Universal Template Approach to Drug Design: Polyamines as Selective Muscarinic Receptor Antagonists

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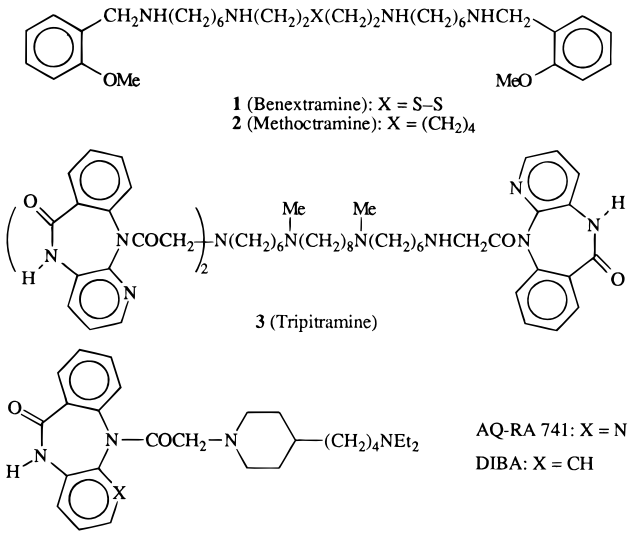
The concept that polyamines may represent a universal template in the receptor recognition process is embodied in the design of new selective muscarinic ligands. Tetraamines **4**−**7** and **16**−**20** and diamine diamides **8**−**15** were synthesized, and their pharmacological profiles at muscarinic receptor subtypes were assessed by functional experiments in isolated guinea pig left atrium (M_2) and ileum (M_3) and by binding assays in rat cortex (M_1), heart (M_2), submaxillary gland (M_3), and NG 108-15 cells (M_4). It has been confirmed that appropriate substituents on the terminal nitrogens of a tetraamine template can tune both affinity and selectivity for muscarinic receptors. The novel tetraamine *C*-tripitramine (**17**) was able to discriminate significantly M_1 and M_2 receptors versus the other subtypes, and in addition it was 100-fold more lipophilic than the lead compound tripitramine. Compound **14** (tripinamide), in which the tetraamine backbone was transformed into a diamine diamide one, retained high affinity for muscarinic subtypes, displaying a binding affinity profile ($M_2 > M_1 > M_4 > M_3$) qualitatively similar to that of tripitramine. Both these ligands, owing to their improved lipophilicity relative to tripitramine and methoctramine, could serve as tools in investigating cholinergic functions in the central nervous system. Furthermore, notwithstanding the fact that the highest affinity was always associated with muscarinic M_2 receptors, for the first time polyamines were shown to display high pA_2 values also toward muscarinic M_3 receptors.

Introduction

The identification of multiple muscarinic receptor subtypes has stimulated the search for ligands with selectivity for a given receptor subtype.[1] Five different subtypes (m_1−m_5) have been identified so far by molecular cloning. Muscarinic receptors that have been characterized pharmacologically and classified as M_1−M_4 appear to correspond to cloned m_1−m_4 receptors. At present, little information is available about the nature and the cellular location of the m_5 subtype.[1]

Nonselective muscarinic receptor antagonists have long been used for treatment of a variety of human diseases. Notwithstanding the enormous therapeutic potential, these compounds have modest clinical benefit owing to a variety of side effects which limit their practical use in favor of newer generations of therapeutic agents.[2] It is possible that many of the unwanted side effects of antimuscarinics are due to their interaction with multiple muscarinic receptor subtypes. Clearly, achievement of selectivity is of paramount importance for receptor subtype characterization and also for the development of therapeutically useful muscarinic drugs.[1−3] For instance, muscarinic M_1 receptor agonists and muscarinic M_2 receptor antagonists may be useful to enhance cognitive function, whereas selective muscarinic M_1 antagonists could have therapeutic potential for gastrointestinal indication and may be useful as cognition-impairing tools for research. Furthermore, muscarinic M_2 and M_3 antagonists have potential for the treatment of bradycardic disorders and of airway obstructions, respectively.

Chart 1



Universal Template Concept

These considerations prompted us to design new antimuscarinics which bear no kinship with previously known classes of muscarinic receptor antagonists. The starting point was the observation that benextramine (**1**) (Chart 1), an irreversible antagonist of both α_1- and α_2-adrenoreceptors, also displayed muscarinic antagonistic activity with a significant selectivity for cardiac

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muscarinic receptors.[4] Using benextramine as the focus, polymethylene tetraamines lacking a disulfide moiety were designed to achieve specific recognition of muscarinic receptors.[5,6] The finding that appropriate chain lengths separating the nitrogen atoms and appropriate substituents can modulate both affinity and selectivity for different receptor systems allowed us to postulate that a polyamine backbone may represent a master key ("passe-partout") in the drug−receptor recognition process.[7] In other words, a polyamine backbone can be considered a universal template on which suitable groups (pharmacophores) can be mounted to achieve selectivity for any given receptor.

The assumption that a polyamine is a universal template stands on the following: It is known that a high percentage of homology exists not only within receptor subtypes but also among different receptor families, which may account for the difficulty to achieve receptor selectivity that remains one of the most fascinating challenges to medicinal chemists. Although receptor homology makes it inherently difficult to achieve receptor selectivity, it may be used indeed to design molecular entities which are able, in principle, to recognize different receptor systems. This working hypothesis derives from the consideration that neurotransmitter receptors are folded polypeptide chains which always contain the same amino acids, albeit in a different proportion and sequence. The sequence of the amino acids constitutes the primary structure which is derived by peptide bonds linking the carboxylate groups to the amino groups. The resulting repeat units in the chain, i.e., the peptide bond and the α carbon, form the backbone. Since the backbone of a protein has only a structural role and cannot be considered a target for selectivity, it is evident that only lateral chains play a major role in drug−receptor binding. Among these functionalities aspartate, glutamate, and aromatic residues may acquire paramount importance for the binding with cationic ligands by way of a cation−anion or a cation−π interaction. Considering that proteins may bear several carboxylate and/or aromatic residues somewhere in their structure, in principle, it is possible to design a lead compound having a polyamine backbone which is able to recognize multiple anionic sites of a given receptor. Thus, such a ligand may interact with all receptor proteins, provided that the distance separating the amine functions of the ligand fits the distance between the carboxylate or aromatic residues of the receptor. In other words, a polyamine could be considered a master key in the drug−receptor recognition process owing to its flexibility that permits it to assume a suitable conformation to allow the interaction between protonated amine functions and receptor anionic sites. It was reported that polyamines, such as spermine and homospermine, are highly protonated at physiological pH. It turned out that spermine and homospermine are 85% and 97% tetracation, respectively, the remainder being the trication.[8] Thus, the ability of a polyamine to interact with biological counterions, that is, a set of carboxylate anions fixed to the backbone of a receptor, could well be related to its cationic properties. Consequently, the distance between the cationic nitrogens of a polyamine becomes critical in the recognition step.

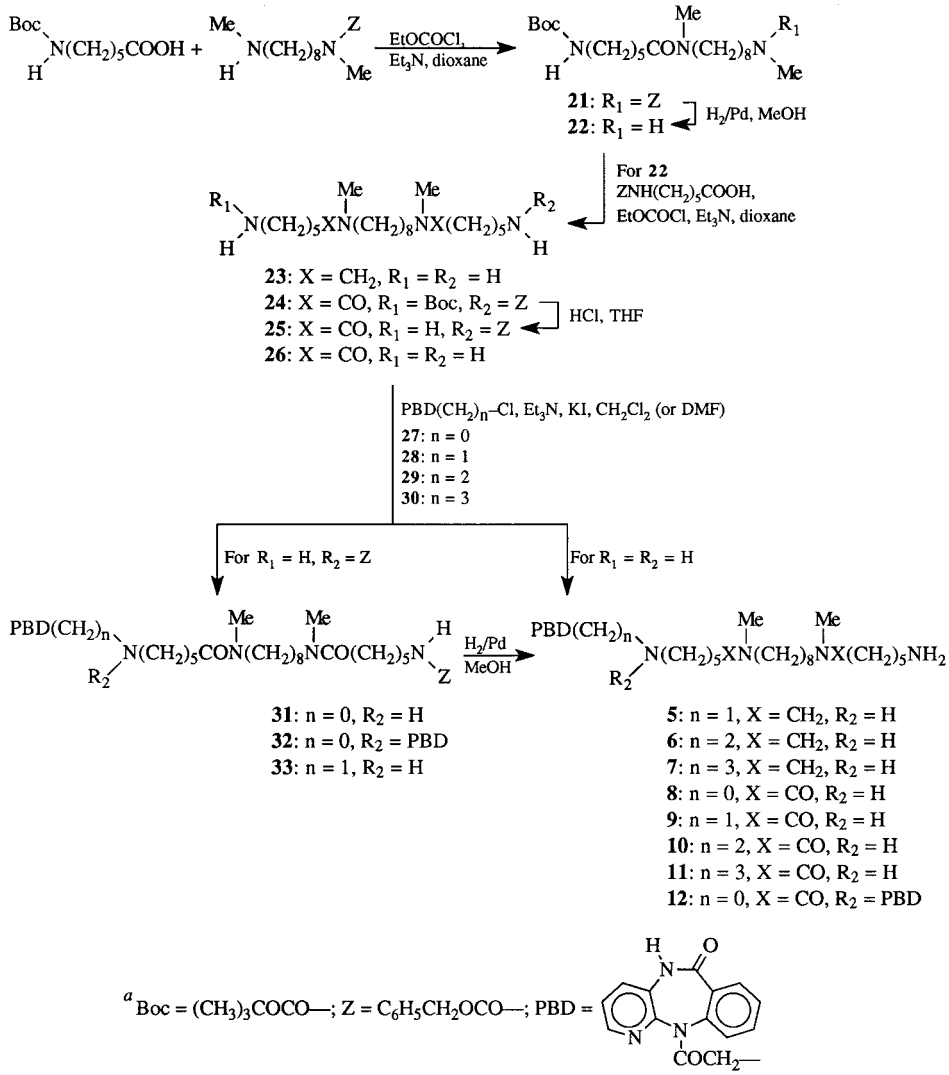
It is known that by increasing the number of interactions that take place between a receptor and a ligand there is more chance to distinguish receptor systems. Thus, an appropriate modification of the chain length separating the nitrogens of a polyamine might give rise to an increase of affinity, whereas the insertion of N-substituents might improve the selectivity as well as the affinity by increasing the overall number of contacts between a drug and a receptor. Many naturally occurring or synthetic polyamines with a linear tetraamine backbone are known. These ligands recognize different receptors which may be either activated or inhibited.[9−11,14]

The development of polymethylene tetraamines as muscarinic receptor antagonists, the prototype of which is methoctramine (**2**) (Chart 1), has been the subject of review articles.[5] The replacement of the terminal 2-methoxybenzyl groups of methoctramine with an 11-acetyl-5,11-dihydropyrido[2,3-*b*][1,4]benzodiazepin-6-one (PBD) moiety led to the discovery of tripitramine (**3**), resulting in the most potent and the most selective muscarinic M_2 receptor antagonist so far available, able to discriminate also between muscarinic M_2 and M_4 receptors[15] but not between M_1 and M_4 subtypes.[16,17] A further modification of methoctramine structure afforded spirotramine that in binding assays displayed an inverse selectivity profile in comparison with both methoctramine and tripitramine, further supporting the hypothesis that a polyamine backbone may represent a universal template for drug design.[18]

The observation that the insertion of one PBD moiety onto only one of the terminal nitrogens of the tetraamine backbone of methoctramine afforded **4**, which displayed an affinity profile similar to that of methoctramine,[17] gave us the opportunity to verify whether the position of the tricyclic moiety relative to the protonated terminal nitrogen may be responsible for receptor subtype selectivity as previously suggested by others to rationalize receptor subtype selectivity displayed by pirenzepine and its analogues.[19] To this end, we synthesized some homologues (**5**−**7**) of the monosubstituted tetraamine **4**, bearing from two to four methylenes between the terminal nitrogen and the tricyclic group. Since the four amine functions of these tetraamines are likely to be mostly protonated at physiological pH, making their use as a tool in improving cognitive function difficult because they will hardly cross the blood brain−barrier, we synthesized the corresponding diamine diamides **8**−**11** of tetraamines **4**−**7** by transforming the two inner amine functions into two amide groups to improve lipophilicity and allow the new compounds to penetrate, albeit to a modest extent, the central nervous system. Furthermore, we synthesized all possible symmetrical and unsymmetrical diamine diamides (**12**−**15**), bearing from one to four PBD groups on the terminal nitrogens, to improve affinity and hopefully selectivity for muscarinic receptor subtypes.

Since the replacement of the pyridine ring of the tricyclic moiety of AQ-RA 741, a potent and selective M_2 antagonist, with a benzene ring afforded the carbon analogue DIBA,[20] which is 1 order of magnitude more potent than the parent compound, we thought it of interest to perform the same structural modification on the tricyclic system of tripitramine. Thus, we designed tetraamines **16** and **17** (*C*-tripitramine), which bear on their terminal nitrogens the more lipophilic 5-acetyl-10,11-dihydrodibenzo[*b*,*e*][1,4]diazepin-11-one (DBD) tri-

Scheme 1[a]



cyclic ring system of DIBA rather than PBD moieties. In addition, to further support the view that the affinity profile of methoctramine-related tetraamines can be modulated by appropriate substituents on the terminal nitrogens of a tetraamine backbone, we replaced tripitramine PBD moieties with the isomeric 11-acetyl-6,-11-dihydropyrido[2,3-*b*][1,5]benzodiazepin-5-one (inv-PBD) groups, affording tetraamines **18**−**20**.

We describe here the synthesis and the pharmacological profile of tetraamines **4**−**7** and **16**−**20** and diamine diamides **8**−**15** in functional and binding experiments.

Chemistry

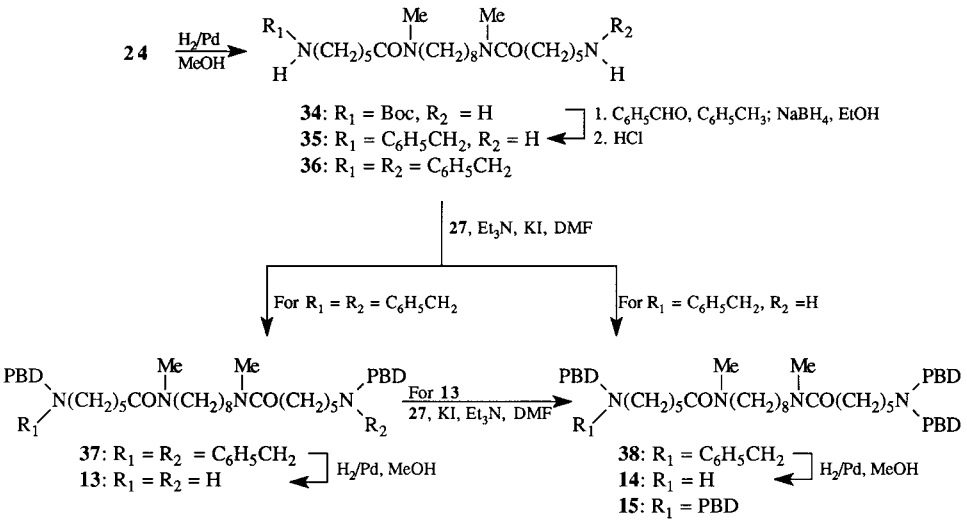
All the compounds were synthesized by standard procedures (Schemes 1−3) and were characterized by IR, ^{1}H NMR, fast atom bombardment (FAB) mass spectra, and elemental analysis.

Tetraamine **23**[21] was alkylated with the appropriate chloride (**28**−**30**)[22] to give the corresponding monosubstituted tetraamines **5**−**7** (Scheme 1). *N*-(*tert*-Butoxycarbonyl)-6-aminocaproic acid was amidated with *N*′-[(benzyloxy)carbonyl]-*N*,*N*′-dimethyl-1,8-octanediamine[23] to give **21**. Removal of the *N*-(benzyloxy)-carbonyl group was achieved by catalytic hydrogenation over 10% palladium on charcoal. Thus, hydrogenolysis of **21** gave **22** which was reacted with *N*-[(benzyloxy)-carbonyl]-6-aminocaproic acid to afford diamine diamide **24**, having the two terminal amine functions protected with different moieties. Removal of the *N*-*tert*-butoxycarbonyl group was achieved by hydrolysis with 6 N HCl. Thus, **24** was transformed into **25** which was treated with **27** or **28**[22] to afford **31** and **32** or **33**, respectively. Diamine diamides **8**, **9**, and **12** were obtained from **31**, **33**, and **32**, respectively, by catalytic hydrogenolysis. Finally, monosubstituted diamine diamides **10** and **11** were prepared by alkylating unsubstituted diamine diamide **26**[21] with **29** and **30**,[22] respectively (Scheme 1).

Diamine diamides **13**−**15** were synthesized following the synthetic pathway shown in Scheme 2. Thus, hydrogenolysis of **24** gave **34** which was transformed into **35** by reaction with benzaldehyde and subsequent reduction of the intermediate Schiff base with $NaBH_4$ followed by removal of the *tert*-butoxycarbonyl group. Alkylation of **35** and **36**[16] with **27**[22] afforded **38** and **37**, respectively, which, in turn, were transformed, upon catalytic hydrogenolysis, into the corresponding **13** and **14**. A further alkylation of **13** with **27**[22] gave **15** (Scheme 2).

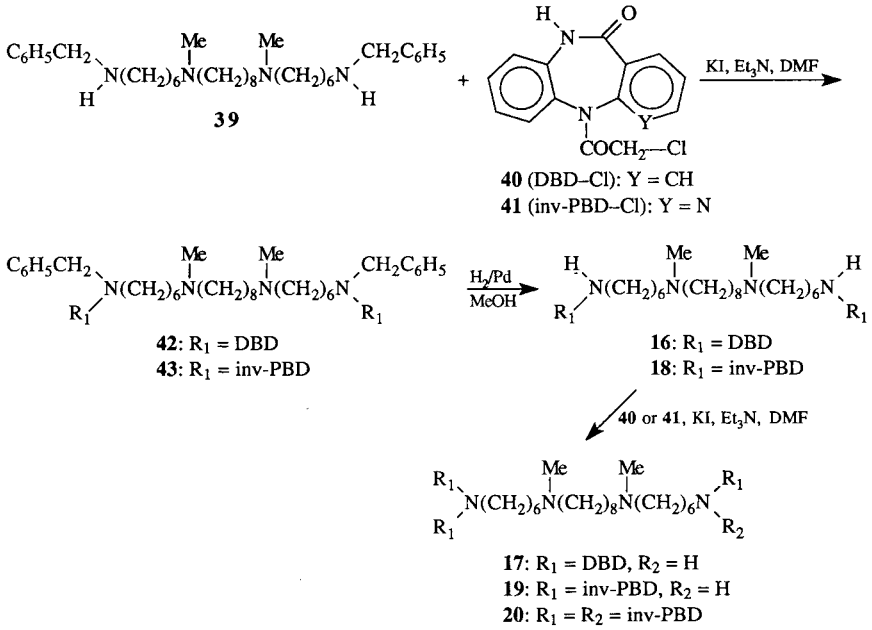
Tetraamines **16**−**20** were obtained as shown in Scheme 3. Alkylation of **39**[16] with **40**[20] and **41**[22] afforded **42** and

Scheme 2[a]



[a] For definition of PBD, see Scheme 1.

Scheme 3



43, respectively, which were transformed, upon hydrogenolysis, into **16** and **18**. A further alkylation of **16** and **18** with **40** and **41**, respectively, gave the corresponding tetraamines **17** and **20**, and **19**.

Biology

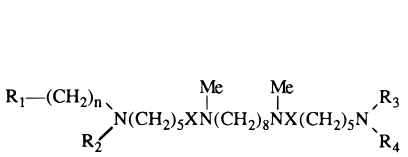
Functional Studies. Functional activity at muscarinic receptor subtypes was determined by the use of the muscarinic M_2 receptor-mediated negative inotropism in driven guinea pig left atria (1 Hz) and muscarinic M_3 receptor-mediated contraction of guinea pig ileum longitudinal muscle. These methods have been described in detail earlier.[17] The agonist was arecaidine propargyl ester (APE). To allow comparison of the results, methoctramine (**2**) and tripitramine (**3**) were used as the standard compounds. The biological results are expressed as pA_2 values determined from Schild plots[24] constrained to slope -1.0,[25] as required by theory. When this method was applied, it was always verified that the experimental data generated a line whose derived slope was not significantly different from unity.

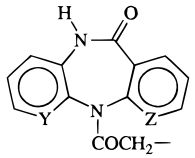
Binding Experiments. The muscarinic receptor subtype selectivity was assessed by employing receptor binding assays as reported previously.[16,17] [^{3}H]-*N*-Methylscopolamine was used to label M_2, M_3, and M_4 muscarinic receptor binding sites of rat heart, submaxillary gland, and NG 108-15 cell homogenates, respectively. [^{3}H]Pirenzepine was the tracer to label M_1 muscarinic receptor binding sites of the rat cerebral cortex. Binding affinities are expressed as pK_i values derived using the Cheng−Prusoff equation.[26] Methoctramine (**2**) and tripitramine (**3**) were used as the standard compounds.

Results and Discussion

The functional activity, expressed as pA_2 values, at peripheral muscarinic M_2 and M_3 receptors of polyamines used in the present study is shown in Table 1 and

Table 1. Antagonist Affinities, Expressed as pA_2 Values, in the Isolated Guinea Pig Left Atrium (M_2) and Longitudinal Ileum (M_3) Muscarinic Receptors





PBD: Y = N, Z = CH
DBD: Y = Z = CH
inv-PBD: Y = CH, Z = N

no.[a]	*n*	X	R_1	R_2	R_3	R_4	pA_2[b] M_2	pA_2[b] M_3
2							7.91 ± 0.03	6.44 ± 0.06
3	0	CH_2	PBD	H	PBD	PBD	9.65 ± 0.08	6.55 ± 0.04
4	0	CH_2	PBD	H	H	H	7.95 ± 0.04	6.56 ± 0.01
5	1	CH_2	PBD	H	H	H	8.57 ± 0.06	7.77 ± 0.02
6	2	CH_2	PBD	H	H	H	9.15 ± 0.08	8.32 ± 0.04
7	3	CH_2	PBD	H	H	H	9.01 ± 0.02	7.71 ± 0.03
8	0	CO	PBD	H	H	H	8.01 ± 0.05	6.36 ± 0.02
9	1	CO	PBD	H	H	H	8.04 ± 0.06	7.74 ± 0.01
10	2	CO	PBD	H	H	H	9.34 ± 0.01	8.73 ± 0.03
11	3	CO	PBD	H	H	H	9.53 ± 0.05	7.86 ± 0.07
12	0	CO	PBD	PBD	H	H	5.25 ± 0.03	5.14 ± 0.04
13	0	CO	PBD	H	H	PBD	9.30 ± 0.01	7.14 ± 0.01
14	0	CO	PBD	H	PBD	PBD	8.36 ± 0.04	6.26 ± 0.08
15	0	CO	PBD	PBD	PBD	PBD	7.75 ± 0.03	5.67 ± 0.01
16	0	CH_2	DBD	H	H	DBD	8.82 ± 0.01	6.95 ± 0.02
17	0	CH_2	DBD	H	DBD	DBD	9.70 ± 0.02	7.35 ± 0.01
18	0	CH_2	inv-PBD	H	H	inv-PBD	8.28 ± 0.02	6.27 ± 0.01
19	0	CH_2	inv-PBD	H	inv-PBD	inv-PBD	8.60 ± 0.04	6.04 ± 0.05
20	0	CH_2	inv-PBD	inv-PBD	inv-PBD	inv-PBD	7.87 ± 0.01	5.79 ± 0.01

[a] **2**, tetrahydrochloride; **3**−**7**, **16**−**20**, tetraoxalates; **8**−**15**, dihydrochlorides. [b] pA_2 values ± SE were calculated from Schild plots,[24] constrained to slope −1.0.[25] pA_2 is the positive value of the intercept of the line derived by plotting log (DR − 1) vs log [antagonist]. The log (DR − 1) was calculated from at least three different antagonist concentrations, and each concentration was tested from four to six times. Dose−ratio (DR) values represent the ratio of the potency of the agonist arecaidine propargyl ester (EC_{50}) in the presence of the antagonist and in its absence. Parallelism of concentration−response curves was checked by linear regression, and the slopes were tested for significance ($p < 0.05$).

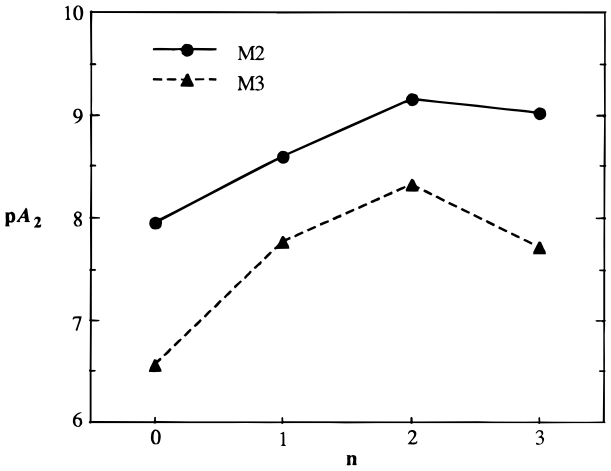


Figure 1. Effect of the carbon chain length separating the PBD moiety and the terminal nitrogen ($n = 0-3$) of tetraamines **4**−**7** on blocking activity of guinea pig left atria (M_2) and guinea pig longitudinal ileum (M_3) muscarinic receptor subtypes. Data from Table 1.

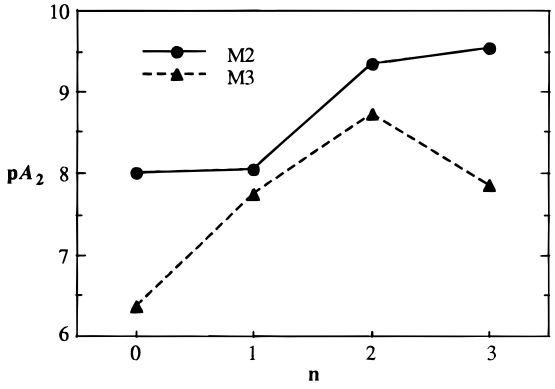


Figure 2. Effect of the carbon chain length separating the PBD moiety and the terminal nitrogen ($n = 0-3$) of diamine diamides **8**−**11** on blocking activity of guinea pig left atria (M_2) and guinea pig longitudinal ileum (M_3) muscarinic receptor subtypes. Data from Table 1.

Figures 1 and 2. To make relevant considerations on structure−activity relationships, we included parent compounds **2** (methoctramine) and **3** (tripitramine) for comparison. All compounds behaved as competitive antagonists as revealed by the slopes of their Schild plots, which were not significantly different from unity ($p > 0.05$) (Table 1).

It can be observed that the effect of lengthening the distance between the tricyclic moiety and the terminal nitrogen atom of **4**, affording **5**−**7**, on affinity is qualitatively similar for both muscarinic M_2 and M_3 receptors; the affinity for these receptors increased with increasing the chain length, reaching a maximum for $n = 2$ (Table 1), although tetraamine **7** carrying an additional methylene also retained high activity (Figure 1). Next, changing the two inner amine functions of tetraamines **4**−**7** into amides, affording the corresponding diamine diamides **8**−**11**, did not alter dramatically the affinity profile at muscarinic M_2 and M_3 receptors, as only a slight variation in the affinity for the M_2 subtype was observed as shown graphically in Figure

2. Diamine diamides **8**−**11** displayed a very high affinity toward muscarinic M_2 receptors, approaching that of tripitramine as revealed by a comparison of pA_2 values of **3** and **11** (9.65 ± 0.08 vs 9.53 ± 0.05). Interestingly enough, notwithstanding the fact that the highest affinity was always associated with muscarinic M_2 receptors, for the first time polyamines were shown to display high pA_2 values also toward muscarinic M_3 receptors. In particular, diamine diamide **10** was more than 2 orders of magnitude more potent than both prototypes, methoctramine (**2**) and tripitramine (**3**), at muscarinic M_3 receptors as revealed by their pA_2 values (**10**, 8.73 ± 0.03; **2**, 6.44 ± 0.06; **3**, 6.55 ± 0.04). In previous related studies polyamines were shown to display invariably pA_2 values of about 6.0−6.5, allowing the conclusion that muscarinic M_3 receptors are less sensitive than muscarinic M_2 receptors to structural modifications performed on tetraamines and that a tetraamine backbone may not be a suitable carrier for optimal interaction with this receptor subtype. Now we have shown that lengthening the distance between the tricyclic ring of the PBD moiety and the terminal amine function of a tetraamine template might represent a first step for the design of tetraamines displaying high affinity and hopefully selectivity for the M_3 subtype. The high affinity displayed by tetraamine **6** for muscarinic M_3 receptors emphasizes once again that a polyamine backbone may represent a universal template for receptor recognition. Affinity and selectivity can be tuned simply by inserting appropriate substituents in the template structure.

The finding that tetraamine **4** and diamine diamide **8** showed a qualitatively and quantitatively similar affinity profile, while being 25- and 45-fold, respectively, more potent at muscarinic M_2 receptors than at muscarinic M_3 receptors, may be relevant (a) to the design of new selective M_2 antagonists based on a diamine diamide rather than a tetraamine backbone and (b) to the understanding of the mode of interaction of methoctramine (**2**)-related polyamines at muscarinic M_2 receptors.

The use of a diamine diamide backbone as template, on which appropriate substituents should be mounted to tune both affinity and selectivity, could afford new M_2 antimuscarinics with improved lipophilic properties relative to those bearing a tetraamine backbone, which might be useful in investigating cognitive function. Since the inclusion of additional PBD moieties on the terminal nitrogens of **4** led to the discovery of tripitramine (**3**), which displayed outstanding properties as an M_2 antagonist, we made the same modification on the terminal nitrogens of **8**, affording diamine diamides **12**−**15**. Surprisingly enough, the affinity of **12**−**15** for muscarinic M_2 receptors did not follow the same trend observed for the corresponding tetraamines obtained through a similar modification performed on tetraamine **4**. An analysis of the results reveals that the unsymmetrical diamine diamide **14** (tripinamide) is significantly less potent (about 20-fold) than the corresponding tetraamine tripitramine (**3**) at muscarinic M_2 receptors while retaining the same affinity for the M_3 subtype. Furthermore, optimum activity was associated with the symmetrically monosubstituted diamine diamide **13** whose pA_2 value at muscarinic M_2 receptors approached that of tripitramine (9.30 ± 0.01 vs 9.65 ± 0.08). The insertion of more than one PBD group on the same terminal nitrogen of a diamine diamide backbone caused, in comparison with **8**, a decrease in affinity, as in **12**, for both muscarinic M_2 and M_3 receptors or, alternatively, did not markedly modify the affinity profile, as in **14** and **15**.

Concerning the mode of interaction of the above polyamines, at a first glance, comparing the results obtained with tetraamines **4**−**7** and diamine diamides **8**−**11**, one could reach the conclusion that both muscarinic M_2 and M_3 receptors may have the same structural requirements and, as a consequence, that tetraamines and diamine diamides may share a common mechanism of receptor interaction. However, taking into account also the diverging effect observed in a previous work[17,27] and in the present investigation by replacing the hydrogens with PBD groups on the terminal nitrogens of **4** and **8**, it becomes reasonable to assume that tetraamines and diamine diamides may have a slightly different mode of interaction with the receptor.

Previously, we advanced that the high affinity of tripitramine for muscarinic M_2 receptors may be the result of an interaction with two different sites thanks to its two differently substituted terminal nitrogens.[17] Thus, the N-substituted terminal nitrogen would interact with the active binding site, whereas the other N,N-disubstituted terminal nitrogen would recognize a second accessory site. The finding that nonsymmetrical polyamines **4**−**11** displayed high affinity for muscarinic M_2 receptors supports the view that the sites where the two terminal nitrogens of these ligands are likely to bind have different structural requirements.

Since the insertion of additional PBD groups on the terminal nitrogens of **8**, affording **12**−**15**, did not improve either affinity or selectivity of tripitramine (**3**) for muscarinic M_2 receptors versus muscarinic M_3 receptors, our attention was focused again on modifying the substituents on the terminal nitrogens of the tetraamine backbone, affording **16**−**20**. An analysis of the results reveals that the replacement of the tricyclic ring system of tripitramine with a DBD moiety, affording the carbon analogues **16** and **17** (*C*-tripitramine), did not improve the affinity for both muscarinic M_2 and M_3 receptors as observed following the same modification performed on AQ-RA 741[20] to afford the carbon analogue DIBA. *C*-Tripitramine (**17**) was as active as tripitramine (**3**) at muscarinic M_2 receptors, whereas it was significantly more potent (6-fold) at the M_3 subtype. Interestingly, the inversion of the *endo*-amide function of the tricyclic moiety of tripitramine, as in **18**−**20**, caused a decrease in affinity for muscarinic M_2 receptors while not affecting that for M_3 receptors, in comparison with tripitramine or its carbon analogue **17**.

The binding affinities, expressed as pK_i values, in rat cortex (M_1), heart (M_2), and submaxillary gland (M_3) and in NG 108-15 cells (M_4) of muscarinic receptor subtypes of tetraamines **4**, **6**, and **16**−**20** and diamine diamides **6**, **8**, **11**, and **14** are shown in Table 2 and, for selected compounds, in Figure 3 in comparison with those of methoctramine (**2**) and tripitramine (**3**). Concerning M_2 and M_3 muscarinic receptor subtypes, it is evident that binding affinities are qualitatively and quantitatively similar to pA_2 values derived from functional experi-

Table 2. Affinity Estimates, Expressed as pK_i Values, in Rat Cortex (M_1), Heart (M_2), and Submaxillary Gland (M_3) and NG 108-15 Cell (M_4) Muscarinic Receptor Subtypes of Polyamines **14**, **17**, and **19** in Comparison with the Prototypes **2** and **3**

	pK_i[a]			
no.	M_1	M_2	M_3	M_4
2 (methoctramine)	7.38 ± 0.10	7.70 ± 0.08	6.02 ± 0.15	7.49 ± 0.14
3 (tripitramine)	8.45 ± 0.13	9.52 ± 0.07	6.83 ± 0.10	7.94 ± 0.12
4	8.36 ± 0.15	8.11 ± 0.09	6.48 ± 0.11	7.80 ± 0.06
6	9.30 ± 0.15	9.43 ± 0.14	8.52 ± 0.22	8.84 ± 0.09
8	6.50 ± 0.07	7.35 ± 0.09	6.80 ± 0.07	7.70 ± 0.08
11	7.20 ± 0.08	8.25 ± 0.08	7.15 ± 0.20	7.20 ± 0.07
14 (tripinamide)	7.30 ± 0.10	8.60 ± 0.07	5.24 ± 0.09	6.80 ± 0.12
16	7.83 ± 0.15	9.25 ± 0.10	6.94 ± 0.06	8.55 ± 0.16
17 (*C*-tripitramine)	9.02 ± 0.20	9.36 ± 0.08	6.96 ± 0.04	7.38 ± 0.08
18	7.84 ± 0.32	7.68 ± 0.22	5.59 ± 0.10	7.44 ± 0.08
19	7.90 ± 0.20	9.04 ± 0.08	6.40 ± 0.07	7.40 ± 0.04
20	7.60 ± 0.07	8.03 ± 0.12	5.60 ± 0.06	6.80 ± 0.08

[a] Values are the mean ± SE of at least three separate experiments performed in triplicate. All Hill numbers (n_H) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived using the Cheng−Prusoff equation.[26] [^{3}H]NMS was used to label muscarinic receptors in rat heart and submaxillary gland and NG 108-15 binding assays, whereas [^{3}H]pirenzepine was the tracer in rat cortex homogenates. Scatchard plots were linear or almost linear in all preparations tested.

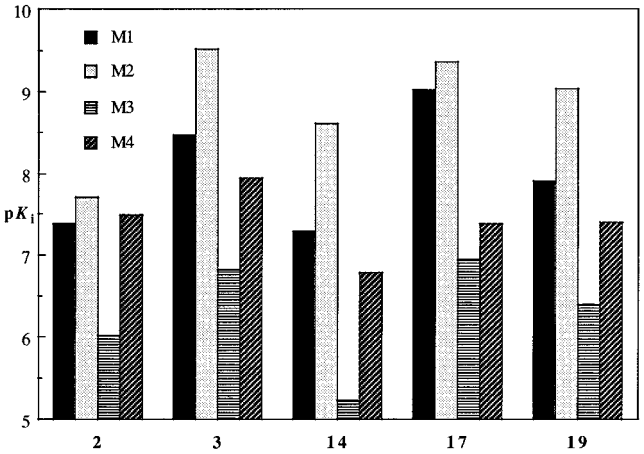


Figure 3. Affinity constants (pK_i) in rat cortex (M_1), heart (M_2), and submaxillary gland (M_3) and NG 108-15 cell (M_4) muscarinic receptor subtypes for **14**, **17**, and **19** in comparison with the prototypes methoctramine (**2**) and tripitramine (**3**).

ments, the only exception being tetraamine **11** and the diamine diamide tripinamide (**14**) at muscarinic M_2 and M_3 receptors, respectively. However, we have no explanation for this discrepancy.

Interestingly, the inversion of the *endo*-amide function of tripitramine, affording **19**, caused a decrease in affinity for all muscarinic receptor subtypes in comparison with tripitramine (**3**). Removal or insertion of one inv-PBD group from a terminal nitrogen atom of **19**, affording **18** and **20**, did not improve the selectivity profile. However, the substitution of the terminal nitrogens of the tetraamine backbone with DBD groups gave different effects on the affinity for muscarinic M_1 and M_4 receptors. *C*-Tripitramine (**17**) resulted as active as tripitramine at muscarinic M_2 and M_3 receptors, while being less potent at the M_4 subtype and more potent at the M_1 subtype. Removal of one DBD group from **17**, affording the symmetrically substituted tetraamine **16**, did not affect the affinity for muscarinic M_2 and M_3 receptors while causing a significant increase in the affinity for the M_4 type and a significant decrease for the M_1 type.

It is evident that an appropriate substitution of the terminal nitrogens of the tetraamine backbone of methoctramine affords potent antimuscarinics that display different selectivity profiles. The insertion of PBD groups on a tetraamine backbone afforded tripitramine which possesses outstanding properties toward muscarinic M_2 receptors in comparison with the parent compound methoctramine.[8−10] However, tripitramine fails to discriminate between muscarinic M_1 and M_4 receptors. Now we have demonstrated that the replacement of the tricyclic ring system of tripitramine with a DBD moiety gives the carbon analogue *C*-tripitramine (**17**) whose selectivity profile makes it possible to overcome the limitation of that displayed by tripitramine owing to a higher (about 100-fold) affinity for muscarinic M_1 and M_2 versus M_3 and M_4 receptors. Furthermore, *C*-tripitramine (**17**) was 100-fold more lipophilic than tripitramine (**3**) as revealed by the log *P* values which were calculated for the free bases by using CLOGP Program.[28] Moreover, changing the inner amine functions of tripitramine into amides afforded tripinamide (**14**) which was significantly less potent than the prototype but retained the same affinity profile as shown in Figure 3. Thus, diamine diamide **14** can serve as a lead for further structural modifications to design new antimuscarinics and also as tool in investigating cholinergic function in the central nervous system, owing to its presumably improved lipophilicity relative to tripitramine or methoctramine.

Conclusions

We have confirmed that appropriate substituents on the terminal nitrogens of a tetraamine template can tune both affinity and selectivity for muscarinic receptors. The insertion of a DBD moiety into a tetraamine backbone afforded the novel tetraamine *C*-tripitramine (**17**), which was able to discriminate significantly muscarinic M_1 and M_2 receptors versus the other subtypes. It is evident that the use of *C*-tripitramine combined with that of tripitramine may represent a valuable tool for the pharmacological identification of muscarinic receptor subtypes. Another additional interesting finding was the observation that the tetraamine backbone which served as a basis for the discovery of both methoctramine and tripitramine can be transformed into a diamine diamide while retaining high affinity for different muscarinic receptor subtypes. Thus, as an example, the diamine diamide tripinamide (**14**) displayed a binding affinity profile ($M_2 > M_1 > M_4 > M_3$) qualitatively similar to, albeit quantitatively different from, that of tripitramine while being significantly more lipophilic which might allow it to cross the blood−brain barrier and to investigate cognition function.

Experimental Section

Chemistry. Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian VXR 300 instruments, respectively. Although the IR and NMR 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 mass spectra were obtained on a VG707EH-F spectrometer. 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. The composition and volumetric ratio of eluting mixtures were as follows: A, $CHCl_3$−EtOH (9:1); B, MeOH−aqueous 28% ammonia (9.7:0.3); C, CH_2Cl_2−EtOAc-EtOH (8.7:1:0.3); D, $CHCl_3$−MeOH−aqueous 28% ammonia (8.5:1.5:0.1); E, $CHCl_3$−MeOH−aqueous 28% ammonia (8.5:1.5:0.15); F, $CHCl_3$−MeOH−aqueous 28% ammonia (9:1:0.1); G, $CHCl_3$−MeOH−aqueous 28% ammonia (5:4.5:0.5); $CHCl_3$−MeOH−aqueous 28% ammonia (9:1:0.07); H, CH_2Cl_2−EtOH−aqueous 28% ammonia (9:1.5:0.15); I, CH_2Cl_2−EtOH−aqueous 28% ammonia (9.1:0.9:0.09); J, CH_2Cl_2−EtOH−aqueous 28% ammonia (9.2:0.8:0.08); K, $CHCl_3$−EtOH−aqueous 28% ammonia (9:1:0.1); L, $CHCl_3$−EtOH−aqueous 28% ammonia (9:1:0.04); M, CH_2Cl_2−MeOH−aqueous 28% ammonia (9:1:0.1). Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F_{254}) that were visualized in an iodine chamber. The term "dried" refers to the use of anhydrous sodium sulfate.

General Procedure for the Synthesis of 5−7, 10, 11, 15, 17, 19, 20, 31−33, 37, 38, 42, and 43. A mixture of an amine and a chloride in a 1:1 (**17**, **19**, **20**, **31**−**33**), 1:2.16 (**37**), 1:2.5 (**42**, **43**), 1:3.7 (**38**), 1.66:1 (**15**), 3.7:1 (**10**, **11**), or 5:1 (**5**−**7**) ratio in dry DMF (**5**−**7**, **10**, **11**, **15**, **17**, **19**, **20**, **37**, **38**, **42**, and **43**) or methylene chloride (**31**−**33**) containing triethylamine (chloride's equivalents) and KI (few crystals) was stirred at room remperature for several days, following the reaction by TLC. Removal of the solvent gave a residue that, unless otherwise specified, was purified by flash chromatography.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]ethyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (5).** It was obtained from **23**[21] and **28**.[22] Eluting with solvent G gave the free base that was transformed into the tetraoxalate salt: 45% yield; mp 118−120 °C (MeOH/*i*-PrOH); MS (FAB) calcd for $C_{37}H_{62}N_7O_2$ 637 $[M + H]^+$, found 637. Anal. ($C_{45}H_{69}N_7O_{18}\cdot 2H_2O$) C, H, N.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]propyl]-7,16-dimethyl-7,-16-diaza-1,22-docosanediamine Tetraoxalate (6).** It was obtained from **23**[21] and **29**.[22] Eluting with solvent G gave the free base that was transformed into the tetraoxalate salt: 20% yield; mp 99−101 °C; MS (FAB) calcd for $C_{38}H_{64}N_7O_2$ 651 $[M + H]^+$, found 651. Anal. ($C_{46}H_{71}N_7O_{18}$) C, H, N.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]butyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (7).** It was obtained from **23**[21] and **30**.[22] Eluting with solvent G gave the free base that was transformed into the tetraoxalate salt: 30% yield; mp 80−85 °C; MS (FAB) calcd for $C_{39}H_{66}N_7O_2$ 664 $[M + H]^+$, found 664. Anal. ($C_{47}H_{73}N_7O_{18}$) C, H, N.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]propyl]-7,16-dimethyl-6,-17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (10).** It was obtained from **26**[21] and **29**.[22] Eluting with solvent B gave the free base that was transformed into the dihydrochloride salt: 25% yield; hygroscopic salt. Anal. ($C_{38}H_{61}Cl_2N_7O_4\cdot H_2O$) C, H, N.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]butyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (11).** It was obtained from **26**[21] and **30**.[22] Eluting with solvent B gave the free base that was transformed into the dihydrochloride salt: 25% yield; hygroscopic salt. Anal. ($C_{39}H_{63}Cl_2N_7O_4\cdot H_2O$) C, H, N.

***N*1,*N*1,*N*22,*N*22-Tetrakis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-7,-16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (15).** It was obtained from **13** (as free base) and **27**.[22] Eluting with solvent J gave an oil that was transformed into the dihydrochloride salt: 12% yield; mp 210 °C dec (from EtOH/ether). Anal. ($C_{78}H_{84}Cl_2N_{16}O_{10}$) C, H, N. The second fraction was **14** (27% yield) that was identical to the compound obtained by another route.

***N*1,*N*1,*N*22-Tris[[(5,10-dihydro-11-oxo-11*H*-dibenzo[*b*,*e*]-[1,4]diazepin-5-yl)carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (17).** It was obtained from **16** (as free base) and **40**.[20] It was purified by flash chromatography, eluting with solvent H, followed by gravity column chromatography. Eluting with toluene−chloroform−methanol−aqueous 28% ammonia (1:9.5:1:0.1) gave **17** as the free base (first fraction) that was transformed into the tetraoxalate salt: 25% yield; mp 150−151 °C (from EtOH/ether); MS (FAB) calcd for $C_{67}H_{81}N_{10}O_6$ 1121 $[M + H]^+$, found 1121. Anal. ($C_{75}H_{88}N_{10}O_{22}$) C, H, N. The second fraction was an unidentified compound.

***N*1,*N*1,*N*22-Tris[[(6,11-dihydro-5-oxo-5*H*-pyrido[2,3-*b*]-[1,4]benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (19) and *N*1,*N*1,*N*22,*N*22-Tetrakis[[(6,11-dihydro-5-oxo-5*H*-pyrido-[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (20).** They were obtained from **18** (as free base) and **41**[22] and purified by gravity column chromatography eluting with solvent E. The first fraction was **20** as the free base that was transformed into the tetraoxalate salt: 20% yield; mp 190−192 °C (from EtOH/ether); MS (FAB) calcd for $C_{78}H_{87}N_{16}O_8$ 1376 $[M + H]^+$, found 1376. Anal. ($C_{86}H_{94}N_{16}O_{24}$) C, H, N. The second fraction was **19** as the free base that was transformed into the tetraoxalate salt: 22% yield; mp 185−187 °C (from EtOH/ether); MS (FAB) calcd for $C_{64}H_{78}N_{13}O_6$ 1125 $[M + H]^+$, found 1125. Anal. ($C_{72}H_{85}N_{13}O_{22}$) C, H, N.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-*N*22-[(benzyloxy)-carbonyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine (31) and *N*1,*N*1-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-*N*22-[(benzyloxy)carbonyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine (32).** They were obtained from **25** and **27**.[22] Eluting with solvent A gave the desired compounds. The first fraction was **32** as an oil (23% yield). The second fraction was **31** (27% yield).

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]ethyl]-*N*22-[(benzyloxy)-carbonyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine (33).** It was obtained from **25** and **28**.[22] Eluting with solvent L gave the desired compound as an oil (43% yield).

***N*1,*N*22-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-*N*1,*N*22-dibenzyl-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine (37).** It was obtained from **36**[16] and **27**.[22] The residue was taken up with methylene chloride, washed with water, and extracted with 3 N HCl. The aqueous layer was made basic with NaOH pellets and extracted with methylene chloride. The extracts were washed with brine, dried, and then evaporated to give **37** as the free base in 97% yield.

***N*1,*N*1,*N*22-Tris[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*]-[1,4]benzodiazepin-11-yl)carbonyl]methyl]-*N*22-benzyl-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (38).** It was obtained from **35** and **27**.[22] The residue was taken up with methylene chloride, washed with water, and extracted with 3 N HCl. The aqueous layer was made basic with NaOH pellets and extracted with methylene chloride. The extracts were washed with brine, dried, and then evaporated to give **38** as the free base which was transformed into the dihydrochloride salt: 25% yield; mp 175−180 °C (from EtOH/ether).

***N*1,*N*22-Bis[[(5,10-dihydro-11-oxo-11*H*-dibenzo[*b*,*e*][1,4]-diazepin-5-yl)carbonyl]methyl]-*N*1,*N*22-dibenzyl-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (42).** It was obtained from **39**[16] and **40**.[22] Eluting with solvent F gave the free base that was transformed into the tetraoxalate salt: 68% yield; mp 180−182 °C (from MeOH/ether).

***N*1,*N*22-Bis[[(6,11-dihydro-5-oxo-5*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-*N*1,*N*22-dibenzyl-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (43).** It was obtained from **39**[16] and **41**.[20] Eluting with solvent D gave the free base that was transformed into the tetraoxalate salt: 46% yield; mp 185−187 °C (MeOH/ether).

General Procedure for the Synthesis of 8, 9, 12, 22, and 34. A solution of the *N*-[(benzyloxy)carbonyl]amine (0.1 mmol) in MeOH (10 mL) was hydrogenated over 10% Pd on charcoal (10% w/w) for 45 min at room temperature and a pressure of 15 psi. Following catalyst removal, the solvent was evaporated, yielding the desired compound as the free base.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]ethyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (8).** It was obtained from **31**. The free base was transformed into the dihydrochloride salt to give a hygroscopic solid: 80% yield. Anal. ($C_{36}H_{57}Cl_2N_7O_4$) C, H, N.

***N*1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]propyl]-7,16-dimethyl-6,-17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (9).** It was obtained from **33** and purified by flash chromatography. Eluting with solvent N gave an oil that was transformed into the dihydrochloride salt to give a hygroscopic solid: 70% yield. Anal. ($C_{37}H_{59}Cl_2N_7O_4 \cdot 1.5H_2O$) C, H, N.

***N*1,*N*1-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-6,-17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (12).** It was obtained from **32**. The free base was transformed into the dihydrochloride salt to give a hygroscopic solid. Anal. ($C_{50}H_{66}Cl_2N_{10}O_6 \cdot H_2O$) C, H, N.

***N*1-(*tert*-Butoxycarbonyl)-*N*15,7-dimethyl-6-oxo-7-aza-1,15-pentadecanediamine (22).** It was obtained from **21** as an oil (75% yield).

***N*1-(*tert*-Butoxycarbonyl)-7,16-dimethyl-6,17-dioxo-7,-16-diaza-1,22-docosanediamine (34).** It was obtained from **24** as an oil in quantitative yield.

General Procedure for the Synthesis of 13, 14, 16, and 18. A solution of *N*-benzylamine hydrochloride (1.0 mmol) in MeOH (30 mL) was hydrogenated over 10% Pd on charcoal (wet, Degussa type E101 NE/W) (0.5 g) overnight at room temperature and a pressure of 75 psi. Following catalyst removal, the solvent was evaporated, yielding a residue that was purified by flash chromatography, unless otherwise specified.

***N*1,*N*22-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-6,-17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (13).** It was obtained from **37** dihydrochloride. Eluting with solvent K gave the free base that was transformed into the dihydrochloride salt: 60% yield; mp 192−194 °C (from EtOH/ether). Anal. ($C_{50}H_{66}Cl_2N_{10}O_6$) C, H, N.

***N*1,*N*1,*N*22-Tris[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*]-[1,4]benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine Dihydrochloride (14).** It was obtained from **38** dihydrochloride. Eluting with solvent I gave the free base that was transformed into the dihydrochloride salt: 40% yield mp 186−188 °C (from EtOH/ether); MS (FAB) calcd for $C_{64}H_{74}N_{13}O_8$ 1152 $[M + H]^+$, found 1152. Anal. ($C_{64}H_{75}Cl_2N_{13}O_8$) C, H, N.

***N*1,*N*22-Bis[[(5,10-dihydro-11-oxo-11*H*-dibenzo[*b*,*e*][1,4]-diazepin-5-yl)carbonyl]methyl]-7,16-dimethyl-7,16-diaza-1,22-docosanediamine Tetraoxalate (16).** It was obtained from **42** (a few drops of 3 N ethanolic HCl added). The residue was mixed with water, made basic with aqueous 28% ammonia, and extracted with chloroform. Removal of dried extracts gave **16** as the free base that was transformed into the tetraoxalate salt: 80% yield; mp 182−184 °C (MeOH/ether); MS (FAB) calcd for $C_{52}H_{71}N_8O_4$ 871 $[M + H]^+$, found 871. Anal. ($C_{60}H_{78}N_8O_{20}$) C, H, N.

***N*1,*N*22-Bis[[(6,11-dihydro-5-oxo-5*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-7,16-dimethyl-7,-16-diaza-1,22-docosanediamine Tetraoxalate (18).** It was obtained from **43** (a few drops of 3 N ethanolic HCl added). The residue was mixed with water, made basic with aqueous 28% ammonia, and extracted with chloroform. Removal of dried extracts gave **18** as the free base that was transformed into the tetraoxalate salt: 80% yield; mp 191−194 °C (from MeOH/ether); MS (FAB) calcd for $C_{50}H_{69}N_{10}O_4$ 873 $[M + H]^+$, found 873. Anal. ($C_{58}H_{76}N_{10}O_{20}$) C, H, N.

***N*1-(*tert*-Butoxycarbonyl)-*N*15-[(benzyloxy)carbonyl]-*N*15,7-dimethyl-6-oxo-7-aza-1,15-pentadecanediamine (21) and *N*1-(*tert*-Butoxycarbonyl)-*N*22-[(benzyloxy)carbonyl]-7,16-dimethyl-6,17-dioxo-7,16-diaza-1,22-docosanediamine (24).** The procedure used for the synthesis of **21** is described. Ethyl chlorocarbonate (1.2 mL, 13 mmol) in dry dioxane (3 mL) was added dropwise to a stirred and cooled (5 °C) solution of *N*-(*tert*-butoxycarbonyl)-6-aminocaproic acid (3.10 g, 13 mmol) and triethylamine (1.3 g, 13 mmol) in dioxane (70 mL), followed after standing for 30 min by the addition of *N*′-[(benzyloxy)carbonyl]-*N*,*N*′-dimethyl-1,8-octanediamine[23] (4.0 g, 13 mmol) in dioxane (20 mL). After stirring at room temperature overnight, the mixture was poured into water (150 mL) and then extracted with methylene chloride (3 × 60 mL). The organic layer was washed with 2 N aqueous $KHSO_4$ (2 × 70 mL), saturated $NaHCO_3$ aqueous solution (2 × 70 mL), and brine. Removal of dried solvents gave a residue which was triturated with cyclohexane to give **21** as an oil: 4.2 g (65% yield).

Similarly, **24** was obtained from *N*-[(benzyloxy)carbonyl]-6-aminocaproic acid and **22**. The residue was purified by flash chromatography. Eluting with solvent C gave **24** as an oil: 65% yield.

***N*1-[(Benzyloxy)carbonyl]-7,16-dimethyl-6,17-dioxo-7,-16-diaza-1,22-docosanediamine (25).** A solution of **24** (2.30 g, 3.63 mmol) in THF (100 mL) was treated with 6 N HCl (20 mL) and stirred at room temperature for 2 h. THF was distilled off under reduced pressure, and the aqueous phase was extracted with ether (3 × 20 mL), made basic with 2 N NaOH, and extracted with methylene chloride (3 × 50 mL). Removal of the dried extracts gave **25** as an oil: 1.9 g (96% yield).

Biology. Functional Antagonism. Guinea pigs of either sex (200−400 g) were sacrificed by cervical dislocation under ketamine anesthesia, and the organs required were set up rapidly under a suitable resting tension in 15-mL organ baths containing physiological salt solution kept at appropriate temperature (see below) and aerated with 5% CO_2−95% O_2 at pH 7.4. Concentration−response curves were constructed by cumulative addition of the agonist.[29] 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 any variation in sensitivity.

Guinea Pig Left Atria. The heart of guinea pigs 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 tension at 35 °C in Tyrode solution of the following composition (mM): NaCl, 136.9; KCl, 5.4; $MgSO_4 \cdot 7H_2O$, 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 to the ileo-caecal junction. The tissue was cleaned, and segments 2−3 cm long of ileum longitudinal muscle were set up under 1-g 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; $KH_2PO_4 \cdot 2H_2O$, 1.19; $NaHCO_3$, 25; glucose, 11. 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 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.

Cell Culture and Binding Assays. The detailed methods have been published previously.[30−32] [^{3}H]-*N*-Methylscopolamine ([^{3}H]NMS; specific activity 79.5 Ci/mmol; NEN Du Pont) was used to evaluate binding sites in rat heart homogenates (expressing M_2 muscarinic receptors; K_d 0.32 ± 0.04 nM; B_{max} 77.8 ± 15.3 fmol/mg of protein), in rat submaxillary gland homogenates (expressing M_3 muscarinic receptors; K_d 0.48 ± 0.03 nM; B_{max} 1102 ± 85 fmol/mg of protein), and in homogenates obtained from NG 108-15 cells (expressing M_4 muscarinic receptors; K_d 0.54 ± 0.03 nM; B_{max} 190 ± 4 fmol/mg of protein). [^{3}H]Pirenzepine (specific activity 86.2 Ci/mmol; NEN Du Pont) was the tracer to label M_1 muscarinic receptor binding sites of the rat cerebral cortex (K_d 2.1 ± 0.2 nM; B_{max} 1.9 ± 0.13 pmol/mg of protein). In competition studies, fixed concentrations of 0.7−0.8 nM [^{3}H]NMS were used in rat heart, rat submaxillary gland, and NG 108-15 cells binding assays, whereas 5 nM was the concentration of [^{3}H]pirenzepine in rat cortex homogenates. Nonspecific binding was assessed in the presence of 10 μM atropine.

Competition binding studies were performed using homogenates of the indicated cells[31,32] or rat tissues[30,32] in incubation buffer (50 mM sodium phosphate, pH 7.4, enriched with 2 mM $MgCl_2$, 1% bovine serum albumin). Homogenates (200 μg of protein for cortex, 500 μg of protein for heart and submaxillary gland, 600 μg of protein for NG 108-15 cells) were incubated for 2 h at 25 °C in 1 mL of incubation buffer and the indicated concentrations of tracer and atropine. Binding assays were terminated by filtration on Whatman GF/C glass-fiber filters previously soaked in 0.1% poly(ethylenimine) and then rinsed four times with 5 mL of ice-cold 50 mM phosphate buffer (pH 7.4). Saturation binding studies were performed as indicated above, in the presence of [^{3}H]NMS (25−4000 pM) or [^{3}H]-pirenzepine (0.08−20 nM) and in the absence or presence of atropine. The results were analyzed according to the method of Scatchard.

Determination of Dissociation Constants. In functional experiments, dose ratios at the EC_{50} values of the agonists were calculated at three to six antagonist concentrations, and each concentration was tested from four to six times. The results are expressed as pA_2 values.[24,25] Data are presented as means ± SE of n experiments. Differences between mean values were tested for significance by Student's t-test.

Binding data were analyzed using LIGAND.[33] Differences in the slope of the curves were determined by the test of parallelism as described by Tallarida and Murray[34] and were not different ($p > 0.05$). Scatchard plots were linear or almost linear in all preparations. All Hill numbers (n_H) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived from the Cheng−Prusoff equation,[26] $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and the equilibrium dissociation constant of [^{3}H]NMS or [^{3}H]pirenzepine, respectively. pK_i values are the mean ± SE of three separate experiments performed in triplicate.

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