Structure−Activity Relationships of Methoctramine-Related Polyamines as Muscular Nicotinic Receptor Noncompetitive Antagonists. 3.[1] Effect of Inserting the Tetraamine Backbone into a Macrocyclic Structure

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The present article expands on the study of another aspect of structure−activity relationships of the polymethylene tetraamines, namely, the effect of inserting the tetraamine backbone into a macrocyclic structure. To this end, compounds **8**−**12** were designed by linking the two terminal nitrogen atoms of prototype methoctramine **2** to an aryl moiety. Alternatively, **2** was first modified to achieve compounds **6** and **7**, which in turn were cyclized by linking the two terminal primary amine functions to a polyphenyl spacer, affording **13**−**20**. All the compounds were tested on muscle-type nAChRs and most of them as well on AChE. Furthermore, selected compounds were tested also on peripheral M_2 and M_3 mAChRs. All these cyclic derivatives, like prototypes, were potent noncompetitive antagonists at both frog and *Torpedo* nAChRs, suggesting that polyamines do not need to be linear or in extended conformation to optimally interact with the nicotinic channel; rather, they may bind in a U-shaped conformation. Relative to muscarinic activity, macrocyclic compounds **10**, **13**, **14**, and **20**, in contrast with the profile displayed by **2**, were almost devoid of affinity. It is derived that an aryl spacer is detrimental to the interaction of polyamines with mAChRs. Finally, all the diamine diamides investigated in this study were much less potent in inhibiting AChE activity than prototype **3**, suggesting that a macrocyclic structure may not be suitable for AChE inhibition.

Introduction

We have demonstrated that polymethylene tetraamines are a versatile tool for the characterization of different receptor systems.[2−6] For example, benextramine[7] (**1**, Figure 1), the prototype tetraamine disulfide for irreversible antagonism of α_1-adrenoreceptors, was shown to antagonize several receptors, including nicotinic receptors (nAChRs),[8] muscarinic receptors (mAChRs),[2,5] neuropeptide Y receptors,[9] and acetylcholinesterase (AChE).[10] The pharmacological profile of **1** was taken as a starting point to develop the universal template approach to the design of polyamines as selective ligands for different biological targets.[4,6,10] Structural modifications performed on the structure of **1** led to the discovery of methoctramine[11] (**2**, Figure 1), a prototype tetraamine for antagonism of mAChRs, and of caproctamine (**3**, Table 1),[10] a prototype diamine diamide for noncompetitive inhibition of AChE. More recently, we have modified the structure of **2** to produce polyamines that have high affinity and selectivity for muscle-type nAChRs.[8,12] Following these structural modifications, a most intriguing finding was that the affinity of polyamines for muscle-type nAChRs is dependent on the type and length of the spacer between the nitrogen atoms and on the substituents on the terminal amine functions as well. The higher homologue

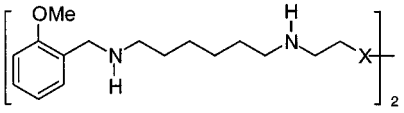

Figure 1. Chemical structure of benextramine (**1**, X = S), methoctramine [**2**, X = $(CH_2)_2$], and homologue **4** [X = $(CH_2)_4$].

4 (Figure 1) of **2** was significantly more potent at the frog rectus nAChR than **2**, while retaining, however, most of the affinity of **2** for M_2 and M_3 mAChRs.[8] The replacement of the flexible 1,12-diaminododecane unit of **4** with a (4″-aminomethyl-[1,1′;4′,1″]terphenyl-4-yl)-methylamine fragment led to **5** (Table 1), which displayed most of the affinity of **4** for nAChRs but lost almost completely the affinity for both M_2 and M_3 mAChRs. Thus, it was demonstrated that flexibility of the spacer between the inner nitrogen atoms of tetraamines is an important determinant of potency with respect to both nAChRs and mAChRs. Consequently, the selectivity for muscle-type nAChRs relative to mAChRs could be achieved by replacing a flexible spacer with a rigid one. Following this observation, we advanced that the inner nitrogen atoms of **5** are unlikely to be less than 15 Å apart because, owing to the rigidity of the terphenyl moiety, the only possibility to alter 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 may be important for the interaction with two anionic sites of the channel

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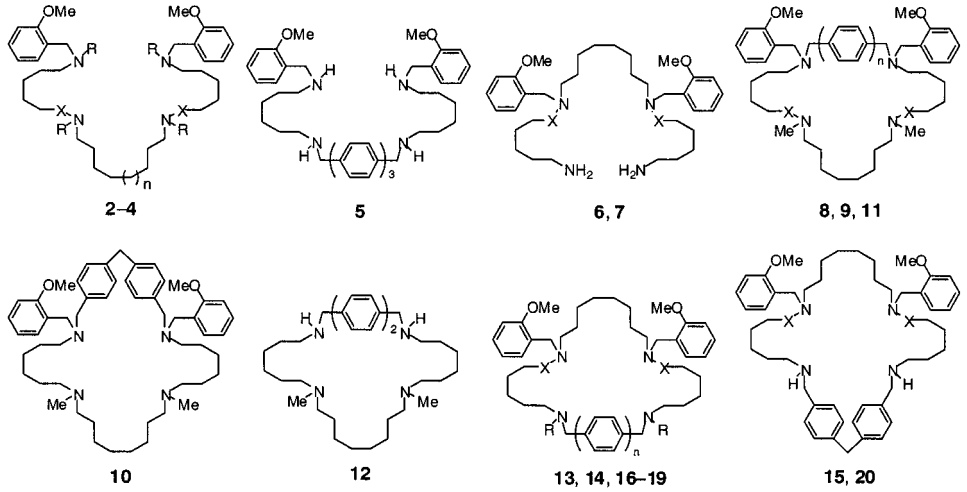
Table 1. Antagonist Affinities, Expressed as pIC_{50}, K_{app}, or pK_B Values, at Nicotinic Acetylcholine Receptors (nAChRs) of the Isolated Frog Rectus Abdominis Muscle (FRA) and *Torpedo* and at Muscarinic Acetylcholine Receptors (mAChRs) of Isolated Guinea Pig Left Atrium (M_2) and Longitudinal Ileum (M_3), Respectively, and Inhibitory Activity, Expressed as pIC_{50} Values, on Acetylcholinesterase (AChE) from Human Erythrocytes of the Compounds Studied



				mAChR		AChE	nAChR	
compd[a]	*n*	X	R	pK_B (M_2)	pK_B (M_3)	pIC_{50}	pIC_{50} (FRA)	pK_{app} (*Torpedo*)
2	1	CH_2	H	7.91 ± 0.03[b]	6.14 ± 0.06[b]	5.27 ± 0.03[c]	5.93 ± 0.03[b]	6.21 ± 0.10[b]
3	1	CO	CH_3	6.39 ± 0.23[c]	5.55 ± 0.12[c]	6.77 ± 0.01[c]		4.99 ± 0.02
4	5	CH_2	H	7.35 ± 0.09[b]	5.98 ± 0.07[b]		7.02 ± 0.04[b]	5.66 ± 0.05[b]
5				<5[d]	<5[d]		6.30 ± 0.02[b]	6.35 ± 0.06[b]
6		CO				4.48 ± 0.01	6.80 ± 0.01	6.46 ± 0.02
7		CH_2					6.48 ± 0.01	6.40 ± 0.01
8	1	CH_2					5.46 ± 0.03	6.43 ± 0.06
9	2	CH_2					6.35 ± 0.01	6.35 ± 0.01
10				<5[d]	<5[d]		6.64 ± 0.01	6.21 ± 0.01
11	2	CO				4.27 ± 0.08	6.28 ± 0.01	5.10 ± 0.01
12							5.37 ± 0.02	<4[e]
13	1	CO	H	<5[d]	<5[d]	5.85 ± 0.03	6.77 ± 0.03	6.47 ± 0.01
14	2	CO	H	<5[d]	<5[d]	5.47 ± 0.04	6.23 ± 0.02	6.64 ± 0.03
15		CO				5.21 ± 0.02	6.62 ± 0.02	6.70 ± 0.01
16	3	CO	H			4.80 ± 0.03		6.03 ± 0.01
17	2	CO	CH_3			3.98 ± 0.04	6.15 ± 0.01	6.01 ± 0.02
18	1	CH_2	H				6.49 ± 0.02	6.01 ± 0.01
19	2	CH_2	H				5.92 ± 0.03	5.48 ± 0.02
20		CH_2		<5	<5		6.72 ± 0.01	6.26 ± 0.01
$TPMP^+$[f]							6.05 ± 0.09[b]	5.70 ± 0.12[b]

[a] **1**, **4**, **5**, **7**, **8**, **12**, **18**−**20**, tetrahydrochlorides; **3**, **11**, **13**−**17**, dihydrochlorides; **6**, dioxalate; **9**, **10**, tetraoxalates. [b] Data from ref 12. [c] Data from ref 10. [d] Not active up to a concentration of 5 μM. [e] Not able to displace bound ethidium up to a concentration of 100 μM. [f] Triphenylmethylphosphonium bromide.

located most likely at a distance comparable with that between the inner nitrogen atoms of **5**.

The present article expands on the study of another aspect of structure−activity relationships of prototype **2**, namely, the effect of inserting the tetraamine backbone into a macrocyclic structure. The rationale for this structural modification stands on the need to gain information on the active conformation of polyamines in the interaction with muscle-type nAChRs. Photolabile compounds have been used to gain insight into the mode of interaction of polymethylene tetraamines with nAChRs. As a most interesting finding, we observed that a radioactive photolabile compound, bearing two identical azido groups on the terminal *N*-aryl-substituted nitrogen atoms, photolabeled nAChR α-subunits, suggesting that each of the two nAChRs α-subunits interacts with one of the two terminal *N*-aryl groups of this compound, resulting in a U-shaped conformation when bound to the lumen of the receptor ion channel.[12] Thus, a cyclic structure should give an answer as to whether a tetraamine interacts with the receptor in an extended or in a folded (U-shaped) conformation. To this end, we designed macrocyclic polyamines **8**−**20**. Compounds **8**−**12** were obtained by linking the two terminal nitrogen atoms of **2** to an aryl moiety. Alternatively, to link to each other the two terminal nitrogen atoms to an octamethylene spacer and the two inner nitrogen atoms to an aryl moiety, prototype **2** was first modified to achieve compounds **6** and **7**, which in turn were cyclized by linking the two terminal primary amine functions to an aryl spacer, affording **13**−**20**. The design strategy for our compounds is shown in Figure 2.

All of the compounds synthesized in this study were tested on muscle-type nAChRs, and most of them were tested on AChE as well. Furthermore, selected compounds were tested also on peripheral M_2 and M_3 mAChRs.

Chemistry

All the newly synthesized compounds were characterized by IR, ^1H NMR, mass spectra, and elemental analysis.

The synthesis of diamine diamide **6** was accomplished using our previously established procedures, as shown

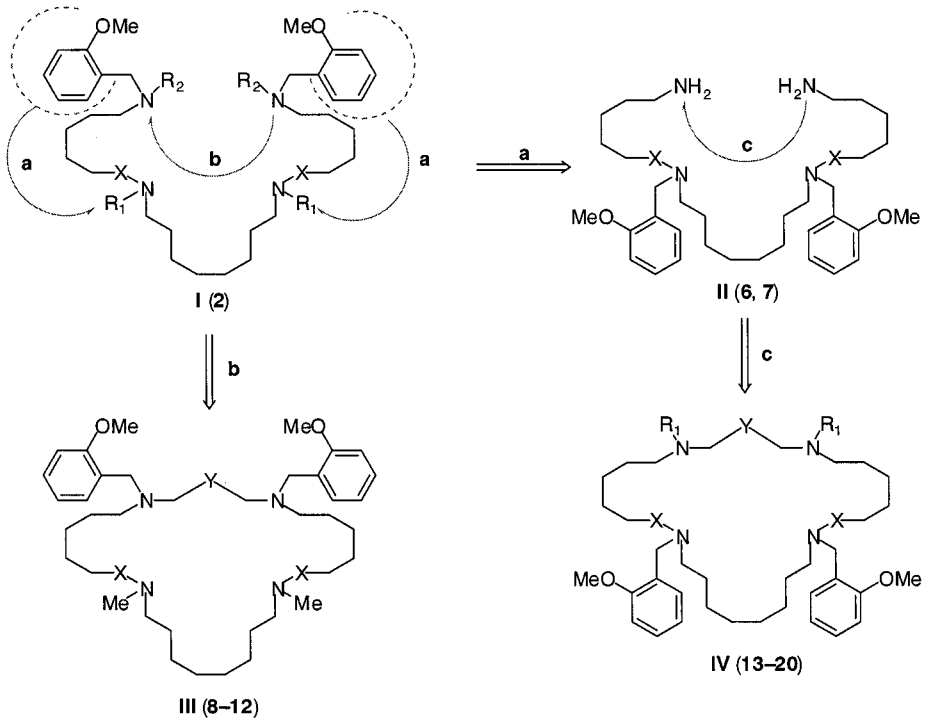
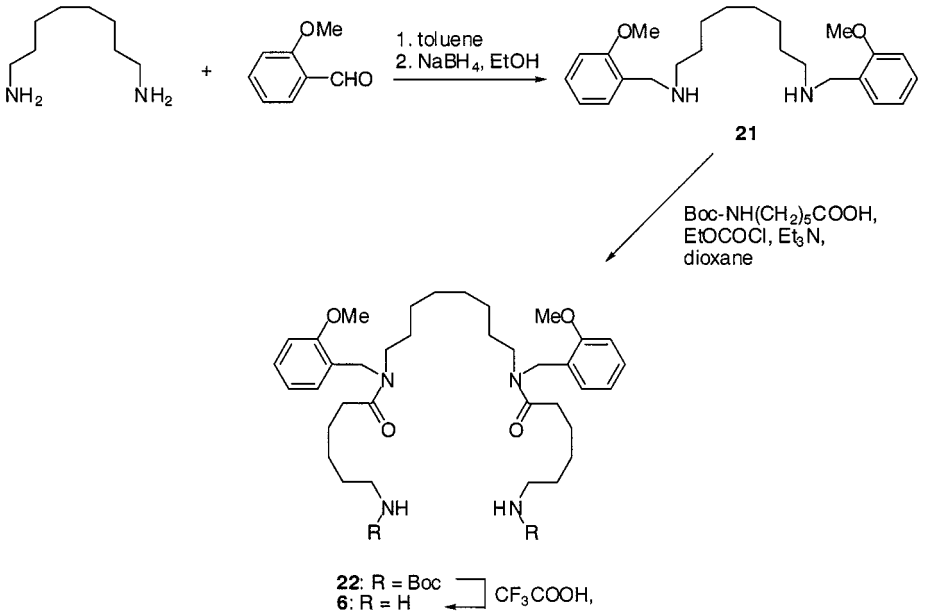


Figure 2. Design strategy for the synthesis of macrocyclic polyamines of the present investigation. First, methoctramine (**2**, structure I, $R_1 = R_2 = H$, $X = CH_2$) has been modified to achieve structure **II** by moving the two methoxybenzyl groups from the terminal to the inner nitrogen atoms (a). Second, the outer nitrogen atoms of structures **I** and **II** have been linked by a suitable spacer to give structures **III** (b) and **IV** (c), respectively.

Scheme 1[a]



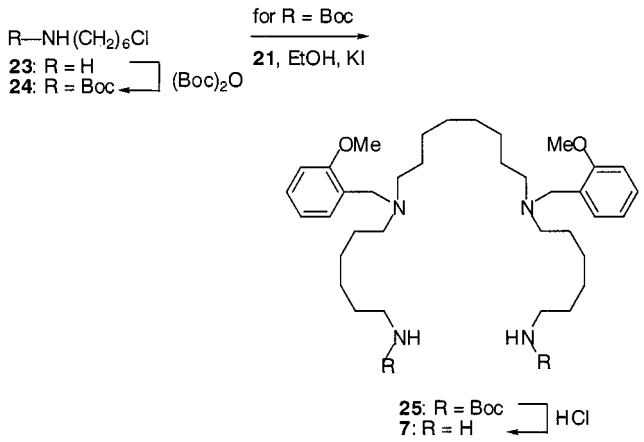
[a] Boc = Me_3COCO-.

in Scheme 1. Thus, *N*-(*tert*-butoxycarbonyl)-6-aminocaproic acid was amidated with diamine **21**, synthesized in turn from 1,8-diaminooctane and 2-methoxybenzaldehyde to give **22**. Removal of the *N*-*tert*-butoxycarbonyl groups of **22** was achieved by acidic hydrolysis in CF_3-COOH.

Reduction of the carbonyl functions of diamine diamide **6** to obtain tetraamine **7** was next attempted. Unfortunately, this transformation, using different reducing agents, led to a complex mixture and to significant amounts of decomposition products. Thus, another synthetic pathway to achieve **7** was then followed (Scheme 2). Alkylation of **21** with **24**, obtained in two steps starting from 6-amino-1-hexanol, afforded **25**. Removal of the protecting groups of **25** by hydrolysis with 6 N HCl gave **7**.

All the polyazamacrocycles listed in Table 1 were obtained in good yields (37−65%), following a one-step synthetic method that includes a first [1 + 1] condensation of the terminal primary amino groups of polyamines with the corresponding dialdehydes, followed by hydrogenation of the intermediate Schiff bases with $NaBH_4$.

Scheme 2[a]



[a] Boc = Me_3COCO-.

Dropwise slow addition of 1 equiv of dialdehydes **28**−**30**[13,14] to a dilute solution containing 1 equiv of polyamine **27**[15] in the presence of $NaBH_4$ afforded intermediate macrocycles **31**, **12**, and **32**, which were subsequently converted to the desired macrocycles **8**−**10** through reductive amination of the secondary amine functions with 2-methoxybenzaldehyde (Scheme 3).

Similarly, cyclization of diamine diamide **26**[15] with **29** provided the required precursor **33**, which was alkylated with 2-methoxybenzyl chloride[16] to give the macrocycle **11**, as illustrated in Scheme 4.

Under the same cyclization conditions, reaction of polyamines **6** and **7** with the different dialdehydes **28**−**30** or **34**[17] provided the final compounds **13**−**16** (Scheme 5) and **18**−**20** (Scheme 6). Alkylation of **14** with formaldehyde and formic acid afforded the *N*,*N*′-dimethyl macrocycle **17** (Scheme 5). While compounds **28**−**30** are soluble in EtOH, *p*-terphenyl-4,4″-dicarboxyaldehyde **34** is highly insoluble, and the use of a different solvent rather than an alcohol then became necessary, affecting the outcome of cyclization, as indicated by the low yield of **16** (22%), which was synthesized using $CHCl_3$ as cosolvent.

Biology

The effects of compounds **10**, **13**, **14**, and **20** on M_2 mAChRs were determined using guinea pig left atria electrically stimulated at 1 Hz.[18,19] The guinea pig ileum longitudinal muscle was used to study their effects on M_3 mAChRs.[18,19] 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).[20]

The inhibitory activity against AChE of compounds **6**, **11**, and **13**−**17** was studied using the method of Ellman et al.[21] The inhibitory activity was expressed as pIC_{50} values that represent the concentration of inhibitor required to decrease enzyme activity by 50%.

The effects of compounds **6**−**15** and **17**−**20** on muscle-type nAChRs were studied using the frog rectus abdominis muscle and carbachol-induced contractions as the measured parameter.[22] 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 **6**−**20** at the noncompetitive binding site of *Torpedo* nAChRs were determined by using the fluorescent noncompetitive inhibitor ethidium in a displacement assay.[23]

Methoctramine (**2**), caproctamine (**3**), analogues **4** and **5**, and triphenylmethylphosphonium bromide ($TPMP^+$) were used as controls, and their pIC_{50}, pK_{app}, and pK_B values were within the error of previous determinations.

Results and Discussion

The newly synthesized compounds **6**−**20** were assayed on nAChRs of isolated frog rectus abdominis muscle and *Torpedo*. Furthermore, selected compounds were evaluated also for their muscarinic M_2 and M_3 antagonist potency (**10**, **13**, **14**, **20**) and for their inhibition of AChE from human erythrocytes (**6**, **11**, **13**−**17**). Polyamines **2**−**5** and $TPMP^+$ were used as standards for a comparison. The results are summarized in Table 1.

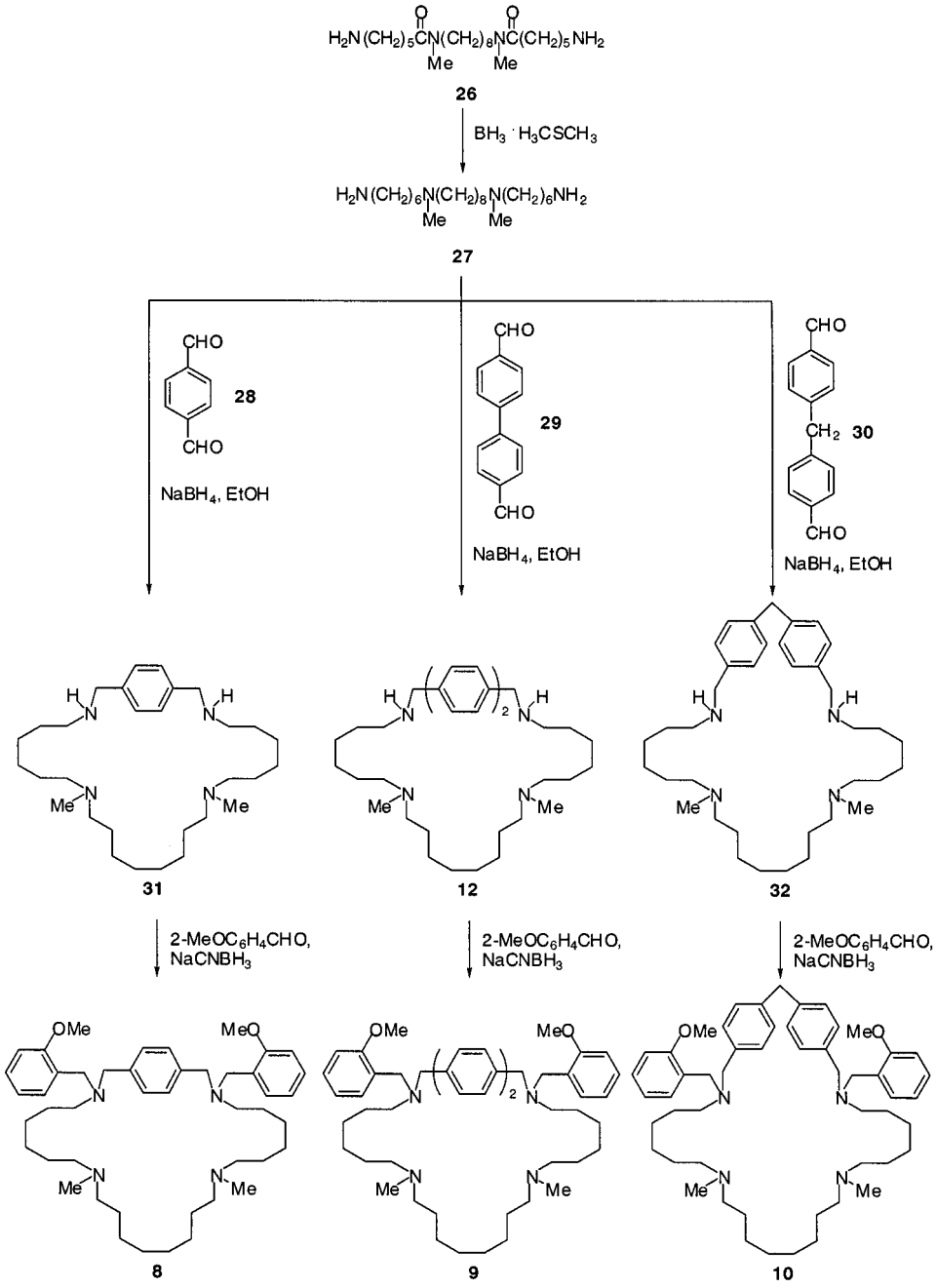
It was observed that over the concentration range investigated all of the compounds were noncompetitive antagonists of muscle-type nAChRs. The maximum response to carbachol was reduced, and the magnitude of this reduction was dependent on the concentration of antagonist. In all cases, washing the muscle in drug-free saline reversed the antagonism. To determine the binding affinities of the various polyamine derivatives at nAChRs, we have used the well-characterized luminal noncompetitive inhibitor ethidium in a fluorescent displacement assay.[23] Ethidium, when bound to the high-affinity site for noncompetitive antagonists of the nAChR in its desensitized state, shows an intensive emission maximum at 590 nm upon excitation at 480 nm.[23,24] Since bound ethidium is displaceable by well-characterized luminal noncompetitive antagonists, ethidium can be used as a reference fluorophor to characterize new ligands of this binding site. It turned out that all of the polyamines investigated in this study, with the exception of compound **12**, compete with bound ethidium, indicating that these compounds also overlap with the high-affinity binding site for noncompetitive antagonists. This observation is in agreement with the noncompetitive mechanism of action observed at the frog rectus muscle nAChR.

Interestingly, analysis of the results reported in Table 1 reveals that the pIC_{50} values obtained at frog rectus nAChRs are comparable with pK_{app} values calculated at *Torpedo* nAChRs because the difference was within ±0.5 log units with the exception of **4**, **8**, **11**, and **12**, whose pIC_{50} and pK_{app} values differed to a larger extent. However, the observed discrepancy between pIC_{50} and pK_{app} values is not surprising because it was already noticed with noncyclic polyamines. We argued that the different potencies displayed by some compounds at the frog rectus relative to the *Torpedo* nAChR might be the result of subtle differences in the mode of interaction of polyamines with the two receptors, owing to structural differences in their ion channel, or simply of a different bioavailability of the compounds at the receptor site.[12]

Taking **2** and **3** as reference compounds, it can be observed how their potency at nAChRs, mAChRs, and AChE can be modified by introducing modifications into their structure.

Moving the two 2-methoxybenzyl groups from the two terminal nitrogen atoms to the inner nitrogen atoms of

Scheme 3

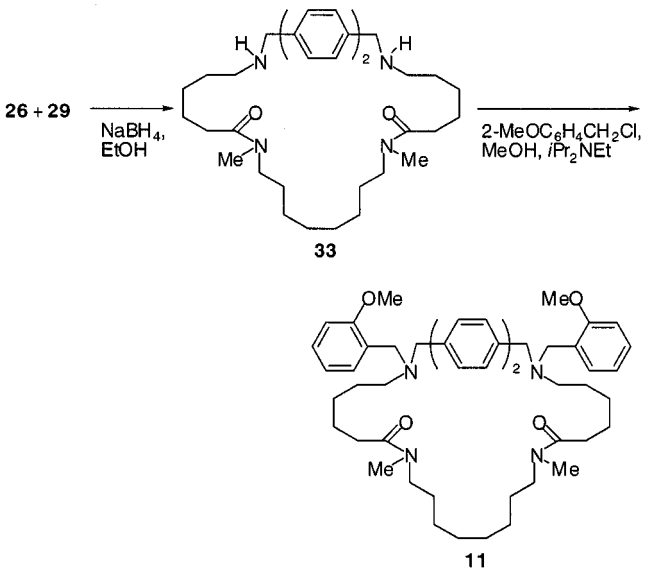


2 afforded polyamines **6** and **7**. These compounds were more potent than prototype **2** at both frog and *Torpedo* nAChRs while being equipotent to each other at *Torpedo* nAChRs, and diamine diamide **6** was more potent than the corresponding tetraamine **7** at frog nAChRs.

Cyclization of **6** and **7** by linking together the two terminal amine functions to an aryl spacer gave **13**−**20**. All these cyclic compounds were potent noncompetitive antagonists at both frog and *Torpedo* nAChRs, suggesting that polyamines do not need to be linear or in extended conformation to optimally interact with the nAChR; rather, they bind in a U-shaped conformation as hypothesized earlier.[12] Analysis of the results reveals that diamine diamides **13**−**15** were more potent than or as active as the corresponding tetraamines **18**−**20** at nAChRs, in line with the results observed for diamine diamide **6** and the corresponding tetraamine **7**. Interestingly, these cyclic compounds displayed an affinity for nAChRs that was comparable to that of open prototypes **6** and **7**. N-Methylation of the two amide functions did not improve the affinity for nAChRs because **14** was more potent than the corresponding *N*,*N*′-dimethyl analogue **17** at both frog and *Torpedo* nAChRs. A di-*p*-tolylmethane spacer, as in **15** and **20**, appears to confer optimum interaction with the nAChR, while a longer spacer, as in **16**, caused a significant decrease in affinity at *Torpedo* nAChRs.

Cyclization of the *N*,*N*′-dimethyl analogue of prototype **2** or of prototype **3** by linking the two terminal amine functions to an aryl spacer gave macrocyclic polyamines **8**−**11**. Again, these compounds displayed affinity for nAChRs that was higher or comparable to that exhibited by prototypes **2** and **3**. However, in this case, diamine diamide **11** was not more potent than the corresponding tetraamine **9**. This finding suggests that the two sets of compounds, that is, **8**−**11** and **13**−**20**,

Scheme 4



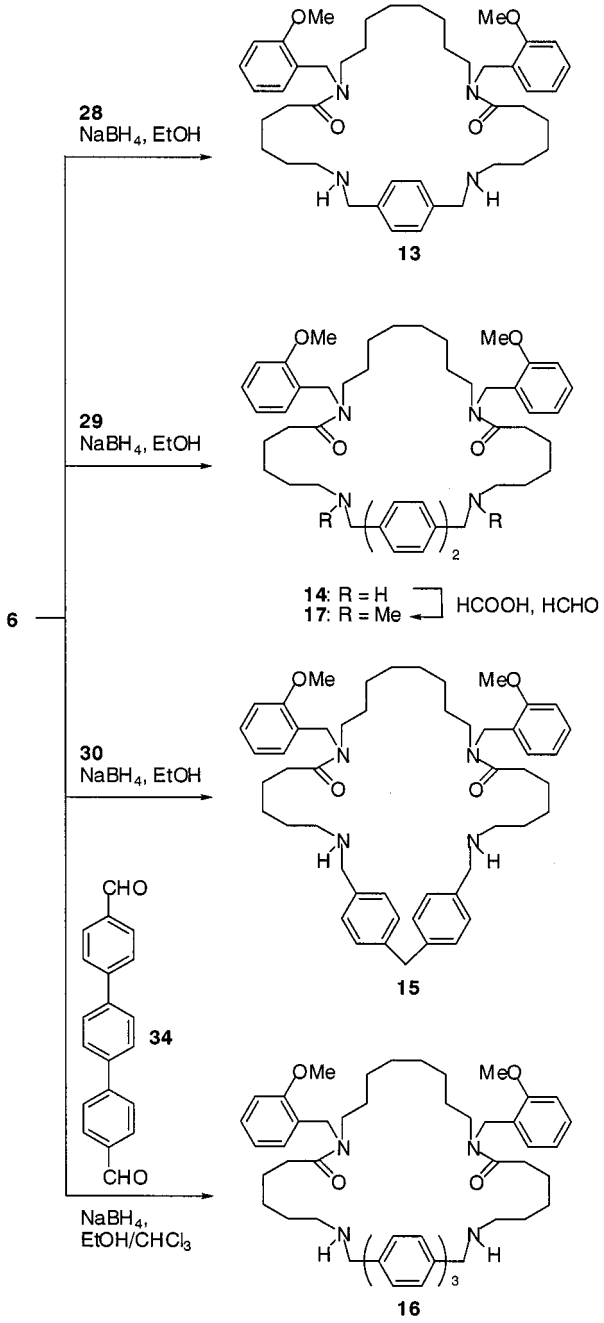
may interact differently with the nAChR. Compound **10**, bearing a di-*p*-tolylmethane spacer, was more potent than both **8** and **9** at frog nAChRs, in line with the results observed for **15** and **20** relative to **13**, **14**, **18**, and **19**. However, **10** was slightly less potent than **8** and **9** at *Torpedo* nAChR. The markedly lower activity of **12** relative to the corresponding 2-methoxybenzyl-bearing analogue **9** suggests clearly that the 2-methoxybenzyl group had a relevant role in receptor binding mechanism.

Prototype **2** was a potent and selective M_2 mAChR antagonist relative to M_3 mAChRs, muscle-type nAChRs, and AChE. In contrast, the higher homologue **4** had high affinity for frog nAChRs that was only slightly lower than the affinity for M_2 mAChRs, whereas analogue **5** displayed significant antagonism at nAChRs while being almost devoid of affinity, at least up to 5 mM concentration, for M_2 and M_3 mAChRs. It is derived that an aryl spacer is detrimental to the interaction of polyamines with mAChRs. This observation was confirmed by the results obtained with cyclic compounds **10**, **13**, **14**, and **20** that were, like **5**, devoid of affinity, up to 5 μM concentration, for M_2 and M_3 mAChRs.

Prototype **3**, which bears two amide functions, displayed higher affinity for AChE relative to mAChRs and *Torpedo* nAChRs. For this reason, we tested at AChE all diamine diamides synthesized in the present study. It turned out that all diamine diamides investigated, unlike **3**, were much less potent in inhibiting AChE activity than muscle-type nAChRs, suggesting that a cyclic structure may not be suitable for AChE inhibition.

In conclusion, we have demonstrated that inserting the tetraamine or the diamine diamide backbone of **2** and **3**, respectively, into a cyclic structure did not negatively affect affinity for muscle-type nAChRs because the newly synthesized polyamines were more potent than or as active as the prototypes, confirming our previous observation that a tetraamine is unlikely to interact with the receptor in an extended conformation. It is derived that the active conformation of polyamines may be a folded one.

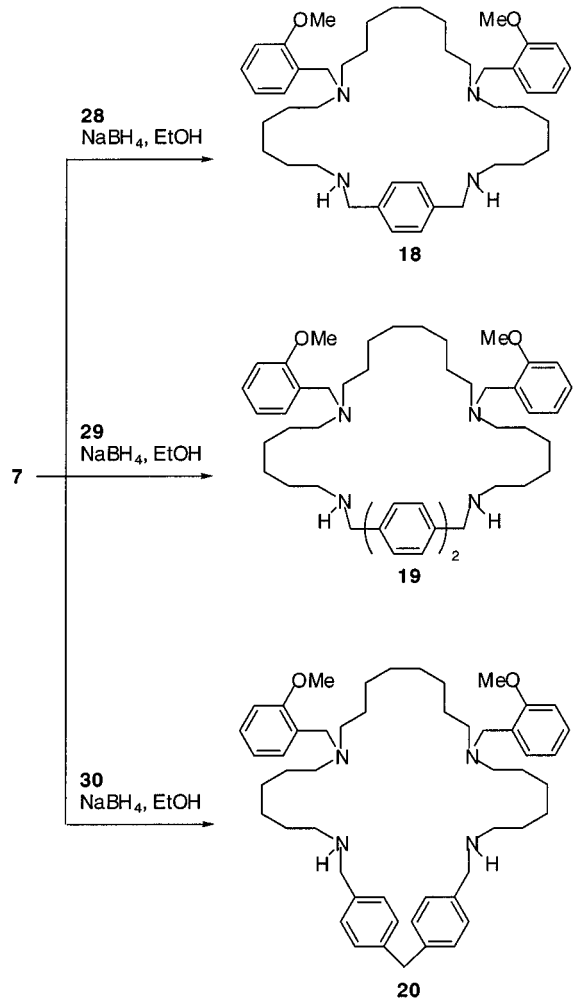
Scheme 5



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 ^{1}H 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), or m (multiplet). Although the 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 ±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) or gravity column (Kieselgel 60, 0.063−0.200 mm; Merck) chromatography. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version

Scheme 6



2.1), a PC integrated software package for systematic names in organic chemistry with the exception of compound **16**, which was named by applying ACD/NAME (Advanced Chemistry Development, Inc.).

***N,N′*-Bis(2-methoxybenzyl)octane-1,8-diamine Dihydrochloride (21).** A solution of 1,8-diaminooctane (3.6 g, 25.0 mmol) and 2-methoxybenzaldehyde (7.49 g, 55.0 mmol) in toluene (100 mL) was refluxed, and the water formed was continuously removed for 3 h. The cooled mixture was filtered and the filtrate evaporated to give the corresponding Schiff base that was dissolved in EtOH (100 mL) and treated with $NaBH_4$ (2.08 g, 55.0 mmol). The mixture was stirred at room temperature for 12 h, cautiously acidified with 6 N HCl, made basic with 2 N NaOH, and finally extracted with chloroform (3 × 50 mL). Removal of dried (Na_2SO_4) solvents afforded, in quantitative yield, **21** as the free base that was converted into the dihydrochloride salt: mp 200−205 °C (from EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.28−1.49 (m, 12), 1.75 (br s, 2, exchangeable with D_2O), 2.58 (t, 4), 3.78 (s, 4), 3.84 (s, 6), 6.84−6.95 (m, 4), 7.20−7.26 (m, 4).

{5-[{8-[(6-*tert*-Butoxycarbonylaminohexanoyl)(2-methoxybenzyl)amino]octyl}(2-methoxybenzyl)carbamoyl]pentyl}carbamic Acid *tert*-Butyl Ester (22). Ethyl chlorocarbonate (2.63 mL, 27.5 mmol) in dry dioxane (5 mL) was added dropwise to a stirred and cooled (5 °C) solution of *N*-(*tert*-butoxycarbonyl)-6-aminocaproic acid (6.35 g, 27.5 mmol) and triethylamine (3.83 mL, 27.5 mmol) in dioxane (120 mL), followed after standing for 30 min by the addition of **21** (5.28 g, 13.7 mmol) in dioxane (30 mL). After being stirred at room temperature overnight, the mixture was evaporated, affording a residue that was purified by gravity chromatography. Eluting with petroleum ether/ethyl acetate/ethanol (5:5:0.1) afforded **22**: 55% yield; ^{1}H NMR ($CDCl_3$) δ 1.19−1.49 (m, 42), 1.60−1.83 (m, 4), 3.01−3.32 (m, 8), 3.79−3.82 (m, 6), 4.44−4.59 (m, 4), 4.70 (br s, 2, exchangeable with D_2O), 6.79−6.99 (m, 6), 7.09−7.28 (m, 2).

6-Aminohexanoic Acid{8-[(6-aminohexanoyl)(2-methoxybenzyl)amino]octyl}(2-methoxybenzyl)amide Dioxalate (6). A solution of **22** (5.73 g, 7.03 mmol) in TFA (40 mL) was stirred at room temperature for 12 h. The solvent was evaporated, yielding a solid that was dissolved in water (100 mL). The obtained solution was then washed with ether (3 × 30 mL), made basic with NaOH pellets, and extracted with $CHCl_3$ (3 × 30 mL). The organic phase was evaporated to give **6** (95% yield) as the free base that was transformed into the dioxalate salt as a white solid: mp 54−56 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.25−1.78 (m, 24 + 4 exchangeable with D_2O), 2.25−2.39 (m, 4), 2.64−2.75 (m, 4), 3.28−3.36 (m, 4), 3.83 (s, 6), 4.47−4.61 (m, 4), 6.83−7.28 (m, 8). Anal. ($C_{40}H_{62}N_4O_{12}$) C, H, N.

6-Chlorohexylamine Hydrochloride (23). A solution of 6-amino-1-hexanol (15.5 g, 0.13 mol) and $SOCl_2$ (43 mL, 0.59 mol) in toluene (100 mL) was refluxed for 1 h. Removal of the solvent under reduced pressure afforded crude **23** in quantitative yield as a hygroscopic solid: ^{1}H NMR ($CDCl_3$) δ 1.44−1.54 (m, 4), 1.74−1.83 (m, 4), 3.01−3.04 (m, 2), 3.55 (t, 2), 8.31 (br s, 3, exchangeable with D_2O).

(6-Chlorohexyl)carbamic Acid *tert*-Butyl Ester (24). To a solution of **23** (10.3 g, 59.85 mmol) in 30 mL of water was added Et_3N (6.6 mL, 89.8 mmol), and the resulting mixture was stirred at room temperature for 15 min. After dilution with THF (60 mL) di-*tert*-butyl dicarbonate (14.54 g, 66.6 mmol) was added portionwise and the reaction mixture was stirred for further 12 h. Separation between the two phases is favored by addition of EtOAc (100 mL) and water (50 mL). The dried organic phase is evaporated to dryness to give a residue that was purified by gravity chromatography. Eluting with petroleum ether/ethyl acetate (8.25:1.75) afforded **24**: 57% yield; ^{1}H NMR ($CDCl_3$) δ 1.31−1.54 (m, 15), 1.75−1.83 (m, 2), 3.12−3.14 (m, 2), 3.55 (t, 2), 4.52 (br s, 1, exchangeable with D_2O).

{6-[{8-[(6-*tert*-Butoxycarbonylaminohexyl)(2-methoxybenzyl)amino]octyl}(2-methoxybenzyl)amino]hexyl}carbamic Acid *tert*-Butyl Ester (25). A mixture of **24** (4.9 g, 20.8 mmol), **21** (2 g, 5.2 mmol), K_2CO_3 (2.87 g, 20.8 mmol), and KI (1.41 g, 8.5 mmol) in absolute ethanol (100 mL) was refluxed for 78 h. Removal of the solvent gave a residue that was purified by flash chromatography. Eluting with chloroform/petroleum ether/ethanol/aqueous 30% ammonia (6:3.4:0.6:0.01) afforded **25** as transparent oil: 13% yield; ^{1}H NMR ($CDCl_3$) δ 1.23−1.50 (m, 46), 2.46 (t, 8), 3.05−3.10 (m, 4), 3.64 (s, 4), 3.82 (s, 6), 4.53 (br s, 2, exchangeable with D_2O), 6.82−6.98 (m, 4), 7.23 (t, 2), 7.42 (d, 2).

***N,N*-Bis(6-aminohexyl)-*N,N*-bis(2-methoxybenzyl)octane-1,8-diamine Tetrahydrochloride (7).** A solution of **25** (200 mg, 0.26 mmol) in 6 N HCl (20 mL) was stirred at room temperature for 2 h. The reaction mixture was then washed with ether (3 × 20 mL), made basic with NaOH pellets, and extracted with chloroform (3 × 20 mL). The organic phase was evaporated to give **7** (90% yield) as the free base that was transformed into the tetrahydrochloride salt as a hygroscopic solid: ^{1}H NMR (free base, $CDCl_3$) δ 1.26−1.70 (m, 28 + 4 exchangeable with D_2O), 2.44 (t, 8), 2.70 (t, 4), 3.59 (s, 4), 3.83 (s, 6), 6.83−6.98 (m, 4), 7.18−7.22 (m, 2), 7.41−7.45 (m, 2). Anal. ($C_{36}H_{66}Cl_4N_4O_2$) C, H, N.

10,19-Dimethyl-3,10,19,26-tetraazabicyclo[26.2.2]dotriaconta-1(31),28(32),29-triene (31). A solution of **28** (45.6 mg, 0.34 mmol) in 100 mL of dry ethanol was added dropwise to a mixture of **27**[15] (130 mg, 0.34 mmol) and molecular sieves (3 Å) over a period of 72 h at room temperature. $NaBH_4$ (29 mg, 0.70 mmol) was then added, and the stirring was continued for further 12 h. Following removal of molecular sieves, the solution was made acidic with 6 N HCl (2 mL). Removal of the solvent gave a residue, which was dissolved in water (40 mL). The solution was washed with ether (3 × 20 mL) to remove nonbasic materials and then was made basic with 5 N NaOH and finally extracted with

chloroform (3 × 20 mL). Removal of the solvent gave a residue that was purified by chromatography. Eluting with methylene chloride/methanol/aqueous 30% ammonia (9:1:0.1) afforded **31**: 60% yield; ^{1}H NMR ($CDCl_3$) δ 1.09−1.66 (m, 28 + 2 exchangeable with D_2O), 2.32 (s, 6), 2.41−2.48 (m, 8), 2.64 (t, 4), 3.82 (s, 4), 7.33 (s, 4).

3,26-Bis(2-methoxybenzyl)-10,19-dimethyl-3,10,19,26-tetraazabicyclo[26.2.2]dotriaconta-1(31),28(32)-triene Tetrahydrochloride (8). A mixture of **31** (130 mg, 0.27 mmol), $NaCNBH_3$ (34 mg, 0.54 mmol), and CH_3COOH (15.4 mL) in absolute ethanol (10 mL) was stirred for 10 min at room temperature, and then 2-methoxybenzaldehyde (150 mg, 1.1 mmol) was added and the stirring was continued overnight. Removal of the solvent gave a residue that was purified by flash chromatography. Eluting with methylene chloride/ethanol/aqueous 30% ammonia (9.5:0.5:0.1) afforded **8** (31% yield) as the free base that was transformed into the tetrahydrochloride salt: mp 74−76 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.28−1.51 (m, 28), 2.22 (s, 6), 2.26−2.47 (m, 12), 3.57 (s, 4), 3.62 (s, 4), 3.81 (s, 6), 6.83−6.99 (m, 4), 7.16−7.32 (m, 6), 7.54−7.57 (m, 2). Anal. ($C_{46}H_{76}Cl_4N_4O_2$) C, H, N.

14,23-Dimethyl-7,14,23,30-tetraazatricyclo[30.2.2.2^{0,0}]-octatriaconta-1(35),2,4,32(36),33,37-hexaene Tetrahydrochloride (12). 12 was obtained from **27**[15] (130 mg, 0.34 mmol) and **29**[13] (71.5 mg, 0.34 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/ethanol/aqueous 30% ammonia (9:1:0.1) gave **12** as the free base that was converted into the tetrahydrochloride salt: 64% yield; mp 212−215 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.28−1.50 (m, 28), 1.91 (br s, 2, exchangeable with D_2O), 2.21−2.33 (m, 14), 2.58 (t, 4), 3.87 (s, 4), 7.39 (d, 4), 7.60 (d, 4). Anal. ($C_{36}H_{64}Cl_4N_4$) C, H, N.

7,30-Bis(2-methoxybenzyl)-14,23-dimethyl-7,14,23,30-tetraazatricyclo[30.2.2.2^{0,0}]octatriaconta-1(35),2,4,32(36),-33,37-hexaene Tetraoxalate (9). **9** was obtained from **12** (90 mg, 0.16 mmol) and 2-methoxybenzaldehyde (98 mg, 0.72 mmol) following the procedure described for **8** and purified by flash chromatography. Eluting with methylene chloride/ethanol/aqueous 30% ammonia (9:1:0.1) gave **9** as the free base that was converted into the tetraoxalate salt: 48% yield; mp 105 °C; ^{1}H NMR (free base, $CDCl_3$) δ 1.16−1.50 (m, 28), 2.16−2.33 (m, 14), 2.43 (t, 4), 3.55 (s, 4), 3.68 (s, 4), 3.82 (s, 6), 6.83−6.87 (m. 2), 6.96 (t, 2), 7.21 (t, 2), 7.40−7.59 (m, 10). Anal. ($C_{60}H_{84}N_4O_{18}$) C, H, N.

15,24-Dimethyl-8,15,24,31-tetraazatricyclo[31.2.2.2^{0,0}]-nonatriaconta-1(36),3,5,33(37),34,38-hexaene (32). **32** was obtained from **27**[15] (130 mg, 0.34 mmol) and **30**[14](76.3 mg, 0.34 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/methanol/aqueous 30% ammonia (9:1:0.1) gave **32** as the free base: 57% yield; ^{1}H NMR ($CDCl_3$) δ 1.24−1.47 (m, 28), 2.01 (br s, 2, exchangeable with D_2O), 2.23 (s, 6), 2.32−2.38 (m, 8), 2.59 (t, 4), 3.75 (s, 4), 3.93 (s, 2), 7.11−7.14 (m, 4), 7.22−7.25 (m, 4).

8,31-Bis(2-methoxybenzyl)-15,24-dimethyl-8,15,24,31-tetraazatricyclo[31.2.2.2^{0,0}]nonatriaconta-1(36),3,5,33-(37),34,38-hexaene Tetraoxalate (10). **10** was obtained from **32** (110 mg, 0.20 mmol) and 2-methoxybenzaldehyde (106 mg, 0.78 mmol) following the procedure described for **8** and purified by flash chromatography. Eluting with methylene chloride/ethanol/aqueous 30% ammonia (9.4:0.6:0.05) gave **10** as the free base that was converted into the tetraoxalate salt: 50% yield; mp 110 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.29−1.93 (m, 28), 2.23 (s, 6), 2.26−2.47 (m, 12), 3.59 (s, 4), 3.63 (s, 4), 3.82 (s, 6), 3.96 (s, 2), 6.84−7.00 (m, 4), 7.12−7.33 (m, 10), 7.53−7.57 (m, 2). Anal. ($C_{61}H_{86}N_4O_{18}$) C, H, N.

14,23-Dimethyl-7,14,23,30-tetrazatricyclo[30.2.2.2^{0,0}]-octatriaconta-1(35),2,4,32(36),33,37-hexaene-13,24-dione (33). 33 was obtained from **26**[15] (140 mg, 0.35 mmol) and **29**[13] (72 mg, 0.34 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.1) gave **33**: 55% yield; ^{1}H NMR ($CDCl_3$) δ 1.20−1.55 (m, 24), 1.75 (br s, 2, exchangeable with D_2O), 2.17−2.23 (m, 4), 2.50−2.57 (m, 4), 2.86 (s, 6), 3.04−3.16 (m, 4), 3.82−3.83 (m, 4), 7.33−7.38 (m, 4), 7.52−7.57 (m, 4).

7,30-Bis(2-methoxybenzyl)-14,23-dimethyl-7,14,23,30-tetraazatricyclo[30.2.2.2^{0,0}]octatriaconta-1(35),2(37),3,5-(38),32(36),33-hexaene-13,24-dione Dihydrochloride (11). A mixture of **30** (110 mg, 0.19 mmol), 2-methoxybenzyl chloride[16] (119.5 mg, 0.76 mmol), and *N*,*N*-diisopropylethylamine (0.13 mL, 0.76 mmol) in MeOH (5 mL) was refluxed for 24 h and then stirred at room temperature for further 48 h. Removal of the solvent gave a residue that was purified by chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (4.5:5:0.5:0.05) afforded **11** (41% yield) as the free base that was transformed into the dihydrochloride: mp 200−205 °C (EtOH/ether); ^{1}H NMR (free base, $CDCl_3$) δ 1.20−1.56 (m, 24), 2.15−2.27 (m, 4), 2.30−2.45 (m, 4), 2.82−2.86 (m, 6), 3.14−3.29 (m, 4), 3.55−3.57 (m, 4), 3.66−3.68 (m, 4), 3.80 (s, 6), 6.83−7.58 (m, 16). Anal. ($C_{52}H_{74}Cl_2N_4O_4$) C, H, N.

10,19-Bis-(2-methoxybenzyl)-3,10,19,26-tetraazabicyclo-[26.2.2]dotriaconta-1(31),28(32),29-triene-9,20-dione Dihydrochloride (13). **13** was obtained from **6** (480 mg, 0.88 mmol) and **28** (118 mg, 0.88 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.1) gave **13** as the free base that was converted into the dihydrochloride salt: 38% yield; mp 185−187 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$,) δ 1.23−1.45 (m, 24 + 2 exchangeable D_2O), 2.36−2.40 (m, 8), 2.57 (t, 4), 3.55 (t, 4), 3.77 (s, 4), 3.79 (s, 6), 6.81−6.93 (m, 4), 7.16−7.27 (m, 6), 7.39−7.41 (m, 2). MALDI-MS calcd for $C_{44}H_{65}N_4O_4$ 713.49 $(M + H)^+$, found 713.51. Anal. ($C_{44}H_{66}Cl_2N_4O_4$) C, H, N.

14,23-Bis(2-methoxybenzyl)-7,14,23,30-tetraazatricyclo-[30.2.2.2^{0,0}]octatriaconta-1(35),2,4,32(36),33,37-hexaene-13,24-dione Dihydrochloride (14). **14** was obtained from **6** (210 mg, 0.34 mmol) and **29**[13] (71.5 mg, 0.34 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.1) gave **14** as the free base that was converted into the dihydrochloride salt: 56% yield; mp 280−285 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.04−1.57 (m, 24 + 2 exchangeable D_2O), 2.24−2.31 (m, 4), 2.39−2.69 (m, 4), 3.22−3.30 (m, 4), 3.79−3.84 (m, 10), 4.43−4.57 (m, 4), 6.83−7.56 (m, 16). MALDI-MS calcd for $C_{50}H_{69}N_4O_4$ 789.52 $(M + H)^+$, found 789.62. Anal. ($C_{50}H_{70}Cl_2N_4O_4$) C, H, N.

15,24-Bis(2-methoxybenzyl)-8,15,24,31-tetraazatricyclo-[31.2.2.2^{0,0}]nonatriaconta-1(36),3,5,33(37),34,38-hexaene-14,25-dione Dihydrochloride (15). 15 was obtained from **6** (230 mg, 0.375 mmol) and **30**[14] (84 mg, 0.375 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.08) gave **15** as the free base that was converted into the dihydrochloride salt: 49% yield; mp 121 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.23−1.58 (m, 24), 2.10 (br s, 2, exchangeable with D_2O), 2.22−2.40 (m, 4), 2.55−2.68 (m, 4), 3.21 (t, 2), 3.32 (t, 2), 3.76−3.82 (m, 10), 3.95 (s, 2), 4.45 (s, 2), 4.59 (s, 2), 6.84−7.27 (m, 16). MALDI-MS calcd for $C_{51}H_{71}N_4O_4$ 803.54 $(M + H)^+$, found 803.59. Anal. ($C_{51}H_{72}Cl_2N_4O_4$) C, H, N.

18,27-Di(2-metossifenil)-11,18,27,34-tetraazatetracyclo-[34.2.2.2^{2,5}.2^{6,9}]tetratetraconta-1(38),2,4,6,8,36,39,41,43-nonaene-17,28-dione Dihydrochloride (16). **16** was obtained from **6** (171 mg, 0.28 mmol) and **34**[17] (80 mg, 0.28 mmol) following the procedure described for **31** and purified by flash chromatography. In this case, the aldehyde was dissolved in 150 mL of $CHCl_3$. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.05) gave **16** as the free base that was converted into the dihydrochloride salt: 22% yield; mp 185−187 °C (EtOH/Et_2O); ^{1}H NMR ($CDCl_3$, free base) δ 1.26−1.46 (m, 24), 2.01 (br s, 2, exchangeable with D_2O), 2.29−2.33 (m, 4), 2.49−2.63 (m, 4), 3.08−3.31

(m, 4), 3.78−3.84 (m, 10), 4.42−4.58 (m, 4), 6.86−7.60 (m, 20). Anal. ($C_{56}H_{74}Cl_2N_4O_4$) C, H, N.

14,23-Bis(2-methoxybenzyl)-7,30-dimethyl-7,14,23,30-tetraazatricyclo[30.2.2.2^{0,0}]octatriaconta-1(35),2,4,32(36),33,37-hexaene-13,24-dione Dihydrochloride (17). Formic acid (96%, 1.4 mL, 37 mmol) was added dropwise to **14** (200 mg, 0.25 mmol), and then 40% formaldehyde (1.2 mL, 17.4 mmol) was added to the resulting mixture, which was heated at 100 °C for 18 h, cooled (5 °C), made basic with 40% aqueous NaOH, and extracted with $CHCl_3$ (3 × 30 mL). Removal of dried solvents gave a residue that was purified by gravity chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.1) gave **17** as the free base that was converted into the dihydrochloride salt: 30% yield; mp 205−215 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.12−1.32 (m, 12), 1.32−1.56 (m, 12), 2.16−2.23 (m, 14), 2.40−2.42 (m, 4), 3.14−3.30 (m, 4), 3.55 (s, 4), 3.82 (s, 6), 6.85−7.59 (m, 16). Anal. ($C_{52}H_{74}Cl_2N_4O_4$) C, H, N.

10,19-Bis(2-methoxybenzyl)-3,10,19,26-tetraazabicyclo-[26.2.2]dotriaconta-1(31),28(32),29-triene Tetrahydrochloride (18). 18 was obtained from **7** (200 mg, 0.34 mmol) and **28** (46.6 mg, 0.34 mmol) following the procedure described for **31** and purified by gravity chromatography. Eluting with methanol/aqueous 30% ammonia (10:0.1) gave **18** as the free base that was converted into the tetrahydrochloride salt: 37% yield; mp 80−84 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.23−1.45 (m, 28), 1.75 (br s, 2, exchangeable with D_2O), 2.36−2.42 (m, 8), 2.55−2.60 (m, 4), 3.55 (s, 4), 3.77 (s, 4), 3.80 (s, 6), 6.81−6.93 (m, 4), 7.16−7.27 (m, 6), 7.40 (d, 2). Anal. ($C_{44}H_{72}Cl_4N_4O_2$) C, H, N.

14,23-Bis(2-methoxybenzyl)-7,14,23,30-tetraazatricyclo-[30.2.2.2^{0,0}]octatriaconta-1(35),2,4,32(36),33,37-hexaene Tetrahydrochloride (19). **19** was obtained from **7** (260 mg, 0.44 mmol) and **29**[13] (93 mg, 0.44 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methylene chloride/petroleum ether/ethanol/aqueous 30% ammonia (8:1:1:0.1) gave **19** as the free base that was converted into the tetrahydrochloride salt: 40% yield; mp 214−218 °C (EtOH/Et_2O); ^{1}H NMR (free base, $CDCl_3$) δ 1.18−1.50 (m, 28), 1.86 (br s, 2, exchangeable with D_2O), 2.37 (t, 8), 2.52 (t, 4), 3.54 (s, 4), 3.78 (s, 6), 3.83 (s, 4), 6.82 (d, 2), 6.90 (t, 2), 7.18 (t, 2), 7.35−7.38 (m, 6), 7.57 (d, 4). MALDI-MS calcd for $C_{50}H_{73}N_4O_2$ 761.51 $(M + H)^+$, found 761.57. Anal. ($C_{50}H_{76}Cl_4N_4O_2$) C, H, N.

15,24-Bis(2-methoxybenzyl)-8,15,24,31-tetraazatricyclo-[31.2.2.2^{0,0}]nonatriaconta-1(36),3,5,33(37),34,38-hexaene Tetrahydrochloride (20). **20** was obtained from **7** (200 mg, 0.34 mmol) and **30**[14] (76 mg, 0.34 mmol) following the procedure described for **31** and purified by flash chromatography. Eluting with methanol/aqueous 30% ammonia (10:0.1) gave **20** as the free base that was converted into the tetrahydrochloride salt as a hygroscopic solid: 38% yield; ^{1}H NMR (free base, $CDCl_3$) δ 1.25−1.52 (m, 28), 1.72 (br s, 2, exchangeable with D_2O), 2.24 (t, 8), 2.59 (t, 4), 3.58 (s, 4), 3.77 (s, 4), 3.82 (s, 6), 3.97 (s, 2), 6.82−6.96 (m, 4), 7.13−7.27 (m, 10), 7.41−7.44 (m, 2). Anal. ($C_{51}H_{78}Cl_4N_4O_2$) C, H, N.

Biology. Functional Antagonism. Male guinea pigs (200−300 g) and frogs (10−20 g) were sacrificed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 mL organ baths containing physiological salt solution (PSS) kept at an appropriate temperature (see below) and aerated with 5% CO_2/95% O_2. Concentration−response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately 3-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 connected to a MacLab system PowerLab/800. 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 Ileum Longitudinal Muscle. The 2 cm long portions of terminal ileum were taken at about 5 cm from the ileo−caecal junction. The tissue was cleaned, and the ileum longitudinal muscle was separated from the underlying circular muscle and set up at 37 °C in organ baths containing PSS of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.52; $MgSO_4 \cdot 7H_2O$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 23.8; glucose, 11.7. Tension changes were recorded isotonically. 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 arecaidine propargyl ester (APE) (0.01−0.5 μM) were obtained at 30 min intervals, the first one being discarded and the second one taken as the control. Following incubation with the antagonist for 60 min, a new concentration−response curve to the agonist was obtained.

Guinea Pig Left Atria. The guinea pig heart was rapidly removed, and right and left atria were separated out. The left atria were mounted at 30 °C in PSS of the same composition used for the ileum. Tissues were stimulated through platinum electrodes by square-wave pulses (1 ms, 1 Hz, 5−10 V) (Tetra Stimulus, N. Zagnoni). Inotropic activity was recorded isometrically. Tissues were equilibrated for 2 h, and cumulative concentration−response curves to APE (0.01−1 μM) were constructed. Following incubation with the antagonist for 60 min, a new concentration−response curve to APE 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 for 60 min. Two cumulative concentration−response curves to carbachol (1−100 μM) were constructed at 1 h intervals, the first one being discarded and the second one taken as the control. Following incubation with the antagonist for 60 min, a new concentration−response curve to the agonist was obtained.

Fluorescence Titration. nAChR-rich membranes were prepared from frozen *Torpedo californica* electric organ as described earlier.[25] All fluorescence spectra were recorded using an Aminco Bowman spectrometer series 2 (Rochester). 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.

Inhibition of Acetylcholinesterase. AChE (0.5 UI/mg) derived from human erythrocytes was purchased from Sigma Chemical (Italy). Buffer components and other chemicals were of the highest purity commercially available. The method of Ellman et al. was followed.[21] A 0.037 M acetylthiocoline iodide solution was prepared in water, 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was dissolved in pH 7.0 phosphate buffer, and 0.15% (w/v) $NaHCO_3$ was added. AChE solution was prepared by dissolving 20 units in 5 mL of 0.2% aqueous gelatine with sonication at 35 °C. A 1:1 dilution with water was performed before use to get the enzyme activity between 0.13 and 0.100 AU/min. Stock solutions of the test compounds (1 mM) were prepared in water, as well as caproctamine (**3**) reference stock solution. The assay solutions were prepared by diluting the stock solutions in water. Five different concentrations of each compound were used in order to obtain inhibition of AChE activity between 20% and 80%. The assay solution consisted of a 0.1 M phosphate buffer, pH 8.0, with the addition of 340 μM DTNB, 0.035 UI/mL AChE, and 550 μM acetylthiocoline iodide. The final assay volume was 1 mL. Test compounds were added to the assay solution and preincubated with the enzyme for 20 min, the addition of substrate following. Initial rate assays were performed at 37 °C with a Jasco Uvidec-610 double-beam spectrophotometer; the rate of increase in the absorbance at 412 nm was followed for 5 min. Assays were done with a blank containing all components except AChE in order to account for nonenzymatic reaction. The reaction rates were compared,

and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate.

Data Analysis. The percent inhibition of AChE activity due to the presence of increasing test compound concentration was calculated by the following expression,

$$\frac{100 - v_i}{v_o} \times 100$$

where v_i is the rate calculated in the presence of inhibitor and v_o is the enzyme activity. Inhibition curves were obtained for each compound by plotting the percent inhibition versus the logarithm of the inhibitor concentration in the assay solution. The linear regression parameters were determined for each curve, and the IC_{50} was extrapolated.

Antagonism of mAChRs, expressed as pK_B values, was estimated according to the equation $pK_B = \log(DR - 1) - \log$ [antagonist], where DR is the ratio between individual EC_{50} values in the presence and in the absence of antagonists.[20] 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. The newly synthesized compounds were tested at only one concentration (5 μM) in triplicate.

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.[26]

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.[24]

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