residue that was purified by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (9:1:0.07) afforded **54** as an oil: 70% yield; ¹H NMR (CDCl₃) δ 1.11–1.48 (complex m, 42), 1.53–1.63 (m, 2), 2.01 (br s, 2, exchangeable with D₂O), 2.22–2.35 (m, 8), 2.66 (t, 2), 2.98–3.26 (m, 6), 3.48 (s, 4), 4.54 (br s, 1, exchangeable with D₂O), 7.18–7.31 (m, 10).

[6-(Benzyl-{6-[benzyl-(6-*tert*-butoxycarbonylamino-hexyl)amino]hexyl}{3-[2-butrylamino-3-(4-phenoxyphenyl)propionylamino]propyl}carbamic Acid *tert*-Butyl Ester (56). A solution of **54** (0.35 g, 0.49 mmol) in MeOH (10 mL) was added dropwise with stirring at room temperature to a suspension of **55**²⁸ (0.25 g, 0.54 mmol) in MeOH (10 mL). After 1 h, the reaction mixture was evaporated to give a residue that was purified by flash chromatography. Elution with a step gradient system of CH₂Cl₂/EtOH (9.5:0.5 to 9:1) gave **56** as an oil: 67% yield; ¹H NMR (CDCl₃) δ 0.88 (t, 3), 1.21–1.44 (complex m, 44), 1.58–1.63 (m, 2), 2.18–2.22 (m, 2), 2.35–2.39 (m, 8), 3.02–3.15 (m, 10), 3.53 (s, 4), 4.52 (br s, 1, exchangeable with D₂O), 4.62–4.69 (m, 1), 5.01 (s, 2), 6.16 (br s, 1, exchangeable with D₂O), 6.87 (d, 2), 7.10 (d, 2), 7.26–7.39 (m, 15).

N-[1-{3-[6-((6-Aminoethyl)benzylamino)hexyl]-benzylamino]hexylamino]propylcarbonyl-2-(4-phenoxyphenyl)ethyl]butyramide Tetratetrafluoroacetate (57). A solution of **56** (0.34 g, 0.33 mmol) and CF₃COOH (0.5 mL) in dry CH₂Cl₂ (6 mL) was stirred at room temperature for 2 h. Removal of the solvent gave **57**: quantitative yield; ¹H NMR (CD₃OD) δ 0.84 (t, 3), 1.28–1.80 (complex m, 28), 2.17 (t, 2), 2.85–3.22 (complex m, 18), 4.35 (s, 4), 4.38–4.45 (m, 1), 5.04 (s, 2), 6.92 (d, 2), 7.16 (d, 2), 7.31–7.49 (m, 15).

N-[1-(3-{6-[6-(6-Aminoethylamino)hexylamino]hexylamino]propylcarbonyl-2-(4-hydroxyphenyl)ethyl]butyramide Tetratetrafluoroacetate (14). A solution of **57** (0.28 g, 0.32 mmol) in MeOH (15 mL) was hydrogenated over 10% Pd on charcoal (wet, Degussa type E101 NE/W) (28 mg) for 3 h. Following catalyst removal, the solvent was evaporated, yielding **14** as a foam solid: ¹H NMR (CD₃OD) δ 0.85 (t, 3), 1.43–1.80 (complex m, 28), 2.16 (t, 2), 2.83–3.00 (m, 16), 3.20–3.24 (m, 2), 4.38–4.41 (m, 1), 6.70 (d, 2), 7.04 (d, 2). Anal. (C₄₂H₆₈F₁₂N₆O₁₁) C, H, N.

N-{2-(4-Hydroxyphenyl)-1-[3-(6-[6-(2-methoxybenzylamino)hexylamino]hexylamino)propylcarbonyl]ethyl]butyramide Tetrahydrochloride (15). It was synthesized from **14** (free base) (0.11 g, 0.18 mmol) and 2-methoxybenzaldehyde (27 mg, 0.20 mmol) following the procedure described for **5**. Removal of the dried solvent gave a residue that was purified by flash chromatography. Elution with CHCl₃/MeOH/aqueous 28% ammonia (5:4.5:0.6) afforded crude **15** that was transformed into the tetrahydrochloride salt: 65% yield; mp 195–198 °C; ¹H NMR (D₂O) δ 0.83 (t, 3), 1.34–1.82 (complex m, 28), 2.25 (t, 2), 2.83 (t, 2), 2.99–3.10 (m, 14), 3.20–3.28 (m, 2), 3.95 (s, 3), 4.28 (s, 2), 4.46 (t, 1), 6.90 (d, 2), 7.07–7.22 (m, 4), 7.41 (d, 1), 7.55 (t, 1). Anal. (C₄₂H₇₆Cl₄N₆O₄) C, H, N.

(6-*tert*-Butoxycarbonylaminoethyl)-(12-{*tert*-butoxycarbonyl-[6-(2-cyanoethylamino)hexyl]amino}dodecylcarbamic Acid *tert*-Butyl Ester (58). A solution of acrylonitrile (0.042 mL, 0.64 mmol) in CHCl₃ (2 mL) was added dropwise to **38** (0.45 g, 0.643 mmol) in CHCl₃ (40 mL). After the mixture was stirred for 48 h, removal of the solvent gave a residue that was purified by flash chromatography. Elution with EtOAc/EtOH (9.5:0.4) gave **58**: 60% yield; ¹H NMR (CDCl₃) δ 1.15–1.55 (complex m, 63), 1.67 (br s, 1, exchangeable with D₂O), 2.49 (t, 2), 2.60 (t, 2), 2.90 (t, 2), 3.0–3.20 (m, 10), 4.71 (br, s, 1, exchangeable with D₂O).

[6-(*tert*-Butoxycarbonyl-12-{*tert*-butoxycarbonyl-(6-*tert*-butoxycarbonylaminoethyl)amino]dodecyl}amino)-hexyl-(2-cyanoethyl)carbamic Acid *tert*-Butyl Ester (59). It was obtained in a quantitative yield from **58** (0.20 g, 0.27 mmol) and di-*tert*-butyl dicarbonate (65 mg, 0.30 mmol) following the procedure described for **43**: oil; ¹H NMR (CDCl₃) δ 1.05–1.50 (complex m, 72), 2.45–2.58 (m, 2), 2.90–3.13 (m, 10), 3.17 (t, 2), 3.37 (t, 2), 4.55 (br s, 1, exchangeable with D₂O).

(3-Aminopropyl)-[6-(*tert*-butoxycarbonyl-12-{*tert*-butoxycarbonyl-(6-*tert*-butoxycarbonylaminoethyl)amino]dodecyl}amino)hexyl]carbamic Acid *tert*-Butyl Ester (60). It was obtained by reduction of **59** (0.55 g, 0.65 mmol) with Raney Ni (nickel sponge; suspension in water) as described for **33**: 75% yield; ¹H NMR (CDCl₃) δ 1.12–1.70 (complex m, 72), 1.76 (br s, 2, exchangeable with D₂O), 1.87–

1.95 (m, 2), 2.60–2.75 (m, 2), 3.0–3.30 (m, 12), 3.57 (t, 2), 4.71 (br s, 1, exchangeable with D₂O).

[6-(*tert*-Butoxycarbonyl-12-{*tert*-butoxycarbonyl-(6-*tert*-butoxycarbonylaminoethyl)amino]dodecyl}amino)-hexyl]-{3-[2-butrylamino-3-(4-phenoxyphenyl)propionylamino]propyl}carbamic Acid *tert*-Butyl Ester (61). A solution of **60** (0.41 g, 0.48 mmol) in CHCl₃ (10 mL) was added dropwise to a suspension of **55**²⁸ (0.22 g, 0.48 mmol) in MeOH (10 mL). After the mixture was stirred overnight, removal of the solvent gave a residue that was purified by flash chromatography. Elution with a step gradient system of EtOAc/petroleum ether (5:5 to 7:3) afforded **61**: 50% yield; ¹H NMR (CDCl₃) δ 0.85 (t, 3), 1.18–1.52 (complex m, 72), 1.59 (q, 2), 1.88–1.92 (m, 2), 2.10–2.20 (m, 2), 2.95–3.20 (m, 18), 4.60–4.68 (m, 1), 5.0 (s, 2), 6.19 (d, 1, exchangeable with D₂O), 6.86 (d, 2), 7.10 (d, 2), 7.18 (br s, 1, exchangeable with D₂O), 7.28–7.40 (m, 5).

N-[1-(3-{6-[12-(6-Aminoethylamino)dodecylamino]hexylamino]propylcarbonyl-2-(4-phenoxyphenyl)ethyl]butyramide Tetratetrafluoroacetate (62). A solution of **61** (0.27 g, 0.229 mmol) in CHCl₃ (2.5 mL) and CF₃COOH (0.5 mL) was stirred for 2 h. Removal of the solvent gave a residue that was triturated with ether to afford in a quantitative yield **62** as a tetratetrafluoroacetate salt: ¹H NMR (CD₃OD) δ 0.82 (t, 3), 1.20–1.80 (complex m, 40), 2.16 (t, 2), 2.80–3.10 (m, 16), 3.12–3.35 (m, 2), 4.45 (t, 1), 5.0 (s, 2), 6.89 (d, 2), 7.16 (d, 2), 7.22–7.43 (m, 5).

N-[1-(3-{6-[12-(6-Aminoethylamino)dodecylamino]hexylamino]propylcarbonyl-2-(4-hydroxyphenyl)ethyl]butyramide Tetratetrafluoroacetate (16). A solution of **62** (0.26 g, 0.21 mmol) in MeOH (10 mL) was hydrogenated over 10% Pd on charcoal (wet, Degussa type E101 NE/W) (26 mg) for 2 h. Following catalyst removal, the solvent was evaporated, affording **16** as a tetratetrafluoroacetate salt: quantitative yield; ¹H NMR (CD₃OD) δ 0.86 (t, 3), 1.22–1.83 (complex m, 40), 2.20 (t, 2), 2.78–3.08 (m, 16), 3.18–3.30 (m, 2), 4.38–4.42 (m, 1), 6.73 (d, 2), 7.07 (d, 2), 8.19 (d, 1, exchangeable with D₂O). Anal. (C₄₈H₈₀F₁₂N₆O₁₁) C, H, N.

N-{2-(4-Hydroxyphenyl)-1-[3-(6-[12-(6-(2-methoxybenzylamino)hexylamino]dodecylamino)hexylamino)propylcarbonyl]ethyl]butyramide Tetrahydrochloride (17). It was obtained starting from **16** (free base) (0.11 g, 0.16 mmol) and 2-methoxybenzaldehyde (0.024 g, 0.176 mmol) following the procedure described for **5**. The residue was purified by flash chromatography. Elution with a step gradient system of CHCl₃/MeOH/aqueous 28% ammonia (5:4.5:0.4 to 5:4.5:0.5) afforded **17** as free base that was transformed into the tetrahydrochloride salt: 60% yield; mp 205–207 °C (from EtOH/Et₂O); ¹H NMR (CD₃OD) δ 0.84 (t, 3), 1.30–1.81 (complex m, 40), 2.18 (t, 2), 2.80–2.82 (m, 2), 2.90–3.08 (m, 14), 3.15–3.22 (m, 2), 3.92 (s, 3), 4.20 (s, 2), 4.38 (t, 1), 6.70 (d, 2), 7.0–7.13 (m, 4), 7.38–7.50 (m, 2), 8.2 (t, 1, exchangeable with D₂O). Anal. (C₄₈H₈₈Cl₄N₆O₄) C, H, N.

(6-{[3-(4-Azido-2-hydroxybenzoylamino)propyl]-*tert*-butoxycarbonylamino}hexyl)-{8-[(6-{[3-(4-azido-2-hydroxybenzoylamino)propyl]-*tert*-butoxycarbonylamino}hexyl)-*tert*-butoxycarbonylamino]octyl}carbamic Acid *tert*-Butyl Ester (63). A solution of 4-azidosalicylic acid *N*-hydroxysuccinimide ester²⁹ (0.14 g, 0.50 mmol) in dry CHCl₃ (5 mL) was added dropwise to a stirred solution of **45** (0.20 g, 0.23 mmol) in distilled CHCl₃ (10 mL). After the mixture was stirred for 2 h at room temperature, the solvent was evaporated to give a residue that was purified by flash chromatography. Elution with petroleum ether/acetone (8.5:2) afforded **63** as an oil: 55% yield; ¹H NMR (CDCl₃) δ 1.22–1.58 (complex m, 64), 1.72–1.78 (m, 4), 3.10–3.19 (m, 16), 3.36–3.43 (m, 4), 6.50–6.62 (m, 4), 7.62 (d, 2), 8.40 (br s, 2, exchangeable with D₂O).

(6-{[3-(4-Azido-2-hydroxybenzoylamino)propyl]-*tert*-butoxycarbonylamino}hexyl)-{12-[(6-{[3-(4-azido-2-hydroxybenzoylamino)propyl]-*tert*-butoxycarbonylamino}hexyl)-*tert*-butoxycarbonylamino]dodecyl}carbamic Acid *tert*-Butyl Ester (64). It was obtained from 4-azidosalicylic acid *N*-hydroxysuccinimide ester²⁹ (0.20 g, 0.72 mmol) and **46**

(0.30 g, 0.33 mmol) following the procedure described for **63**. It was purified first by gravity chromatography (eluting system, petroleum ether/acetone (8:2)) and then by flash chromatography. Elution with petroleum ether/acetone (9:1) gave **64**: 30% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.10–1.62 (complex m, 72), 1.63–1.82 (m, 4), 3.10–3.22 (m, 12), 3.25–3.50 (m, 8), 6.50–6.63 (m, 4), 7.65 (d, 2), 8.42 (br s, 2, exchangeable with D_2O).

N-[3-((6-((8-((3-[(4-Azido-2-hydroxybenzoyl)amino]propyl)amino)hexyl)amino)octyl)amino)hexyl)amino]propyl-4-azido-2-hydroxybenzamide Tetratetrafluoroacetate (**19**). It was obtained as a foam solid from **63** (0.14 g, 0.115 mmol) and CF_3COOH (0.30 mL) following the procedure reported for **57**: quantitative yield; $^1\text{H NMR}$ (CD_3OD) δ 1.38–1.44 (complex m, 16), 1.62–1.76 (m, 12), 1.95–2.02 (m, 4), 2.90–3.05 (complex m, 16), 3.49 (t, 4), 6.50–6.61 (m, 4), 7.78 (d, 2). Anal. ($\text{C}_{48}\text{H}_{70}\text{F}_{12}\text{N}_{12}\text{O}_{12}$) C, H, N.

N-[3-((6-((12-((3-[(4-Azido-2-hydroxybenzoyl)amino]propyl)amino)hexyl)amino)dodecyl)amino)hexyl)amino]propyl-4-azido-2-hydroxybenzamide Tetratetrafluoroacetate (**20**). It was obtained as a foam solid from **64** (0.12 g, 0.092 mmol) and CF_3COOH (0.30 mL) following the procedure reported for **57**: quantitative yield; $^1\text{H NMR}$ (CD_3OD) δ 1.30–1.82 (complex m, 36), 1.92–2.11 (m, 4), 2.92–3.18 (complex m, 16), 3.53 (t, 4), 6.58–6.70 (m, 4), 7.82 (d, 2). Anal. ($\text{C}_{52}\text{H}_{78}\text{F}_{12}\text{N}_{12}\text{O}_{12}$) C, H, N.

[8-(*tert*-Butoxycarbonyl-6-*tert*-butoxycarbonyl-(2-cyanoethyl)amino)hexyl]amino]octyl-6-*tert*-butoxycarbonyl-(2-methoxybenzyl)amino]hexyl]carbamic Acid *tert*-Butyl Ester (**66**). It was obtained in quantitative yield from **65**¹ (0.31 g, 0.6 mmol) and di-*tert*-butyl dicarbonate (0.58 g, 2.64 mmol) following the procedure described for **43**: $^1\text{H NMR}$ (CDCl_3) δ 1.08–1.43 (complex m, 64), 2.42–2.53 (m, 2), 3.93–3.18 (m, 12), 3.38 (t, 2), 3.72 (s, 3), 4.38 (d, 2), 6.72–6.83 (m, 2), 7.02–7.18 (m, 2).

[8-((6-((3-Aminopropyl)-*tert*-butoxycarbonylamino)hexyl)-*tert*-butoxycarbonylamino)octyl]-6-*tert*-butoxycarbonyl-(2-methoxybenzyl)amino]hexyl]carbamic Acid *tert*-Butyl Ester (**67**). This compound was obtained in a quantitative yield as an oil by reduction of **66** (0.49 g, 0.58 mmol) with Raney Ni (nickel sponge; suspension in water) (0.15 g) as described for **33**: $^1\text{H NMR}$ (CDCl_3) δ 1.12–1.52 (complex m, 64), 1.56–1.62 (m, 2 + 2 exchangeable with D_2O), 2.61 (t, 2), 3.02–3.22 (m, 14), 3.77 (s, 3), 4.38 (d, 2), 6.78 (d, 1), 6.82 (t, 1), 7.03–7.18 (m, 2).

[8-*tert*-Butoxycarbonyl-6-*tert*-butoxycarbonyl-3-[(7-methoxy-2-oxo-2*H*-chromene-3-carbonyl)amino]propyl]amino]hexyl]amino]octyl-6-*tert*-butoxycarbonyl-(2-methoxybenzyl)amino]hexyl]carbamic Acid *tert*-Butyl Ester (**68**). A solution of 2,5-dioxotetrahydro-1*H*-1-pyrrolyl-7-methoxy-2-oxo-2*H*-3-chromene carboxylate (53 mg, 0.17 mmol) in dry DMF (4 mL) was added dropwise with stirring at room temperature to a solution of **67** (0.14 g, 0.15 mmol) in dry DMF (6 mL). After 2 h, the reaction mixture was evaporated to give a residue that was purified by flash chromatography. Elution with petroleum ether/acetone/toluene (7:2:1) afforded **68** as an oil: 82% yield; $^1\text{H NMR}$ (CDCl_3) δ 0.80–1.48 (complex m, 64), 1.81 (t, 2), 3.05–3.28 (m, 14), 3.38–3.46 (m, 2), 3.79 (s, 3), 3.88 (s, 3), 4.41 (d, 2), 6.80–7.19 (m, 6), 7.55 (d, 1), 8.80 (s, 1).

7-Methoxy-2-oxo-2*H*-chromene-3-carboxylic Acid [3-6-8-((2-Methoxybenzylamino)hexylamino)octylamino]hexylamino]propyl]amide Tetratetrafluoroacetate (**21**). It was synthesized in a quantitative yield from **68** (0.14 g, 0.12 mmol) and CF_3COOH (0.4 mL) as described for **57**: mp 157–159 °C; $^1\text{H NMR}$ (CD_3OD) δ 1.37–1.41 (complex m, 16), 1.68–1.70 (complex m, 12), 1.97–2.03 (m, 2), 2.94–3.06 (m, 14), 3.54 (t, 2), 3.90 (s, 3), 3.93 (s, 3), 4.18 (s, 2), 6.97–7.09 (m, 4), 7.37–7.43 (m, 2), 7.71 (d, 1), 8.77 (s, 1). Anal. ($\text{C}_{50}\text{H}_{71}\text{F}_{12}\text{N}_5\text{O}_{13}$) C, H, N.

[8-*tert*-Butoxycarbonyl-6-*tert*-butoxycarbonylamino]hexyl]amino]octyl-6-*tert*-butoxycarbonyl-(2-cyanoethyl)amino]hexyl]carbamic Acid *tert*-Butyl Ester (**69**). It was synthesized from **37** (0.60 g, 0.93 mmol) and acryloni-

trile (0.06 mL, 0.93 mmol) following the procedure described for **41** and purified by flash chromatography. Elution with a step gradient system of petroleum ether/EtOAc (5:5 to 9:1) gave **69** as an oil: 70% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.11–1.46 (complex m, 55), 1.81 (br s, 1, exchangeable with D_2O), 2.51 (t, 2), 2.62 (t, 2), 2.91 (t, 2), 3.08–3.14 (m, 10), 4.60 (br s, 1, exchangeable with D_2O).

[6-((3-Aminopropyl)-*tert*-butoxycarbonylamino)hexyl]-8-*tert*-butoxycarbonyl-(6-*tert*-butoxycarbonylamino)hexyl]amino]octyl]carbamic Acid *tert*-Butyl Ester (**70**). This compound was obtained by reduction of **69** (0.44 g, 0.63 mmol) with Raney Ni (nickel sponge; suspension in water) (0.15 g) as described for **33**. Removal of the dried solvent gave **70** as an oil: 90% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.61 (complex m, 57 + 3 exchangeable with D_2O), 2.50–2.75 (m, 6), 2.93–3.18 (m, 10), 4.81 (br s, 1, exchangeable with D_2O).

[8-*tert*-Butoxycarbonyl-6-*tert*-butoxycarbonylamino]hexyl]amino]octyl-6-*tert*-butoxycarbonyl-3-(2,2,2-trifluoroacetyl)amino]propyl]amino]hexyl]carbamic Acid *tert*-Butyl Ester (**71**). Ethyl trifluoroacetate (0.07 mL, 0.57 mmol) was added to a stirred and cooled (-80 °C) solution of **70** (0.4 g, 0.57 mmol) in MeOH (30 mL). Stirring was continued for 1 h at -80 °C and then to 0 °C over 1 h. Removal of the solvent gave a residue that was purified by flash chromatography. Elution with $\text{CHCl}_3/\text{MeOH}$ (9:1) afforded **71** as an oil: 90% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.51 (complex m, 55 + 1 exchangeable with D_2O), 1.63–1.68 (m, 2), 2.68 (t, 2), 2.88 (t, 2), 3.04–3.18 (m, 10), 3.41–3.48 (m, 2), 4.61 (br s, 1, exchangeable with D_2O).

N-(3-6-8-((6-Aminohexylamino)octylamino)hexylamino]propyl-2,2,2-trifluoroacetamide (**72**). CF_3COOH (2 mL) was added to a stirred solution of **71** (0.40 g, 0.50 mmol) in dry CH_2Cl_2 (5 mL). After the mixture was stirred for 2 h at room temperature, the solvent was removed to give a residue that was taken up in 0.5 N NaOH and immediately extracted with CHCl_3 (1×30 mL). Removal of the dried solvent gave **72** as a clear oil: 56% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.51 (complex m, 28 + 5 exchangeable with D_2O), 1.62–1.69 (m, 2), 2.48–2.56 (m, 10), 2.64 (t, 2), 2.78 (t, 2), 3.43 (t, 2).

2,2,2-Trifluoro-*N*-(3-6-8-((5-iodo-2-methoxybenzyl)amino)hexylamino)octylamino]hexylamino]propyl]acetamide (**73**). A solution of **72** (0.14 g, 0.28 mmol) and 5-iodo-2-methoxybenzaldehyde³⁰ (74 mg, 0.28 mmol) in toluene (30 mL) was heated under reflux, and the water formed was continuously removed for 5 h. The cooled mixture was evaporated to give the corresponding Schiff base that was dissolved in EtOH (25 mL) and treated with NaBH_4 (11 mg, 0.29 mmol). After the mixture was stirred at 0 °C for 4 h, the solvent was evaporated to give a residue that was treated with petroleum ether (3×20 mL). Removal of the combined organic layers afforded **73** as an oil: 60% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.41 (complex m, 28 + 4 exchangeable with D_2O), 1.61–1.66 (m, 2), 2.43–2.55 (m, 12), 2.78 (t, 2), 3.41 (t, 2), 3.63 (s, 2), 3.74 (s, 3), 6.58 (d, 1), 7.42–7.51 (m, 2).

[8-*tert*-Butoxycarbonyl-6-*tert*-butoxycarbonyl-(5-iodo-2-methoxybenzyl)amino]hexyl]amino]octyl-6-*tert*-butoxycarbonyl-3-(2,2,2-trifluoroacetyl)amino]propyl]amino]hexyl]carbamic Acid *tert*-Butyl Ester (**74**). It was obtained from **73** (0.13 g, 0.17 mmol) and di-*tert*-butyl dicarbonate (168 mg, 0.17 mmol) following the procedure described for **43** and purified by flash chromatography. Elution with a step gradient system of $\text{CHCl}_3/\text{toluene}/\text{EtOH}$ (5:4.5:0.5 to 5:4.5:1.1) gave **74** as an oil: 45% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.41 (complex m, 64), 1.61–1.78 (m, 2), 3.05–3.38 (m, 16), 3.80 (s, 3), 4.38 (d, 2), 6.62 (d, 1), 7.38–7.58 (m, 2), 8.19 (br s, 1, exchangeable with D_2O).

[6-((3-Aminopropyl)-*tert*-butoxycarbonylamino)hexyl]-8-*tert*-butoxycarbonyl-6-*tert*-butoxycarbonyl-(5-iodo-2-methoxybenzyl)amino]hexyl]amino]octyl]carbamic Acid *tert*-Butyl Ester (**75**). Aqueous 28% ammonia was added to a vigorously stirred solution of **74** (90 mg, 0.07 mmol) in MeOH (20 mL) until the pH was increased to 11. After the mixture was stirred at room temperature overnight, the solvent was evaporated to give a residue that was dissolved

in water (20 mL) and extracted with CHCl_3 (3×30 mL). Removal of the dried solvent gave **75** in quantitative yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.44 (complex m, 64 + 2 exchangeable with D_2O), 1.58–1.66 (m, 2), 2.63 (t, 2), 3.03–3.22 (m, 14), 3.78 (s, 3), 4.31 (d, 2), 6.59 (d, 1), 7.38–7.51 (m, 2).

[8-(tert-Butoxycarbonyl)-{6-[tert-butoxycarbonyl-(5-iodo-2-methoxybenzyl)amino]hexyl}amino]octyl]-[6-(tert-butoxycarbonyl)-{3-[(7-methoxy-2-oxo-2H-chromene-3-carbonyl)amino]propyl}amino]hexyl]carbamic Acid tert-Butyl Ester (76**).** A solution of 2,5-dioxotetrahydro-1H-pyrrolyl-7-methoxy-2-oxo-2H-3-chromene carboxylate (27 mg, 0.084 mmol) in dry DMF (2 mL) was added dropwise at room temperature to a solution of **75** (80 mg, 0.077 mmol) in dry DMF (5 mL). After the mixture was stirred overnight, the reaction mixture was evaporated to give a residue that was purified by flash chromatography. Elution with petroleum ether/acetone/toluene (7:2:1) afforded **76** as an oil: 72% yield; $^1\text{H NMR}$ (CDCl_3) δ 1.18–1.51 (complex m, 64), 1.81 (t, 2), 3.02–3.23 (m, 14), 3.41–3.48 (m, 2), 3.77 (s, 3), 3.90 (s, 3), 4.38 (d, 2), 6.60 (d, 1), 6.82–6.96 (m, 2), 7.38–7.51 (m, 2), 7.59 (d, 1), 8.81 (s, 1).

7-Methoxy-2-oxo-2H-chromene-3-carboxylic Acid [3-(6-{8-[6-(5-Iodo-2-methoxybenzylamino)hexylamino]octylamino]hexylamino]propyl]amide Tetratrifluoroacetate (22**).** It was obtained in quantitative yield as a foam solid from **76** (80 mg, 0.055 mmol) and CF_3COOH (0.25 mL) following the procedure reported for **57**: $^1\text{H NMR}$ (CD_3OD) δ 1.37–1.43 (complex m, 16), 1.62–1.76 (complex m, 12), 1.97–2.04 (m, 2), 2.94–3.05 (m, 14), 3.55 (t, 2), 3.88 (s, 3), 3.94 (s, 3), 4.18 (s, 2), 6.94–7.03 (m, 3), 7.73–7.79 (m, 3), 8.80 (s, 1). Anal. ($\text{C}_{50}\text{H}_{70}\text{F}_{12}\text{I}_2\text{N}_5\text{O}_{13}$) C, H, N.

Synthesis and Purification of $^{125}\text{I}_2$ -19**.** Compound **19** was radioactively iodinated with ^{125}I using the chloramine T method.³¹ A total of 60 μL of chloramine T (2.3 mg/mL in 100 mM NaPi, pH 7.4) was added in the dark to a solution containing 50 μL of **19** (5 mg/mL), 140 μL of K^{125}I (30 000 cpm/nmol), and 380 μL of 100 mM NaPi, pH 7.4. After 5 min of incubation at room temperature, the iodination reaction was stopped by applying the solution onto a reverse-phase high-performance liquid chromatography (RP-HPLC) column (Vydac C_{18} , Waters model 626, Eschborn, Germany). For elution of the di- ^{125}I derivative, the following linear gradient (1 mL/min) was applied: solvent A (aqueous solution containing 0.1% trifluoroacetic acid (TFA)) and solvent B (acetonitrile containing 0.085% TFA). The UV absorption of the eluent was determined at 305 nm, and the radioactivity of each fraction was detected using a γ -counter. $^{125}\text{I}_2$ -**19** was characterized by matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS).

Biology. Functional Antagonism. Guinea pigs of either sex (200–400 g) and frogs (10–20 g) were sacrificed under ketamine or ether anesthesia, respectively, and the organs required were set up rapidly under a suitable resting tension in 15 mL organ baths containing physiological salt solution kept at the appropriate temperature (see below) and aerated with 5% $\text{CO}_2/95\%$ O_2 at pH 7.4. Concentration–response curves were constructed by cumulative addition of the agonist. The concentration of agonist in the organ bath was increased approximately 5-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Contractions were recorded by means of a force displacement transducer (FT.03 Grass and 7003 Basile) connected to a four-channel pen recorder (Battaglia-Rangoni KV 380). In all cases, parallel experiments in which tissues did not receive any antagonist were run in order to check for variations in sensitivity.

Guinea Pig Left Atria. The guinea pig heart was rapidly removed and washed by perfusion through the aorta with oxygenated physiological salt solution, and right and left atria were separated out. The left atria were mounted under 0.2–0.3 g of tension at 35 °C in Tyrode solution of the following composition (mM): NaCl, 136.9; KCl, 5.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; CaCl_2 , 2.52; NaH_2PO_4 , 0.4; NaHCO_3 , 11.9; glucose, 5.5. Tissues were stimulated through platinum electrodes by square-wave

pulses (0.6–0.8 ms, 1 Hz, 1–5 V). Inotropic activity was recorded isometrically. Tissues were equilibrated for 1 h, and cumulative concentration–response curves to arecaidine propargyl ester (APE) (0.01–1 μM) were constructed. Following incubation with the antagonist for 60 min, a new concentration–response curve to APE was obtained.

Guinea Pig Ileum Longitudinal Muscle. The terminal portion of the ileum was excised after discarding the 8–10 cm nearest the ileo–caecal junction. The tissue was cleaned, and segments 2–3 cm long of ileum longitudinal muscle were separated from the underlying circular muscle and set up under 1 g of tension at 37 °C in organ baths containing Tyrode solution of the following composition (mM): NaCl, 118; KCl, 4.75; CaCl_2 , 2.54; MgSO_4 , 1.2; $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.19; NaHCO_3 , 25; glucose, 11. Tension changes were recorded isotonicly. Tissues were allowed to equilibrate for at least 30 min during which time the bathing solution was changed every 10 min. Concentration–response curves to APE (0.01–0.5 μM) were obtained at 30 min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 60 min, a new concentration–response curve to the agonist was obtained.

Frog Rectus Abdominis Muscle. The rectus abdominis muscle of frogs was set up at room temperature in Clark frog Ringer solution of the following composition (mM): NaCl, 111; KCl, 1.88; CaCl_2 , 1.08; NaH_2PO_4 , 0.08; NaHCO_3 , 2.38; glucose, 11.1. The tissues were equilibrated under 1 g of tension for 60 min. Two cumulative concentration–response curves to carbachol (1–100 μM) were constructed at 45 min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 30 min a new concentration–response curve to the agonist was obtained.

$^{125}\text{I}_2$ -19** Binding Assays.** nAChR-rich membranes were prepared from frozen *Torpedo californica* electric organ as described earlier.³⁷ Increasing concentrations of $^{125}\text{I}_2$ -**19** (80 000 cpm/nmol) were added to a constant amount of nAChR-rich membranes (0.3 mg/mL protein, diluted in 50 mM NaCl, 0.1% TritonX-100, 10 mM Na_3PO_4 , pH 7.5, total volume per sample, 200 μL) and were incubated for 30 min at room temperature. Nonspecific binding was determined from bound $^{125}\text{I}_2$ -**19** in the presence of a 100-fold molar excess of I_2 -**19**. Incubations were terminated by vacuum filtration through Whatman GF/B filters presoaked in (0.3%) polyethylenimine. The filters were washed three times with 4 mL aliquots of 50 mM NaCl, 0.1% TritonX-100, 10 mM Na_3PO_4 , pH 7.5. [^{125}I] α -BTX binding assays and the calculation of binding sites have been performed as described previously.³⁶

Photo-Cross-Linking Experiments. nAChR-rich membranes (25 μg) were diluted in 0.1 M NaPi, pH 7.4, to a final receptor concentration of 140 nM. The radioactive $^{125}\text{I}_2$ -**19** (10 μM ; 1 Mio cpm/nmol) was mixed with the sample solution, incubated for 30 min at room temperature, and irradiated with UV light of 254 nm (distance, 15 cm; quartz lamp; Desaga, Heidelberg, Germany) at 254 nm for 30 s. Unbound $^{125}\text{I}_2$ -**19** was separated from $^{125}\text{I}_2$ -**19** bound to nAChR-rich membranes by centrifugation (15 000g, 15 min). The pellet was dissolved and separated by SDS–PAGE using a 10% SDS–PAGE.³⁸ The stained gel was dried, and radioactive receptor subunits were visualized by autoradiography.

Fluorescence Titration. All fluorescence spectra were recorded using an Aminco Bowman spectrometer series 2 (Rochester, NY). For fluorescence titration experiments aliquots of competing ligand were added stepwise to a solution containing nAChR-rich membranes (1 μM receptor concentration), ethidium (7 μM), and carbachol (1 mM) in 50 mM NaPi, pH 7.4. Ethidium fluorescence was measured by employing an excitation wavelength of 480 nm (slit widths, 4 nm/4 nm) while monitoring the emission from 540 to 740 nm.

Data Analysis. Antagonism of mAChRs, expressed as pK_B values, was estimated according to the Furchgott equation: $K_B = [\text{antagonist}]/(\text{DR} - 1)$, where DR is the ratio between individual EC_{50} values in the presence and in the absence of antagonists.³² The potency of the agonist, i.e., the concentration

resulting in 50% of the maximum response (EC_{50}), was estimated graphically from the individual concentration–response curves after checking for parallelism of the curves. Antagonism of nAChRs was estimated by determining the concentration of the noncompetitive antagonist, which inhibited 50% of the maximum response to the agonist. Three different antagonist concentrations were used, and each concentration was tested at least four times. Data were analyzed using a pharmacological computer program.³⁹

Dissociation constants (K_{app} values) for nonfluorescent competing ligands were derived from analysis of their capacity to displace the fluorescent ligand, ethidium. For calculations of K_{app} values, fluorescence data were plotted according to a logarithmic formula described by Herz et al.³⁴

Acknowledgment. Grants from the University of Bologna, the European Community (Grant BMH4-CT97-2395), MURST, the Deutsche Forschungsgemeinschaft (Grant DFG, Sfb 449), and the Fonds der Chemischen Industrie supported this research.

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JM011067F