Design, Synthesis, and Biological Activity of Methoctramine-Related Tetraamines Bearing an 11-Acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one Moiety: Structural Requirements for Optimum Occupancy of Muscarinic Receptor Subtypes As Revealed by Symmetrical and Unsymmetrical Polyamines

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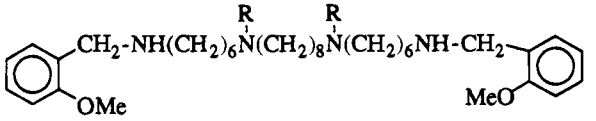
Tetraamines **5**–**13** and diamines **14**–**17** as well as monoamine **18** were synthesized, and their biological profiles at muscarinic receptor subtypes were assessed by functional experiments in isolated guinea pig left atrium (M_2) and ileum (M_3) and by binding assays in rat cortex (M_1), heart (M_2), and submaxillary gland (M_3) homogenates and NG 108–15 cells (M_4). An appropriate number and type of substituents on the terminal nitrogens of a tetraamine backbone afforded compounds, such as tripitramine (**8**) and dipitramine (**6**), which are endowed with different affinity and selectivity profiles. Tripitramine, a nonsymmetrical tetraamine, resulted in the most potent and the most selective M_2 muscarinic receptor antagonist so far available ($pA_2 = 9.75 \pm 0.02$; $pK_i = 9.54 \pm 0.08$). However, it failed to discriminate between M_1 and M_4 muscarinic receptor subtypes (selectivity ratio: M_2/M_3, 1600–2200; M_2/M_1, 81; M_2/M_4, 41; M_1/M_3, 28; M_4/M_3, 55; M_4/M_1, 2). Dipitramine, another nonsymmetrical tetraamine bearing two substituents on the same terminal nitrogen, displayed the highest affinity for M_1 muscarinic receptors ($pK_i = 8.60 \pm 0.15$) and was able to differentiate, unlike **8**, all four muscarinic receptor subtypes investigated (selectivity ratio: M_1/M_2, 5; M_1/M_3, 2700; M_1/M_4, 76; M_2/M_3, 260–520; M_2/M_4, 15; M_4/M_3, 35). The results are discussed in terms of a possible mode of interaction of tetraamines with muscarinic receptor subtypes.

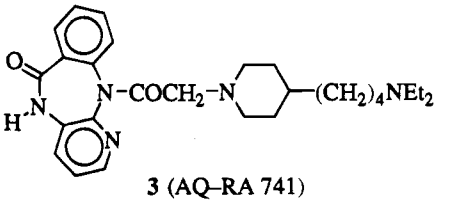
Introduction

Achievement of receptor subtype selectivity is a formidable challenge to medicinal chemists. The task is inherently difficult because the primary structure of the subtypes of a given receptor family may differ in a relatively low number of amino acids, thus complicating prediction of the structural requirements that confer selectivity for one receptor subtype over another. Molecular biology studies have demonstrated that muscarinic receptors are comprised of at least five different subtypes (m_1–m_5) which share about 70% identity of amino acids in the seven transmembrane segments.[1-5] Only four of these subtypes (designated as M_1–M_4 muscarinic receptors) have been so far characterized from a pharmacological point of view.[6] The other cloned receptor (m_5) is not well-characterized yet owing to a lack of selective ligands, which emphasizes clearly the need to acquire knowledge about structural elements for optimum interaction with muscarinic receptor subtypes. In fact, the antagonists currently used to classify and characterize muscarinic receptors lack a clear subtype selectivity.[6,7]

Among selective muscarinic receptor antagonists, polymethylenetetraamines, the prototype of which is methoctramine (**1**), have been thoroughly used as a tool in muscarinic receptor subtypes characterization and classification owing to their high affinity for M_2 receptors, low affinity for M_3 receptors, and intermediate affinity for M_1 receptors.[6,8-10] It was shown that M_2 muscarinic receptor selectivity of methoctramine is dramatically dependent on a tetraamine backbone.[11] Removal of only one amine function, affording a triamine, or changing an amine function into an amide or ether group caused a large drop in selectivity.[11] On the other hand, affinity depends on the type of substituents on the terminal nitrogens.[12,13] Optimum potency toward M_2 muscarinic receptors is associated, among benzyl-type substituents, with a 2-methoxybenzyl (MB) moiety, as in methoctramine, although its replacement by a furylmethyl group, affording furtramine, did not affect both affinity and selectivity.[12] In spite of its marked M_2 selectivity, methoctramine lacks receptor specificity since it inhibits, albeit with modest affinity, other receptor systems, such as α_1- and β_1-adrenoreceptors, and muscular and neuronal nicotinic receptors.[8,14,15] Furthermore, methoctramine, like all other M_2 selective antagonists available to date, fails to discriminate between M_2 and M_4 muscarinic receptors,



1 (methoctramine): R = H
2: R = Me



3 (AQ-RA 741)

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thus preventing a clear pharmacological characterization of muscarinic receptor subtypes.

Design Rationale

These considerations prompted us to modify methoctramine structure in order to improve affinity for M_2 muscarinic receptors such as to better discriminate among muscarinic receptor subtypes.

The starting point of this investigation was the observation that the two amine functions of AQ-RA 741 (11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one, **3**), an analogue of pirenzepine displaying selectivity for M_2 muscarinic receptors,[16] are located at such a distance that they can be superimposed to one inner and one outer nitrogen of methoctramine when comparison is made between the extended conformations of the two prototypes. The observation that diamines, obtained by truncating in two halves the methoctramine structure and which are closely related to AQ-RA 741, resulted in analogues almost devoid of affinity and selectivity toward M_2 muscarinic receptors was another consideration of paramount importance in the design strategy of our compounds.[10,11] Clearly, an 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (PBD) moiety recognizes better than an MB group the binding pocket of M_2 muscarinic receptors. Thus, we thought that the insertion of a tricyclic system on the terminal nitrogens of a tetraamine would improve affinity for M_2 muscarinic receptors. To prevent *N*-alkylation of the two inner nitrogens, we choose as a common backbone a tetraamine bearing on these nitrogens a methyl group. Furthermore, this choice was dictated by a previous finding that methylation of inner nitrogens of methoctramine affording *N*,*N*′-dimethylmethoctramine (**2**) does not affect both affinity and selectivity for M_2 muscarinic receptors.[12]

The finding that replacing a hydrogen of a terminal nitrogen of the tetraamine backbone with a PBD group as in **5** markedly increased affinity and selectivity gave us the opportunity to verify whether polymethylenetetraamines act as divalent ligands, which can be defined as molecules that incorporate two discrete pharmacophores linked through a spacer (Figure 1). It was advanced that the terminal nitrogens of methoctramine may interact with two binding sites having possibly similar if not identical structural requirements.[10] The two pharmacophores might be either the two *N*-(2-methoxybenzyl)-1,6-hexanediamine moieties (pharmacophore a) or the two terminal *N*-(2-methoxybenzyl) groups (pharmacophore b) of methoctramine whereas the spacer between the two pharmacophores is represented by the polymethylene chain which, in turn, could be responsible for selectivity. To test this hypothesis we synthesized all possible symmetrical and unsymmetrical tetraamines bearing one to four PBD groups on the terminal nitrogens (**5**–**9**). Furthermore, a contribution, if any, of MB groups was evaluated by synthesizing tetraamines bearing on the terminal nitrogens either PBD and/or MB groups (**10**–**13**). The observation that tripitramine (**8**), the most potent and selective among polyamines so far available, is an unsymmetrical tetraamine prompted us to synthesize monoamine **18**, which bears two PBD groups, and *N*-substituted (**14** and **16**) and *N*,*N*-disubstituted (**15** and **17**) diamines to verify whether the binding pocket, that is the active site of M_2 muscarinic receptors, may recognize optimally ligands bearing only one, like AQ-RA 741 (**3**), or, alternatively, two PBD groups. This information would help, in turn, in defining the structural requirements needed to recognize the second binding site where polymethylenetetraamines likely bind. The design strategy for our compounds is shown in Figure 1.

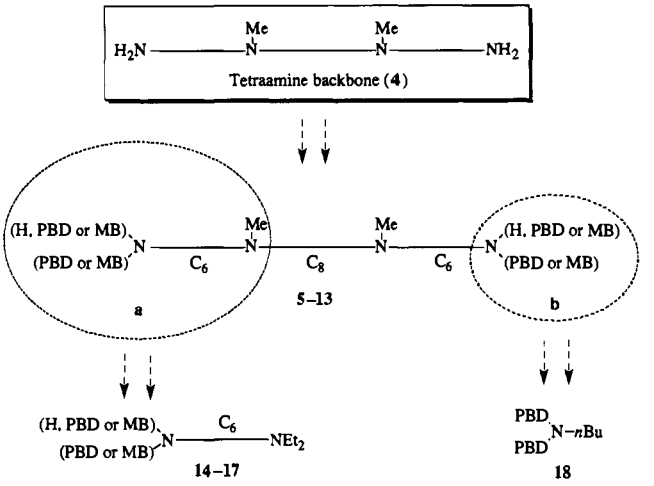


Figure 1. Design strategy for the synthesis of hybrid structures by inserting the structural features of methoctramine (1) and AQ-RA 741 (**3**), that is 2-methoxybenzyl (MB) and 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (PBD) groups, respectively, on the terminal nitrogens of a tetraamine backbone. Circles indicate that *N*-substituted or *N*,*N*-disubstituted 1,6-hexanediamine (a) and *N*,*N*-disubstituted alkylamine (b) are possible pharmacophores of tetraamines considered as divalent ligands. In a divalent ligand two molecules of a pharmacophore are linked by a spanning chain.

We describe here the synthesis and the pharmacological profile of tetraamines **5**–**13**, diamines **14**–**17**, and monoamine **18** in functional and binding experiments.

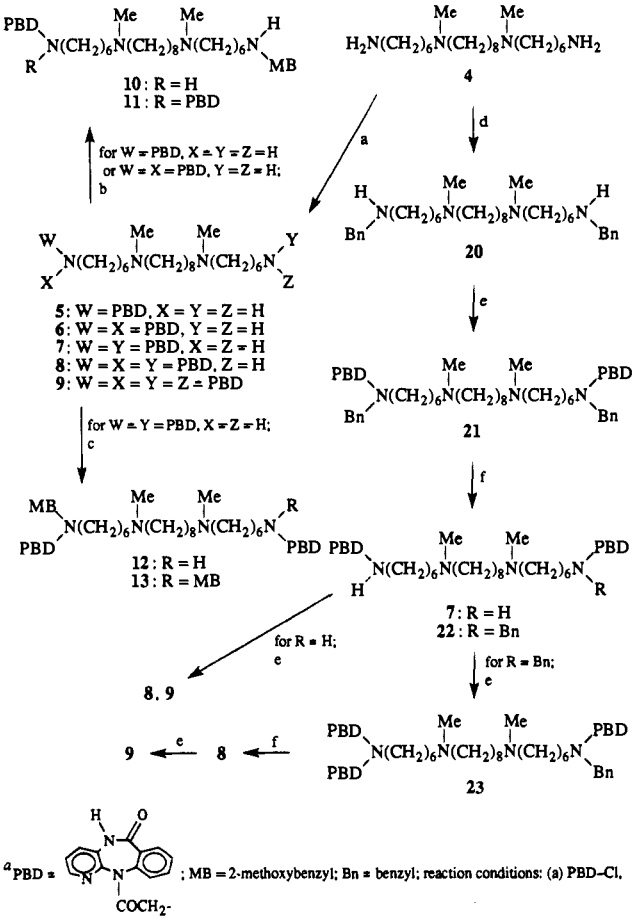
A preliminary communication dealing with tripitramine (**8**) has been published recently.[17]

Chemistry

All the compounds were synthesized by standard procedures (Schemes **1** and **2**) and were characterized by IR, ^{1}H NMR, and elemental analysis.

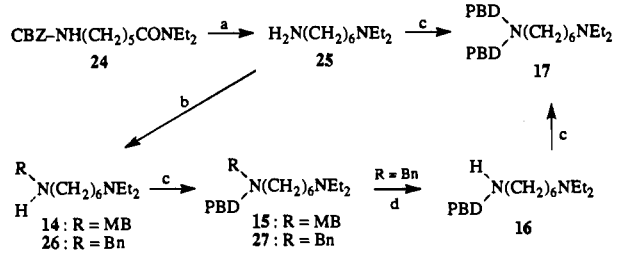
Taking advantage from the fact that amines suffer polyalkylation, we used tetraamine **4** as a key intermediate to synthesize all possible *N*-substituted tetraamines (Scheme **1**). Thus, reaction of **4**[12] with **1** equiv of 11-(chloroacetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-6-one[18] (PBD-Cl) in methylene chloride gave mainly *N*-monosubstituted and *N*,*N*-disubstituted tetraamines **5** and **6** (dipitramine) together with a small amount, revealed on TLC but not isolated, of the other possible tetraamines **7**–**9** which were separated by column chromatography, whereas the reverse was true by performing the reaction with 2 equiv of PBD-Cl in DMF. However, in order to confirm the structure and to improve the yield, tetraamines **7**–**9** were synthesized by an independent synthetic scheme. To this end, the terminal primary amine functions of **4** were protected by benzyl groups through condensation with benzaldehyde and subsequent reduction with $NaBH_4$ of inter-

Scheme 1. Synthesis of Symmetrical and Unsymmetrical Tetraamines **5**–**13**[a]



[a] PBD = ; MB = 2-methoxybenzyl; Bn = benzyl; reaction conditions: (a) PBD–Cl, CH_2Cl_2 (for **5** and **6**) or DMF (for **7**–**9**), rt, 72 h; (b) 2-$MeOC_6H_4CHO$, EtOH, rt, 30 min; then $NaBH_4$, EtOH, rt, 4 h; (c) MB–Cl, DMF, NEt_3, rt, 6 h; (d) C_6H_5CHO, benzene, reflux, 6 h; then $NaBH_4$, EtOH, rt, 3 h; (e) PBD–Cl, DMF, NEt_3, KI, rt, 72 h; (f) catalytic hydrogenation over 10% Pd on charcoal at 5 atm, EtOH, 3N etanolic HCl (few drops), 50 °C, 12 h.

Scheme 2. Synthesis of Diamines **14**–**17**[a]



[a] PBD, MB and Bn as in Scheme I; CBZ = $C_6H_5CH_2OCO$–; reaction conditions: (a) catalytic hydrogenation over 10% Pd on charcoal, EtOH, rt, 1 h; (b) 2-$MeOC_6H_4CHO$ or C_6H_5CHO, benzene, reflux, 7 h; then $NaBH_4$, EtOH, rt, 3 h; (c) PBD–Cl, DMF, rt, 72 h; (d) catalytic hydrogenation over 10% Pd on charcoal, MeOH, 3N ethanolic HCl (few drops), rt, 12 h.

mediate Schiff base to give **20**. Resulting *N,N'*-dibenzyl tetraamine **20** was alkylated with 2 equiv of PBD-Cl to give **21**. Removal of *N*-benzyl groups under controlled conditions by catalytic hydrogenation over 10% palladium on charcoal afforded a mixture of **7** and *N*-benzyl tetraamine **22**. Reaction of **22** with **1** equiv of PBD-Cl gave the intermediate *N*-benzyl tetraamine **23** which, in turn, was converted to tripitramine (**8**) by catalytic hydrogenation. Tetrasubstitued tetraamine **9** was obtained from **8** by alkylation with PBD-Cl. Alternatively, alkylation of **7** with **1** equiv of PBD-Cl in methylene chloride gave a mixture of **8** and **9** which was separated by column chromatography. The structures of **5** and **6** were confirmed by the introduction of a 2-methoxybenzyl (MB) group on their terminal primary amine function through condensation with 2-methoxybenzaldehyde and subsequent reduction with $NaBH_4$ of the intermediate Schiff base to give **10** or **11**. Tetraamine **13** was obtained by reaction of **7** with 2 equiv of 2-methoxybenzyl chloride, whereas the reaction with **1** equiv of 2-methoxybenzyl chloride gave a mixture of **12** and **13** which was separated by column chromatography.

N-[(Benzyloxy)carbonyl]-6-aminocaproic acid was amidated with diethylamine to give **24** (Scheme 2). The protecting group was removed by hydrogenolysis followed by the reduction of the amide group with borane–methyl sulfide complex to yield diamine **25**. The benzyl or 2-methoxybenzyl group was easily introduced by condensation of **25** with benzaldehyde or 2-methoxybenzaldehyde and subsequent reduction of the intermediate Schiff bases to yield **26** and **14** which, in turn, were alkylated with PBD-Cl to give **27** and **15**, respectively. Removal of the *N*-benzyl group of **27** by hydrogenolysis gave **16** which was transformed into **17** by alkylation with PBD-Cl. Finally, amine **18** was synthesized by alkylation of *n*-butylamine with PBD-Cl.

Biology

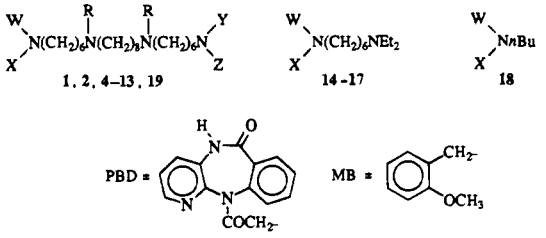
Functional Studies. The biological activity of compounds listed in Table **1** at peripheral muscarinic receptors was assessed by antagonism of carbachol-induced contractions of isolated guinea pig ileum (M_3 receptors) and by antagonism of carbachol-induced inhibition of electrically stimulated guinea pig left atria (M_2 receptors). In order to allow comparison of the results, methoctramine (**1**), its *N,N'*-dimethyl analogue (**2**), and AQ-RA 741 (**3**) were used as the standard compounds together with the unsubstituted tetraamine **4**[12] and the mono 2-methoxybenzyl analogue (**19**)[11] of methoctramine (**1**). The biological results were expressed as pA_2 values determined from Schild plots[19] constrained to slope −1.0,[20] 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. However, for compounds with low potency, dissociation constants derived from only one concentration were calculated according to van Rossum.[21]

Binding Experiments. Receptor subtype selectivity of selected tetraamines was further determined by employing receptor binding assays. [^{3}H]-*N*-Methylscopolamine ([^{3}H]NMS) was used to label M_2, M_3, and M_4 muscarinic receptors binding sites of rat heart, rat submaxillary gland, and NG 108-15 cell homogenates, respectively. [^{3}H]Pirenzepine was the tracer to label M_1 muscarinic receptors binding sites of the rat cerebral cortex. Binding affinities were expressed as pK_i values derived using the Cheng–Prusoff equation.[22] Methoctramine (**1**), AQ-RA 741 (**3**), and pirenzepine were used as the standard compounds.

Results and Discussion

The biological activity, expressed as pA_2 values, at peripheral M_2 and M_3 muscarinic receptors of compounds used in the present study is shown in Table **1**. In order to make relevant considerations on structure–activity relationships prototypes **1**–**4** and **19** were

Table 1. pA_2 Values in the Isolated Guinea Pig Left Atrium (M_2) and Ileum (M_3) Muscarinic Receptors



no.[a]	R	W	X	Y	Z	pA_2[b] M_2	pA_2[b] M_3	selectivity ratio[c] M_2/M_3
1 (methoctramine)	H	MB	H	MB	H	7.82 ± 0.01	6.32 ± 0.07	32
2	Me	MB	H	MB	H	7.90 ± 0.02	6.22 ± 0.04	48
3 (AQ-RA 741)						8.03 ± 0.02	7.0 ± 0.02	11
4	Me	H	H	H	H	6.55 ± 0.08	5.12 ± 0.06[d]	27
5	Me	PBD	H	H	H	8.09 ± 0.07	6.18 ± 0.03	81
6 (dipitramine)	Me	PBD	PBD	H	H	8.23 ± 0.04	5.81 ± 0.06	260
7	Me	PBD	H	PBD	H	9.14 ± 0.01	6.90 ± 0.06	170
8 (tripitramine)	Me	PBD	PBD	PBD	H	9.75 ± 0.02	6.55 ± 0.01	1600
9	Me	PBD	PBD	PBD	PBD	7.81 ± 0.05	6.12 ± 0.06	49
10	Me	PBD	H	MB	H	8.51 ± 0.09	6.24 ± 0.02	190
11	Me	PBD	PBD	MB	H	8.34 ± 0.09	6.35 ± 0.04	98
12	Me	PBD	H	MB	PBD	8.80 ± 0.05	6.38 ± 0.04	260
13	Me	PBD	MB	MB	PBD	7.32 ± 0.03	5.69 ± 0.07[d]	26
14		MB	H			5.60 ± 0.02[d]	<5[e]	>4
15		MB	PBD			6.75 ± 0.02[d]	5.90 ± 0.10[d]	7
16		PBD	H			6.60 ± 0.02[d]	6.08 ± 0.05[d]	3
17		PBD	PBD			5.98 ± 0.07[d]	<5[e]	>10
18		PBD	PBD			5.80 ± 0.06[d]	<5[e]	>6
19	H	MB	H	H	H	7.04 ± 0.10	5.81 ± 0.09[d]	17

[a] **1–4**, **19**, tetrahydrochlorides; **5–13**, tetraoxalates; **14–17**, dihydrochlorides; **18**, hydrochloride. [b] pA_2 values ± SE were calculated from Schild plots,[19] constrained to slope −1.0.[20] 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 at least at three different antagonist concentrations, and each concentration was tested from four to eight times. Dose ratio (DR) values represent the ratio of the potency of the agonist carbachol (ED_{50}) in the presence of the antagonist and in its absence. The parallelism of dose–response curves was checked by linear regression, and the slopes were tested for significance ($p < 0.05$). [c] The selectivity ratio is the antilog of the difference between the pA_2 values at left atrium and ileum muscarinic receptors, respectively. [d] Calculated according to Van Rossum[21] at only one concentration (10 μM). [e] Inactive up to a concentration of 10 μM.

included for comparison. All polyamines 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). By taking as a starting point the unsubstituted tetraamine **4**, it is possible to observe how affinity and selectivity for M_2 and M_3 muscarinic receptors can be markedly affected by replacing the hydrogens of the two terminal nitrogens by MB or PBD groups.

An analysis of the results reveals that the unsubstituted tetraamine **4** is a rather weak antimuscarinic, but it has a significant M_2 selectivity. Thus, it appears that a tetraamine backbone plays a role in M_2 muscarinic receptor recognition. It can be seen that insertion of one MB or PBD group affording **19**[11] or **5** gives rise to a marked difference in affinity for M_2 and M_3 muscarinic receptors unlikely due to the presence (**5**) or absence (**19**) of *N*-methyl groups on the inner nitrogens of the tetraamine backbone because we have demonstrated[12] that this structural modification does not affect affinity for at least M_2 and M_3 muscarinic receptor subtypes. Tetraamine **5** was 1 and 0.4 log units more potent than **19** at M_2 and M_3 receptors, respectively. This finding clearly indicates that a PBD serves better than an MB group to recognize M_2 muscarinic receptors.

Next, the insertion of a second PBD or MB group on the terminal unsubstituted nitrogen of **5** affording **7** or **10**, respectively, caused a significant increase in both affinity and selectivity for M_2 muscarinic receptors in comparison to **5** and **19**. Again, however, a PBD group appears superior to an MB group since a comparison of **2**, **10**, and **7** reveals that the potency toward M_2 muscarinic receptors increases significantly by replacing MB groups of **2** with PBD groups (compare **2** with **10** and **7**; $pA_2 = 7.90$, 8.51, and 9.14, respectively), whereas the biological profile at M_3 muscarinic receptors is slightly affected ($pA_2 = 6.22$, 6.24, and 6.90, respectively). Concerning tetraamines bearing both MB and PBD groups, the insertion of an additional PBD group on the terminal nitrogens of **10** giving **11** and **12** did not alter markedly the biological profile at both M_2 and M_3 muscarinic receptors whereas the insertion of two PBD groups on **2** affording **13** resulted in a loss of potency at both muscarinic receptor subtypes. A different result was observed by inserting an additional PBD group on **6** or **7** since **8** resulted in the most potent and the most selective M_2 muscarinic receptor antagonist so far available. The insertion of an additional PBD group in **8**, affording the tetrasubstituted tetraamine **9**, caused a dramatic loss of affinity for M_2 muscarinic receptors although **9** was still as active and selective as methoctramine (**1**) and its *N*,*N*′-dimethyl analogue **2**. Interestingly, among the two series of tetraamines, **5–9** and **10–13**, respectively, the tetrasubstituted ones **9** and **13** were the less potent toward M_2 muscarinic receptors. This finding may be relevant to the under-

standing of the mode of interaction of methoctramine (1)-related polymethylenetetraamines at M_2 muscarinic receptors.

We advanced the hypothesis that polymethylenetetraamines may behave as divalent ligands, which can be defined as molecules that incorporate two discrete pharmacophores linked through a spacer (Figure 1).[10] This reasoning was based on the evident symmetry of polymethylenetetraamines and it was argued that the two 1,6-hexanediamine moieties or simply the two terminal *N*-substituted groups of methoctramine (1) may act as pharmacophores which recognize two similar if not identical sites on M_2 muscarinic receptors separated by a distance corresponding to the length of the spacer between the two pharmacophores of polymethylenetetraamines. The finding that tripitramine (8), the most active and selective member of the series, is, contrary to the parent methoctramine (1), a nonsymmetrical tetraamine suggests clearly that the two possible pharmacophores included in its structure are unlikely to recognize two similar binding sites on M_2 muscarinic receptors. Furthermore, the observation that these two binding sites may have different structural requirements raises the intriguing question of which tripitramine (8) terminus interacts with the M_2 muscarinic receptor active binding site and which terminus recognizes a second binding site. In an attempt to solve this problem, diamines **14**–**17** and amine **18** were investigated. It is evident that all these compounds, which are structurally related to AQ-RA 741 (3), were modest antagonists at both M_2 and M_3 muscarinic receptors (Table 1). This finding parallels that observed[10,11] by truncating in two halves the methoctramine (1) structure, thus confirming the need of four amine functions for optimum interaction at M_2 muscarinic receptors. However, the dramatically lower potency of **17** compared to both **3** and **8** may help in elucidating the possible binding sites of tetraamines. In fact, if the terminal *N*,*N*-disubstituted nitrogen of **8** interacts with the M_2 muscarinic receptor active binding site recognized by **3**, one would expect the diamine **17** to be more active than the diamine **16** due to an additional interaction of the second PBD group, but this was not the case. Similarly, amine **18** was 4 orders of magnitude less potent than tripitramine (8). These results might be interpreted by admitting that tripitramine (8) interacts with the active binding site thanks to its *N*-substituted terminal nitrogen, which is closely related to the AQ-RA 741 (3) structure, and to a second accessory site with the other *N*,*N*-disubstituted terminal nitrogen. Furthermore, the low potency of **17** and **18** for the accessory site might indicate that the occupation of only that site without binding at the primary site is not sufficient to induce a strong antagonism.

This working hypothesis might be a useful tool to study the molecular mechanisms controlling the folding and assembly of muscarinic receptors. It was advanced that, as a general rule, G protein-coupled receptors consist of at least two independent folding domains.[23,24] Studies with cloned muscarinic receptor subtypes have shown two receptor fragments, transmembrane (TM) domains I-V, and VI and VII, respectively, that behave as independent folding domains.[24] Furthermore, it was advanced that multiple domains of the cloned m_2 muscarinic receptor, rather than a distinct sequence element, are involved in conferring high affinity binding to antagonists such as AQ-RA 741 (3).[23] Owing to the high homology of muscarinic receptor subtypes, especially at the level of TM domains where the binding of agonists and antagonists is supposed to take place,[6] it is possible that also extracellular domains might play a role in determining the selectivity of tripitramine (8), in agreement with the hypothesis advanced for related polymethylene tetraamines.[10] Thus, the outstanding M_2 selectivity displayed by tripitramine (8) might be due to an optimal spacer chain length between its two terminal nitrogens that allows an optimal interaction with the two binding domains in M_2 muscarinic receptors, but not in other muscarinic receptor subtypes. Alternatively, it might be speculated that the M_2 selectivity of tripitramine (8) is the result of an interaction with two distinct receptor domains localized on TM I-V and TM VI and VII, respectively. On the basis of the results discussed above, the monosubstituted terminal nitrogen of tripitramine (8) should interact with a domain in TM I-V, which are supposed to bear the active binding site, and the other terminal nitrogen with TM VI and VII, which should contain a second binding site with major structural determinants of ligand binding selectivity.[24] To determine the potential importance, if any, of the different receptor domains for subtype-selective tripitramine (8) binding, it would be of interest to study chimeric muscarinic receptors in which single domains are exchanged, to allow a definition of the structural determinants that contribute to the subtype specificity.

The binding affinities, 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 tetraamines **5**–**13** are shown in Table 2 and Figure 2 in comparison to those of methoctramine (1), AQ-RA 741 (3), and pirenzepine. 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 experiments, the only exception being tetraamines **12** and **13**. However, we have no explanation for this discrepancy. All tetraamines, like methoctramine (1), displayed a much higher affinity for M_2 than for M_3 muscarinic receptors.

A detailed analysis of the binding affinities reveals that the structural modifications performed on tetraamine **4** by inserting PBD and/or MB groups resulted in a different effect at M_1 and M_4 muscarinic receptors. All tetraamines (**5**–**13**) were more potent than both prototypes, methoctramine (1) and AQ-RA 741 (3), at M_1 muscarinic receptors, whereas they were more potent (**7** and **12**), as active as (**5**, **8** and **11**), or less potent (**6**, **9**, and **10**) than **1** and **3** at M_4 muscarinic receptors. Concerning M_1 muscarinic receptors, the insertion of PBD groups on the terminal nitrogens of **4** gave different results from those observed at M_2 muscarinic receptors. In fact, the presence of one (**5**), two (**6** and **7**), or four (**9**) PBD groups on the tetraamine backbone resulted in a significant increase in affinity in comparison to **1**, whereas tripitramine (8), which bears three PBD groups, was almost as active as **1**, in contrast to the observed 50-fold increase in affinity toward M_2 muscarinic receptors. The finding that symmetrical tetraamines **7** and **9** as well as **13** were

Table 2. 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

	pK_i[a]				
no.	M_1	M_2	M_3	M_4	affinity profile[b]
1	7.43 ± 0.11	7.84 ± 0.09	5.96 ± 0.18	7.58 ± 0.13	$M_2 = M_4 = M_1 \gg M_3$
3	6.98 ± 0.18	8.49 ± 0.08	6.60 ± 0.14	7.82 ± 0.15	$M_2 \geq M_4 > M_1 = M_3$
5	8.57 ± 0.14	8.37 ± 0.07	6.33 ± 0.15	7.74 ± 0.08	$M_1 = M_2 \geq M_4 > M_3$
6	8.60 ± 0.15	7.89 ± 0.06	5.17 ± 0.13	6.72 ± 0.10	$M_1 > M_2 > M_4 \gg M_3$
7	8.77 ± 0.14	8.62 ± 0.14	6.76 ± 0.31	8.52 ± 0.07	$M_1 = M_2 = M_4 \gg M_3$
8	7.63 ± 0.09	9.54 ± 0.08	6.19 ± 0.14	7.93 ± 0.11	$M_2 \gg M_4 = M_1 \gg M_3$
9	8.32 ± 0.09	7.66 ± 0.10	6.81 ± 0.08	7.05 ± 0.06	$M_1 \geq M_2 \geq M_4 = M_3$
10	7.55 ± 0.19	8.77 ± 0.09	6.10 ± 0.17	7.24 ± 0.10	$M_2 > M_1 = M_4 > M_3$
11	7.80 ± 0.11	7.73 ± 0.12	5.60 ± 0.17	7.90 ± 0.09	$M_4 = M_1 = M_2 \gg M_3$
12	8.73 ± 0.13	8.80 ± 0.06	d	8.22 ± 0.12	$M_2 = M_1 \geq M_4 \gg M_3$
13	8.90 ± 0.14	8.43 ± 0.08	6.60 ± 0.16	7.80 ± 0.09	$M_1 = M_2 \geq M_4 > M_3$
PZ[c]	8.19 ± 0.08	6.10 ± 0.09	6.76 ± 0.10	7.46 ± 0.17	$M_1 > M_4 \geq M_3 \geq M_2$

[a] Values are the mean ± SE of at least three separate experiments performed in triplicate. All Hill numbers (nH) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived using the Cheng−Prusoff equation.[22] [^{3}H]NMS was used to label muscarinic receptors in rat heart, rat submaxillary gland, and NG 108−15 cell binding assays, whereas [^{3}H]pirenzepine was the tracer in rat cortex homogenates. Scatchard plots were linear or almost linear in all preparations tested. [b] Differences in antagonistic affinities for muscarinic receptor subtypes by a factor of ≤3, >3 ÷ 5, >5 ÷ 20, and >20 are indicated by =, ≥, >, and ≫, respectively. [c] PZ, pirenzepine. [d] It was not possible to calculate the IC_{50} value because the percent inhibition of [^{3}H]NMS specific binding was lower than 50% up to a concentration of 10 μM.

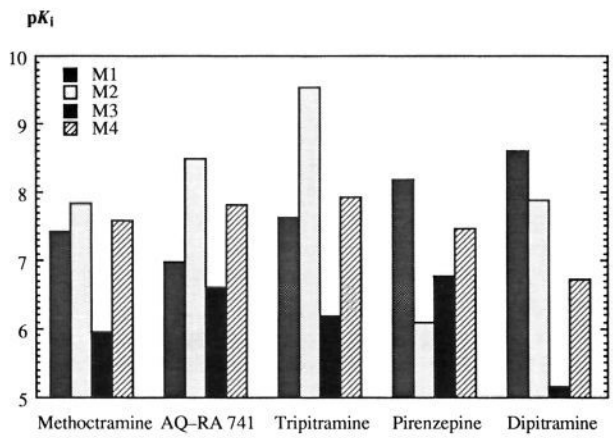


Figure 2. Affinity constants (pK_i) in rat cortex (M_1), heart (M_2), and submaxillary gland (M_3) and NG 108−15 cell (M_4) muscarinic receptor subtypes of tripitramine (**8**) and dipitramine (**6**) in comparison to methoctramine (**1**), AQ-RA 741 (**3**), and pirenzepine.

more potent or at least as active as unsymmetrical tetraamines may indicate that their terminal nitrogens, contrary to the situation observed at M_2 muscarinic receptors, interact with similar M_1 muscarinic receptor binding sites. Another difference between M_1 and M_2 muscarinic receptors is the role of the MB group. The insertion of an MB group on the unsubstituted terminal nitrogen of **5** or **6**, affording **10** and **11**, respectively, resulted in a significant decrease in affinity for M_1 muscarinic receptors, whereas there was an increase (**10**) or retention (**11**) of affinity at M_2 muscarinic receptors. In contrast, the insertion of one or two additional MB groups on **7**, affording **12** and **13**, respectively, resulted in the same effect at both M_1 and M_2 muscarinic receptors. Interestingly, the insertion of an additional PBD group in **7**, leading to tripitramine (**8**), gave a significant increase in affinity at M_2 muscarinic receptors but a 10-fold decrease in affinity for the M_1 subtype.

Concerning M_4 muscarinic receptors, disubstituted tetraamine **7** was the most potent, which may suggest that the terminal nitrogens of tetraamines recognize two similar binding sites on M_4 muscarinic receptors as for M_1 muscarinic receptors. Most interestingly, the structural isomer of **7**, that is dipitramine (**6**), which bears the two PBD groups on the same terminal nitrogen, was the least active at M_4 muscarinic receptors and one of the most potent among tetraamines investigated at M_1 muscarinic receptors. It is evident from Figure 2 that dipitramine (**6**) is able to differentiate significantly all four muscarinic receptors (M_1−M_4) and, in particular, it has an M_1/M_4 selectivity ratio much higher than that of pirenzepine (76 vs 5). In addition, dipitramine (**6**) displayed an outstanding M_1/M_3 selectivity as revealed by the selectivity ratio value of 2700.

Present binding and functional data (Tables 1 and 2) indicate clearly that PBD and MB groups play a key role in both affinity and selectivity for muscarinic receptor subtypes. It is evident that an appropriate substitution of the terminal nitrogens of tetraamine **4** affords potent antimuscarinics that display different selectivity profiles. Among the tetraamines so far investigated, tripitramine (**8**) and dipitramine (**6**) possess outstanding properties in comparison to the standards methoctramine (**1**), AQ-RA 741 (**3**), and pirenzepine in binding assays as graphically shown in Figure 2. Whereas all M_2 selective antagonists available to date bind to M_2 and M_4 muscarinic receptors with similar affinities,[7] tripitramine (**8**) discriminates significantly between these two subtypes with a selectivity ratio value of 41, which is much higher than those of **1** and **3** (2 and 5, respectively). Furthermore, tripitramine (**8**) has an outstanding affinity for M_2 muscarinic receptors, as revealed by its pA_2 and pK_i values of 9.75 ± 0.02 and 9.54 ± 0.08, respectively, that were from 1 to 2 orders of magnitude higher than those of **1** and **3**. In addition, tripitramine (**8**) also distinguishes significantly between the other muscarinic receptor subtypes (M_1−M_3), as revealed by the selectivity ratio values (M_2/M_3, 1600−2200; M_2/M_1, 81). However, tripitramine (**8**) fails to discriminate between M_1 and M_4 muscarinic receptors.

In contrast to tripitramine (**8**), dipitramine (**6**) displayed, among the muscarinic receptor subtypes investigated, the highest affinity toward the M_1 subtype with a pK_i value of 8.60 ± 0.15, which was higher than that observed for pirenzepine (pK_i, 8.19 ± 0.08). Furthermore, dipitramine (**6**) possesses a unique binding profile

since it is able to discriminate, unlike **8**, between M_1 and M_4 muscarinic receptors as well as among all other muscarinic receptor subtypes investigated as revealed by the selectivity ratio values (M_1/M_2, 5; M_1/M_3, 2700; M_1/M_4, 76; M_2/M_3, 260–520; M_2/M_4, 15; M_4/M_3, 35). Thus, dipitramine (**6**) appears to be superior to pirenzepine in discriminating M_1 and M_4, and M_1 and M_3 muscarinic receptor subtypes. The fact that the antagonists currently used to classify muscarinic receptors lack a clear subtype selectivity may explain the difficulties in characterizing with classic pharmacological studies the five cloned muscarinic receptors. The results presented in this paper clearly show that the use of tripitramine (**8**) combined with that of dipitramine (**6**), which is able to discriminate between M_1 and M_4, might eventually allow the pharmacological identification of muscarinic receptor subtypes.

Conclusions

The most striking result of the present investigation was the finding that an appropriate number of PBD groups on the terminal nitrogens of a tetraamine backbone leads to compounds, such as tripitramine (**8**) and dipitramine (**6**), which are able to differentiate among muscarinic receptor subtypes while displaying an inverse affinity profile. To our knowledge, tripitramine (**8**) represents, until now, the most potent and the most selective M_2 muscarinic receptor antagonist in both functional (M_2 and M_3) and binding assays (M_1–M_4), whereas dipitramine (**6**) is the first antagonist able to discriminate in binding assays all four pharmacologically characterized muscarinic receptor subtypes (M_1–M_4). Another additional, interesting finding is the observation that those structural manipulations which affect affinity for M_1, M_2, and M_4 muscarinic receptors are not able to increase affinity toward M_3 muscarinic receptors. It seems that a tetraamine backbone does not represent a suitable carrier for optimal interaction with the latter receptor subtype.

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. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), 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. R_f values were determined with silica gel TLC plates (Kieselgel 60 F_{254}, 0.25-mm layer thickness, Merck). The term "dried" refers to the use of anhydrous sodium sulfate.

Synthesis of Tetraamines 20–23. These were synthesized as described in a preliminary communication.[17]

1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (5), 1,1-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (6), 1,24-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (7), 1,1,24-Tris[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (8), and 1,1,24,24-Tetrakis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (9). These were obtained by different methods as it follows.

A. A mixture of tetraamine **4**[12] (as free base) (0.5 g, 1.35 mmol) and PBD-Cl[18] (0.39 g, 1.35 mmol) in CH_2Cl_2 (120 mL) was stirred at room temperature for 36 h. Removal of the solvent gave a residue that was purified by flash chromatography using chloroform/methanol/aqueous 30% ammonia (8:1.8:0.2) as the eluting solvent. The first fraction was **6** as the free base that was transformed into the tetraoxalate salt and crystallized from EtOH/ether: 13% yield; R_f 0.07; mp 132–134 °C, ^{1}H NMR (DMSO-d_6) δ 1.03–1.41 (m, 18), 1.45–1.72 (m, 10), 2.32–2.50 (m, 2), 2.67 (s, 6), 2.71–2.81 (m, 2), 2.83–3.05 (m, 10), 3.23 (br s, 1), 3.29–3.41 (m, 1), 3.55 (br s, 10, exchangeable with D_2O), 7.30–7.52 (m, 6), 7.59–7.72 (m, 4), 7.79 (d, 2), 8.10–8.20 (m, 1), 8.21–8.34 (m, 1), 10.73–10.91 (m, 2, exchangeable with D_2O). Anal. ($C_{50}H_{68}N_{10}O_4 \cdot 4H_2C_2O_4 \cdot 2H_2O$) C, H, N.

The second fraction was **5** as the free base that was transformed into the tetraoxalate salt and crystallized from EtOH/MeOH: 20% yield; R_f 0.18; mp 143–145 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.10–1.78 (m, 28), 2.59–3.12 (m, 18), 3.62 (d, 1), 4.23 (d, 1), 4.77 (br s, 11, exchangeable with D_2O), 7.36–7.95 (m, 6), 8.32 (d, 1), 10.98 (br s, 1, exchangeable with D_2O). Anal. ($C_{36}H_{59}N_7O_2 \cdot 4H_2C_2O_4$) C, H, N.

B. A mixture of tetraamine **4**[12] (as free base) (1.2 g, 3.24 mmol) and PBD-Cl[18] (1.9 g, 6.6 mmol) in DMF (60 mL) was stirred at room temperature for 36 h. Removal of the solvent gave a residue that was purified by gravity column chromatography using chloroform/methanol/triethylamine (8.5:1.2:0.2) as the eluting solvent. The first fraction was **9** as the free base that was transformed into the tetraoxalate salt and crystallized from EtOH/ether: 10% yield; R_f 0.31. The melting point was indefinite; fusion started at 168 °C and was complete at 201 °C; ^{1}H NMR (DMSO-d_6) δ 1.08–1.35 (m, 20), 1.53–1.69 (m, 8), 2.40–2.53 (m, 4), 2.73 (s, 6), 2.86 (d, 2), 2.92–3.12 (m, 8), 3.23–3.33 (m, 3), 3.37–3.47 (m, 3), 4.71 (br s, 8, exchangeable with D_2O), 7.13–7.86 (m, 24), 8.20 (br s, 2), 8.33 (br s, 2), 10.73–10.92 (m, 4, exchangeable with D_2O). Anal. ($C_{78}H_{86}N_{16}O_8 \cdot 4H_2C_2O_4 \cdot 8H_2O$) C, H. N.

The second fraction was **8** as the free base that was identical to that obtained following method D: 14% yield; R_f 0.26.

The third fraction was **7** as the free base that was identical to that obtained following method C: 6% yield; R_f 0.21.

C. A procedure described in a preliminary communication was followed.[17] Thus, alkylation of tetraamine **20** with PBD-Cl gave **21**. Removal of *N*-benzyl groups by catalytic hydrogenation afforded a mixture of **7** and **22**. Tetraamine **7** was identical to that obtained following method B.

D. A procedure described in a preliminary communication was followed.[17] Thus, debenzylation of **23** gave tetraamine **8** which was identical to that obtained following method B.

E. A mixture of **8** (as free base) (0.11 g, 0.1 mmol) and PBD-Cl (0.043 g, 0.15 mmol) in dry DMF (5 mL) was stirred at room temperature for 18 h. Removal of the solvent gave a residue that was purified by flash chromatography eluting with chloroform/methanol/aqueous 30% ammonia (9:0.8:0.1) to give **9** as the free base (70% yield) that was identical to that obtained following method B.

F. A mixture of **7** (as free base) (0.22 g, 0.25 mmol) and PBD-Cl (0.072 g, 0.22 mmol) in DMF (5 mL) was stirred at room temperature for 18 h. Removal of the solvent gave a residue that was purified by gravity column chromatography eluting with chloroform/methanol/aqueous 30% ammonia (9:0.5:0.1). The first fraction was **9** as the free base (22% yield) that was identical to that obtained following methods B and E.

The second fraction was **8** as the free base (19% yield) that was identical to that obtained following methods B and D.

The third fraction was unreacted **7** (0.04 g).

1-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-24-(2-methoxybenzyl)-1,8,17,24-tetraazatetracosane Tetraoxalate (10). A solution of **5** (as free base) (0.12 g, 0.19 mmol) and 2-methoxybenzaldehyde (0.08 g, 0.6 mmol) in EtOH (20 mL) was stirred at room temperature for 30 min. The mixture was cooled at 5 °C, $NaBH_4$ (0.01 g, 0.25 mmol) was added, and then the mixture was stirred at room temperature for 4 h. The mixture was acidified with 2 N HCl (2 mL) and the solvent was evaporated under reduced pressure to give a residue that was taken up in water and washed with CH_2Cl_2 (3 × 20 mL) to remove nonbasic materials. The aqueous layer was made basic with 2 N NaOH and the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The extracts were washed with a saturated NaCl aqueous solution, dried, and then evaporated to give **10** as the free base which was transformed into the tetraoxalate salt and crystallized from EtOH/MeOH: 80% yield; mp 113–115 °C dec; ^1H NMR (DMSO-d_6) δ 1.10–1.69 (m, 28), 2.78 (d, 6), 2.77–3.03 (m, 12), 3.59 (d, 1), 3.78 (s, 3), 3.89 (br s, 10, exchangeable with D_2O), 4.08 (s, 2), 4.24 (d, 1), 6.97 (t, 1), 7.07 (d, 1), 7.35–7.87 (m, 8), 8.31 (d, 1), 10.95 (br s, 1, exchangeable with D_2O). Anal. ($C_{44}H_{67}N_7O_3 \cdot 4H_2C_2O_4 \cdot H_2O$) C, H, N.

1,1-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-24-(2-methoxybenzyl)-1,8,17,24-tetraazatetracosane Tetraoxalate (11). This was synthesized from **6** (as free base) and 2-methoxybenzaldehyde following the procedure described for **10**: 80% yield; mp 140–145 °C (from EtOH/ether); ^1H NMR (DMSO-d_6) δ 1.03–1.42 (m, 18), 1.45–1.75 (m, 10), 2.35–2.50 (m, 2), 2.65–3.10 (m, 18), 3.21 (br s, 1), 3.28–3.43 (m, 1), 3.82 (s, 3), 4.11 (s, 2), 5.51 (br s, 9, exchangeable with D_2O), 6.97 (t, 1), 7.07 (d, 1), 7.30–7.71 (m, 12), 7.79 (d, 2), 8.10–8.20 (m, 1), 8.21–8.34 (m, 1), 10.69–10.88 (m, 2, exchangeable with D_2O). Anal. ($C_{58}H_{76}N_{10}O_5 \cdot 4H_2C_2O_4 \cdot 2H_2O$) C, H, N.

1,24-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-1-(2-methoxybenzyl)-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (12) and 1,1-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-1,-24-bis(2-methoxybenzyl)-8,17-dimethyl-1,8,17,24-tetraazatetracosane Tetraoxalate (13). A. A solution of **7** (as free base) (0.35 g, 0.4 mmol), triethylamine (0.036 g, 0.35 mmol), and 2-methoxybenzyl chloride[25] (0.055 g, 0.35 mmol) in dry DMF was stirred at room temperature for 6 h. Removal of the solvent afforded a residue that was purified by gravity column chromatography eluting with methanol/chloroform/aqueous 30% ammonia (0.8:9:0.15). The first fraction was **13** as the free base that was transformed into the tetraoxalate salt and crystallized from EtOH/ether: 10% yield; mp 115–117 °C; ^1H NMR (DMSO-d_6) δ 1.10–1.45 (m, 20), 1.45–1.73 (m, 8), 2.30–2.48 (m, 4), 2.65 (s, 6), 2.90–3.08 (m, 8), 3.40–3.48 (d, 2), 3.48–3.55 (d, 2), 3.55–3.65 (m, 4), 3.66 (s, 6), 5.40 (br s, 8, exchangeable with D_2O), 6.82–6.95 (m, 4), 7.18 (d, 2), 7.21 (t, 2), 7.36–7.60 (m, 6), 7.60–7.75 (m, 4), 7.79 (d, 2), 8.12–8.23 (m, 2), 10.85 (br s, 2, exchangeable with D_2O). Anal. ($C_{66}H_{84}N_{10}O_6 \cdot 4H_2C_2O_4 \cdot 2H_2O$) C, H, N.

The second fraction was **12** as the free base which was transformed into the tetraoxalate salt and crystallized from EtOH/ether: 17% yield; mp 116–120 °C; ^1H NMR (DMSO-d_6) δ 1.10–1.42 (m, 18), 1.42–1.78 (m, 10), 2.29–2.41 (m, 2), 2.71 (d, 6), 2.80–3.08 (m, 10), 3.28–3.42 (m, 2), 3.44–3.52 (m, 2), 3.65 (s, 3), 3.80 (d, 1), 4.40 (d, 1), 4.50 (br s, 9, exchangeable with D_2O), 6.80–7.00 (m, 2), 7.00–7.14 (m, 1), 7.14–7.25 (m, 1), 7.35–7.90 (m, 12), 8.12–8.20 (m, 1), 8.25–8.38 (m, 1), 10.80–11.08 (m, 2, exchangeable with D_2O). Anal. ($C_{58}H_{76}N_{10}O_5 \cdot 4H_2C_2O_4 \cdot 2H_2O$) C, H, N.

B. A solution of **2**[12] (as free base) (0.2 g, 0.33 mmol), triethylamine (0.07 g, 0.7 mmol), and PBD-Cl (0.2 g, 0.7 mmol) in dry DMF was stirred at room temperature for 72 h. Removal of the solvent gave a residue that was purified by flash chromatography. Eluting with methanol/chloroform/aqueous 30% ammonia (0.8:9:0.15) gave 0.16 g (44% yield) of **13** as the free base which was identical to that obtained following method A.

***N,N*-Diethyl-1,6-hexanediamine Dihydrochloride (25).** Ethyl chlorocarbonate (1.34 g, 12 mmol) in dioxane (5 mL) was added dropwise to a stirred and cooled (5 °C) solution of *N*-[(benzyloxy)carbonyl]-6-aminocaproic acid (3.0 g, 11.3 mmol) and triethylamine (1.25 g, 12 mmol) in dioxane (50 mL), followed after standing for 30 min by the addition of diethylamine (0.82 g, 11.3 mmol) in dioxane (20 mL). After standing for 18 h, the mixture was poured onto water (ice added) (250 mL) and then extracted with chloroform (3 × 60 mL). The organic layer was washed with water (2 × 20 mL), 2 N NaOH (2 × 20 mL), 2 N HCl (2 × 20 mL), and finally aqueous saturated NaCl solution. Removal of dried solvents gave 2.6 g (72% yield) of crude **24** which was dissolved in EtOH (100 mL) and hydrogenated over 10% Pd on charcoal (0.5 g) for 1 h at room temperature and a pressure of 30 psi. Following catalyst removal, the solvent was evaporated, yielding 1.51 g of crude 6-aminocaproic acid *N,N*-diethyl amide, which was dissolved in dry diglyme (50 mL) and treated with a 10 M solution of BH_3·MeSMe (3 mL) in dry diglyme (10 mL). After 14 h at 120 °C under a stream of dry nitrogen, excess borane was destroyed by careful addition of MeOH (15 mL). The resulting mixture was left to stand for 3 h, treated with HCl gas for 20 min, and then heated at 120 °C for 4 h. Removal of the solvent gave a residue which was crystallized from EtOH/ether to give **25**: 1.4 g (70% yield); mp 170–172 °C; ^1H NMR (free base; $CDCl_3$) δ 0.98 (t, 6), 1.20–1.35 (m, 4), 1.35–1.50 (m, 4), 1.72 (br s, 2), 2.38 (t, 2), 2.51 (q, 4), 2.65 (t, 2). Anal. ($C_{10}H_{24}N_2 \cdot 2HCl$) C, H, N.

***N,N*-Diethyl-*N'*-(2-methoxybenzyl)-1,6-hexanediamine Dihydrochloride (14).** A solution of **25** (as free base) (0.15 g, 0.87 mmol) and 2-methoxybenzaldehyde (0.13 g, 0.96 mmol) in benzene (20 mL) was heated under reflux and the water formed continuously removed for 7 h. The cooled mixture was filtered and the filtrate evaporated to give the corresponding Schiff base that was dissolved in EtOH (10 mL) and treated with $NaBH_4$ (0.04 g, 0.96 mmol). The mixture was stirred at room temperature for 3 h, then it was acidified with 6 N HCl and evaporated to dryness. The residue was mixed with water (20 mL) and the mixture extracted with ether (3 × 10 mL) in order to remove nonbasic materials. The aqueous layer was made basic with 2 N NaOH and the mixture extracted with chloroform (4 × 20 mL). Removal of dried extracts gave **14** as the free base that was characterized as the dihydrochloride salt: 95% yield; mp 152–153 °C (from EtOH/2-PrOH); ^1H NMR (DMSO-d_6) δ 1.20 (t, 6), 1.25–1.42 (m, 4), 1.55–1.80 (m, 4), 2.83 (t, 3), 2.95 (t, 2), 3.05 (q, 4), 3.82 (s, 3), 4.08 (s, 2), 6.98 (t, 1), 7.10 (d, 1), 7.40 (t, 1), 7.50 (d, 1), 9.17 (br s, 2, exchangeable with D_2O). 10.52 (br s, 1, exchangeable with D_2O). Anal. ($C_{18}H_{32}N_2O \cdot 2HCl$) C, H, N.

***N*-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-*N*-(2-methoxybenzyl)-*N',N'*-diethyl-1,6-hexanediamine Dihydrochloride (15).** A solution of **14** (as free base) (0.09 g, 0.03 mmol) and PBD-Cl (0.13 g, 0.45 mmol) in DMF (3 mL) was stirred at room temperature for 72 h. Removal of the solvent gave a residue that was purified by gravity column chromatography. Eluting with chloroform/methanol/aqueous 30% ammonia (8.5:0.8:0.1) gave 0.15 g of **15** as the free base that was transformed into the dihydrochloride salt and crystallized from EtOH/ether: mp 192–195 °C; ^1H NMR (DMSO-d_6) δ 1.20 (t, 6), 1.27–1.46 (m, 4), 1.45–1.82 (m, 4), 2.85–3.25 (m, 4), 3.05 (q, 4), 3.52–3.80 (m, 2), 3.80 (s, 3), 4.23–4.54 (m, 2), 6.92–7.95 (m, 10), 8.27 (d, 1), 9.75 (br s, 1, exchangeable with D_2O), 10.45 (br s, 1, exchangeable with D_2O), 10.98 (br s, 1, exchangeable with D_2O). Anal. ($C_{32}H_{41}N_5O_3 \cdot 2HCl$) C, H, N.

***N,N*-Diethyl-*N'*-benzyl-1,6-hexanediamine (26).** This was synthesized as the free base in 85% yield from **25** and benzaldehyde following the procedure described for **14**. It was used in the next step without further purification.

***N*-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-11-yl)carbonyl]methyl]-*N*-benzyl-*N',N'*-diethyl-1,6-hexanediamine (27).** This was synthesized from **26** and PBD-Cl following the procedure described for **15**. It was purified as the free base by flash chromatography eluting with methanol/chloroform/aqueous 30% ammonia (0.8:9:0.1) for yield of 86%. It was used in the next step without further purification.

***N*-[[(5,11-Dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]benzo-**

diazepin-11-yl)carbonyl]methyl]-*N',N'*-diethyl-1,6-hexanediamine Dihydrochloride (16). A solution of **27** (0.44 g, 0.85 mmol) in MeOH (20 mL, a few drops of 3 N ethanolic HCl added) was hydrogenated over 10% Pd on charcoal (0.1 g) at room temperature for 12 h. Following catalyst removal, the solvent was evaporated, yielding crude **16** as the free base which was transformed into the dihydrochloride salt and crystallized from EtOH/ether: 0.39 g (92% yield); mp 202–207 °C; ^{1}H NMR (DMSO-d_6) δ 1.15 (t, 6), 1.20–1.35 (m, 4), 1.44–1.55 (m, 4), 2.78–3.00 (m, 4), 3.06 (q, 4), 3.65 (d, 1), 4.25 (d, 1), 7.40–7.86 (m, 6), 8.32 (d, 1), 8.97 (br s, 1, exchangeable with D_2O), 9.20 (br s, 1, exchangeable with D_2O), 10.30 (br s, 1, exchangeable with D_2O), 10.98 (br s, 1, exchangeable with D_2O). Anal. ($C_{24}H_{33}N_5O_2$·2HCl) C, H, N.

***N,N*-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-*N',N'*-diethyl-1,6-hexanediamine Dihydrochloride (17). A.** A solution of **25** (as free base) (0.5 g, 2.3 mmol) and PBD-Cl (1.66 g, 5.8 mmol) in DMF (10 mL) was stirred at room temperature for 12 h. Removal of the solvent gave a residue that was purified by gravity column chromatography. Eluting with methanol/chloroform/aqueous 30% ammonia (1.2:8.5:0.2) gave 1.54 g of **17** as the free base that was transformed into the dihydrochloride salt and crystallized from EtOH/ether: 80% yield; mp 198–201 °C; ^{1}H NMR (DMSO-d_6) δ 1.22 (t, 6), 1.10–1.40 (m, 4), 1.45–1.75 (m, 4), 2.84–2.98 (m, 2), 3.05 (q, 4), 3.00–3.25 (m, 2), 3.65–4.00 (m, 2), 4.50–4.80 (m, 2), 7.45–7.98 (m, 12), 8.32 (d, 2), 10.58 (br s, 2, exchangeable with D_2O), 11.05 (br s, 2, exchangeable with D_2O). Anal. ($C_{38}H_{42}N_8O_4$·2HCl) C, H, N.

B. Compound **17** as the free base was also synthesized in 70% yield starting from **16** (0.21 g, 0.5 mmol) and PBD-Cl (0.18 g, 0.65 mmol) in DMF following the procedure described in method A.

***N,N*-Bis[[(5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-*n*-butylamine Hydrochloride (18).** A mixture of *n*-butylamine (0.073 g, 1 mmol), PBD-Cl (0.575 g, 2 mmol), and triethylamine (0.3 g, 2 mmol) in CH_2Cl_2 (20 mL) as stirred at room temperature for 72 h. The formed solid was filtered, washed with CH_2Cl_2 and ether, and then transformed into the hydrochloride salt: 73% yield; mp 235–237 °C (from EtOH/ether); ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3), 1.00–1.60 (m, 4), 3.00–3.25 (m, 2), 3.65–4.05 (m, 2), 4.45–4.80 (m, 2), 7.40–7.98 (m, 12), 8.30 (d, 2), 9.40 (br s, 1, exchangeable with D_2O), 10.98 (br s, 2, exchangeable with D_2O). Anal. ($C_{32}H_{29}N_7O_4$·HCl) C, H, N.

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. Dose–response curves were constructed by cumulative addition of the agonist.[21] 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 hearts of guinea pigs were 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; $MgSO_4$·$7H_2O$, 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 dose–response curves to carbachol (0.01–1 μM) were constructed. Following incubation with the antagonist for 60 min, a new dose–response curve to carbachol was obtained.

Guinea Pig Ileum. 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 of approximately 2 cm 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·$2H_2O$, 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. Dose–response curves to carbachol (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 dose–response curve to the agonist was obtained.

Cell Culture and Binding Assays. The detailed methods have been published previously.[26–28] [^{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), submaxillary gland homogenates (expressing M_3 muscarinic receptors; K_d 0.48 ± 0.03 nM; B_{max} 1102 ± 85 fmol/mg of protein), and homogenates obtained from NG 108–15 cells (expressing M_4 muscarinic receptors; K_d 0.54 ± 0.03 nM; B_{max} 19 ± 4 fmol/ mg of protein). [^{3}H]-Pirenzepine (specific activity 86.2 Ci/mmol; NEN Du Pont) was the tracer used to label M_1 muscarinic receptors binding sites of the rat cerebral cortex (K_d 2.1 ± 0.2 nM; B_{max} 49 ± 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 cell binding assays, whereas 5 nM was the concentration of [^{3}H]pirenzepine in rat cortex cortex homogenates. Nonspecific binding was assessed in the presence of 10 μM atropine.

Determination of Dissociation Constants. In functional experiments, dose ratios at the EC_{50} values of the agonists were calculated at one to six antagonist concentrations and each concentration was tested from four to eight times. The results were expressed as pA_2 values.[19,20] For certain compounds (**4, 13–19**) pA_2 values were calcualted at only one concentration and tested at least five times, according to the method of Van Rossum.[21] 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.[29] Differences in the slope of the curves were determined by the test of parallelism described by Tallarida and Murray[30] and were not different ($p > 0.05$). Scatchard plots were linear or almost linear in all preparations. All Hill numbers (nH) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived from the Cheng–Prusoff equation,[22] $K_i = IC_{50}/(1 + L/K_d)$, 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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