Synthesis and Biological Activity of Some Methoctramine-Related Tetraamines Bearing a 11-Acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-6-one Moiety as Antimuscarinics: A Second Generation of Highly Selective M_2 Muscarinic Receptor Antagonists

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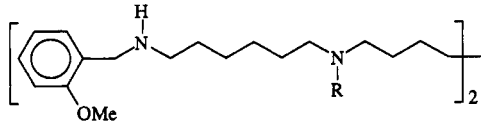
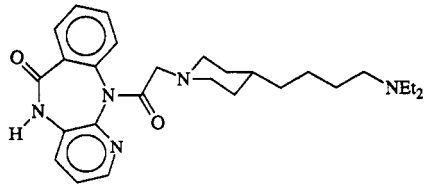
Methoctramine[1] (*N*,*N*′-bis[6-[(2-methoxybenzyl)-amino]hexyl]-1,8-octanediamine; **1**) is one of the most selective antimuscarinics available and proved to be a useful probe for characterizing muscarinic receptor subtypes.[2-4] Its M_2/M_3 selectivity ratio is about 100, but it fails to discriminate to the same extent between M_2 and M_1 and M_2 and M_4 muscarinic receptors.[4-6] Selective M_2 muscarinic receptor antagonists could be useful for the treatment of bradycardic disorders without concomitant side effects on other parasympathetically innervated organs.[7] Furthermore, the use of selective M_2 antagonists could be a useful strategy to improve memory and learning since it seems clear that M_2 muscarinic receptors are selectively lost in brain from Alzheimer patients.[8-10] Evidence has been obtained on the exhistence of functional M_2 autoreceptors in the brain. Consequently, it may be possible to improve learning and memory by blocking M_2 inhibitory autoreceptors which should increase acetylcholine levels in the synaptic cleft.[10]



1 (Methoctramine): R = H

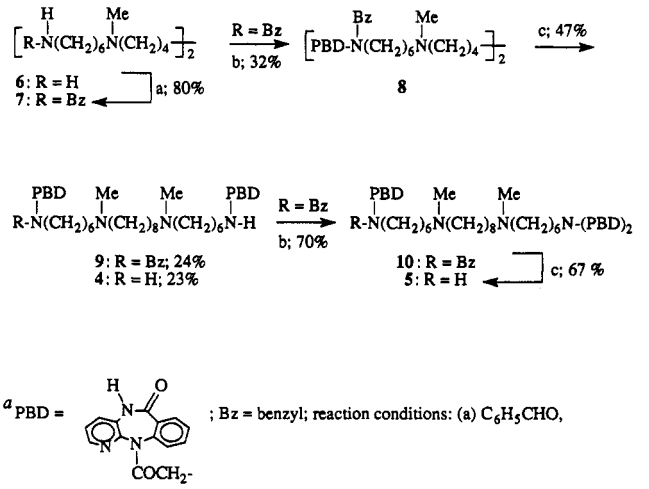
2: R = Me



3 (AQ-RA 741)

The selectivity of methoctramine for M_2 muscarinic receptors was shown to be dramatically dependent on a tetraamine backbone[11] whereas affinity depends on the type of substituent on the terminal nitrogens.[12,13] Since methoctramine is a relatively potent M_2 muscarinic receptor antagonist, an improvement of its affinity would not represent a major achievement unless there is also a concomitant increase in selectivity. The objective of this study was to improve the selectivity toward M_2 muscarinic receptors by modifying the substituents on the terminal nitrogens of methoctramine. The starting point was the observation that 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,[7] could be superimposed to the structure of methoctramine. In fact, a preliminary analysis of the stereomodels of **1** and **3** in their extended conformations revealed that the distances between the two basic nitrogens of **3** and between outer and inner nitrogens of **1** are very similar. In addition, structure–activity relationship studies among polyamines related to methoctramine showed that diamines, obtained by truncating in two halves methoctramine structure, are almost devoid of affinity and selectivity toward M_2 muscarinic receptors.[11] This finding clearly indicates that, contrary to 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-6-one moiety of diamine **3**, a 2-methoxybenzyl group on one nitrogen of a diamine does not contribute to both affinity and selectivity. Thus, we thought that the insertion of a tricyclic system on the terminal nitrogens of a tetraamine would improve affinity and hopefully selectivity for M_2 muscarinic receptors. To this end, we describe here the synthesis and the pharmacological profile of tetraamines **4** and **5** (tripitramine) in functional and binding experiments.

Scheme I. Synthesis of Tetraamines **4** and **5**[a]



[a] PBD = ; Bz = benzyl; reaction conditions: (a) C_6H_5CHO, benzene, reflux, 6 h; then $NaBH_4$, EtOH, rt, 3 h; (b) PBD-Cl, DMF, NEt_3, KI, rt, 3 days; (c) catalytic hydrogenation over 10% palladium on charcoal at 5 atm, EtOH, 3N ethanolic HCl (few drops), 50 °C, 12 h.

The choice of a tetraamine, bearing on the two inner nitrogens a methyl group, as a common backbone was dictated by the fact that N-methylation prevents from unwanted alkylation on these nitrogens and because the *N*,*N*′-dimethyl analogue **2**[12] of methoctramine showed a biological profile similar to that of the parent compound.

Chemistry. The compounds used in this investigation were synthesized by standard procedures as shown in Scheme I.[14]

The benzyl substituent on the terminal primary amine function was easily introduced by condensation of **6**,[12] as free base, with benzaldehyde and subsequent reduction with $NaBH_4$ of the intermediate Schiff base to give **7** in 80% yield.[15] Tetraamine **7** was alkylated with 11-(chloroacetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]-

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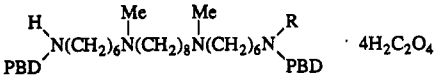
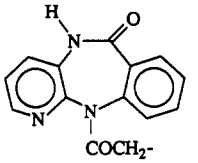
Table I. pA_2 Values in the Isolated Guinea Pig Left Atrium (M_2) and Ileum (M_3) Muscarinic Receptors and Affinity Constants (pK_i) in Rat Cortex (M_1), Heart (M_2) and Submaxillary Gland (M_3) and NG 108-15 Cells (M_4) Muscarinic Receptor Subtypes



PBD =



								selectivity ratio[c]			
		pA_2[a]		pK_i[b]							
no.	R	atrium, M_2	ileum, M_3	cortex, M_1	heart, M_2	sub. gland, M_3	NG 108-15, M_4	atrium/ ileum	heart/ cortex	heart/ gland	heart/ NG108-15
1		7.82 ± 0.01	6.32 ± 0.07	7.43 ± 0.11	7.84 ± 0.09	5.96 ± 0.18	7.58 ± 0.13	32	3	81	2
2		7.90 ± 0.02	6.22 ± 0.04					48			
3[d]		8.5	6.6	7.6	8.3	6.8	8.0	79	5	32	2
4	H	9.14 ± 0.01	6.90 ± 0.06					174			
5	PBD	9.75 ± 0.02	6.55 ± 0.01	7.63 ● 0.09	9.54 ± 0.08	6.19 ± 0.14	7.93 ● 0.11	1585	81	2239	41
PZ[e]				8.19 ± 0.08	6.10 ± 0.09	6.76 ± 0.10	7.46 ± 0.17		0.008	0.2	0.04

[a] pA_2 values ± SE were calculated from Schild plots,[21] constrained to slope −1.0.[22] 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 three different antagonist concentrations (atrium: 30, 300, and 3000 nM; ileum: 1, 0.5, and 10 μM), and each concentration was tested at least five times following the protocol described in ref 12. 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. Parallelism of dose–response curves was checked by linear regression, and the slopes were tested for significance ($p < 0.05$). [b] Values are the mean ± SE of at least three separate experiments performed in triplicate. All Hill number (nH) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived using 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. Scatchard plots were linear or almost linear in all preparation tested. In competition studies, fixed concentrations of 0.7–0.8 nM [^{3}H]NMS were used in rat heart and submaxillary gland, and NG 108-15 binding assays, whereas 5 nM was the concentration of [^{3}H]pirenzepine in rat cortex homogenates. Nonspecific binding was assessed in the presence of 1 μM atropine. [c] The selectivity ratio is the antilog of the difference between the pA_2 (or pK_i) values at left atrium (or heart) and ileum (or cortex, submaxillary gland and NG 108-15) muscarinic receptors, respectively. [d] Data from ref 7. [e] PZ, pirenzepine.

benzodiazepin-6-one[16] (PBD-Cl) to give 8.[17] Removal of *N*-benzyl groups was achieved by catalytic hydrogenation over 10% palladium on charcoal. Thus, debenzylation of 8 gave 4 and 9 as free bases.[18] Similarly, tetraamine 5[19] was obtained through debenzylation of 10[20] that, in turn, was synthesized by reacting 9 and PBD-Cl.

Biological Activity. The biological profile in functional experiments of tetraamines 4 and 5 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 atrium (M_2 receptors) following a described procedure.[13] To allow comparison of the results, methoctramine (1) and its *N*,*N*′-dimethyl analogue 2 were used as the standard compounds. The biological results were expressed as pA_2 values determined from Schild plots[21] constrained to slope −1.0,[22] 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 ($p > 0.05$).

We chose to further examine the receptor subtype selectivity by employing receptor binding assays. The detailed methods have been published previously.[23–25] [^{3}H]-*N*-Methylscopolamine ([^{3}H]NMS; specific activity 79.5 Ci/mmol) was used to label M_2, M_3, and M_4 muscarinic receptors binding sites of rat heart (K_d 0.32 ± 0.042 nM; B_{max} 77.8 ± 15.3 fmol/mg of protein) and submaxillary gland (K_d 0.485 ± 0.035 nM; B_{max} 1102 ± 85 fmol/mg of protein), and NG 108–15 cells homogenates (K_d 0.544 ± 0.032 nM; B_{max} 19 ± 4 fmol/mg of protein), respectively. [^{3}H]Pirenzepine (specific activity 86.2 Ci/mmol) was the tracer to label M_1 muscarinic receptors binding sites of the rat cerebral cortex (K_d 2.15 ± 0.19 nM; B_{max} 49 ± 13 pmol/mg of protein). Binding affinities were expressed as pK_i values. Methoctramine and pirenzepine were used as the standard compounds.

Results and Discussion. The results, expressed as pA_2 and pK_i values, of tetraamines 4 and 5 are shown in Table I together with those of standard compounds 1–3 and pirenzepine. It can be seen that replacing 2-methoxybenzyl groups of 2 by 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one moieties affording 4 or replacing a hydrogen atom of 4 by an additional tricyclic system as in 5 (tripitramine) alters markedly both affinity and selectivity toward M_2 muscarinic receptors. It is also evident that these structural modifications do not alter the biological profile at M_3 muscarinic receptors. This finding clearly indicates that the insertion of tricyclic systems on the terminal nitrogens of methoctramine (1) is highly effective toward M_2 muscarinic receptors whereas the affinity for M_3 muscarinic receptors is almost unchanged compared to the prototypes 1–3. Interestingly, the results obtained with functional experiments parallel those derived from binding assays. However, the most striking result of the present investigation is the selectivity toward M_2 muscarinic receptors displayed by 5 which resulted in markedly increased selectivity compared to those of methoctramine (1) and AQ-RA 741 (3).

Methoctramine (1) and AQ-RA 741 (3) are classified as selective M_2 muscarinic receptor antagonists with a selective profile $M_2 \geq M_4 \geq M_1 > M_3$, that is, these drugs are not capable to discriminate significantly between M_2 and M_4, M_2 and M_1, and M_4 and M_1. An analysis of affinity constants in Table I reveals that 5 possesses a remarkable affinity profile for muscarinic receptor subtypes ($M_2 > M_4 \geq M_1 > M_3$). Clearly 5 is capable to distinguish among M_2 and all other muscarinic receptor subtypes investigated, M_1 to M_4, as well as between M_1 and M_3, and M_4 and M_3 muscarinic receptors. The outstanding properties of 5

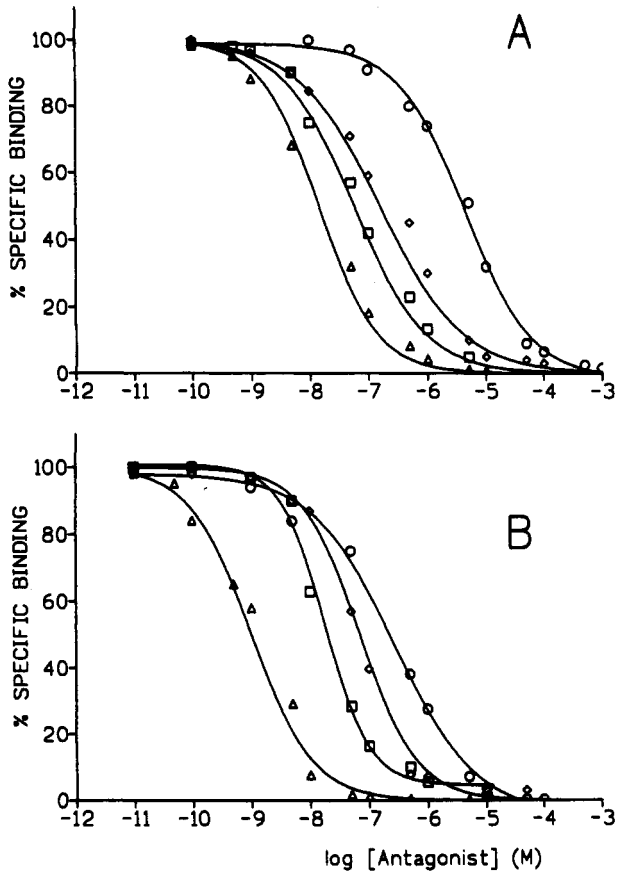


Figure 1. Representative experiments showing 1 (panel A) and 5 (panel B) competition binding curves for muscarinic receptors of rat heart (△), cortex (◇) and submaxillary gland (○) and for NG108-15 cells (□). Cortical receptors were labeled with [^{3}H]-pirenzepine and all the others with [^{3}H]NMS. Data were analyzed using LIGAND.[27] Differences in slopes of the curves were determined by the test of parallelism as described by Tallarida and Murray[28] and were not different ($p > 0.05$).

are graphically shown in Figure 1 in comparison to methoctramine (1). Whereas all M_2 selective antagonists available to date bind to M_2 and M_4 receptors with similar affinities,[6] tripitramine (5) discriminates significantly between these two subtypes with a selectivity ratio of 41. The fact that the antagonists currently used to classify muscarinic receptors lack a clear subtype selectivity may explain the difficulties to characterize with classic pharmacological studies the five cloned muscarinic receptors. The results presented in this paper clearly show that the use of tripitramine (5) combined with that of other selective antagonists, mainly pirenzepine which is able to discriminate between M_1 and M_4, might eventually allow the pharmacological identification of muscarinic receptor subtypes.

To our knowledge, 5 (tripitramine) represents, until now, the most potent and the most selective muscarinic receptor antagonists in both functional (M_2 and M_3) and binding (M_1 to M_4) assays. Tripitramine (5) emerges as a powerful tool for the characterization of muscarinic receptor subtypes but also as lead compound for the design of new drugs for the treatment of bradycardic disorders and hopefully of Alzheimer's disease.

Our future work in this area will include studies directed at gaining a better understanding of the intriguing trends noted above. In addition, we hope to develop relevant structure–activity relationships for muscarinic receptor subtypes through the synthesis of N-substituted polymethylenetetraamines related to those presented in this paper. These studies should provide a complete description of the structural requirements for selective antagonism of muscarinic receptor subtypes.

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(14) ^{1}H NMR spectra were recorded on a Varian VXR 300 instrument. 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), or m (multiplet). The elemental compositions of the compounds agreed to within ±0.4% of the calculated value, unless otherwise specified. 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 (Kieselgel 40, 0.040–0.063 mm, Merck) by flash chromatography eluting with chloroform–methanol–32% ammonia (8.5:1:0.15).

(15) For the synthesis of 7, the experimental procedure was similar to that described in detail in ref 12 for *N*-aryl-substituted tetraamines. Tetrahydrochloride salt: mp 238–245 °C (from EtOH/*i*-PrOH).

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(17) A mixture of 7 (2 mmol), PBD-Cl (4 mmol), Et_3N (4 mmol), and KI (0.1 g) in dry DMF (10 mL) was stirred at room temperature for 3 days. Removal of the solvent gave a residue that was purified by chromatography, affording a 32% yield of 8 as free base (mp 64–73 °C).

(18) Tetraamine **8** (0.64 g) in EtOH (100 mL) (few drops of 3 N ethanolic HCl added) was hydrogenated at 5 atm with 10% Pd/C (0.1 g) at 50 °C for 12 h. The mixture was filtered and evaporated. The residue was purified by chromatography. The first fraction was unreacted **8** (0.1 g). The second fraction was **9** (0.12 g; 24% yield). The third fraction was **4** (0.1 g; 23% yield). It was transformed into the tetraoxalate salt: mp 182–183 °C (from EtOH/*i*-PrOH); ^{1}H NMR (DMSO-d_6) δ 1.24–1.35 (16, m), 1.55–1.70 (12, m), 2.73 (6, s), 2.84–3.06 (12, m), 3.68 (2, d, J = 17 Hz), 4.31 (2, d, J = 17 Hz), 7.50 (2, d), 7.55–7.63 (3, m), 7.73–7.85 (5, m), 7.87 (2, d), 8.36 (2, d), 10.91–11.07 (2, m). Anal. ($C_{58}H_{76}N_{10}O_{20}\cdot 2H_2O$) C, H; N: calcd, 11.03; found: 10.38.

(19) This compound was obtained in 67% yield from **10** following the procedure described for **4**. It was characterized as tetraoxalate salt: mp 192–193 °C (from EtOH/*i*-PrOH); ^{1}H NMR (DMSO-d_6) δ 1.18–1.36 (18, m), 1.56–1.75 (10, m), 2.39–2.60 (4, m), 2.73 (6, d), 2.84–3.08 (10, m), 3.28 (1, br s), 3.39–3.46 (1, m), 3.69 (1, d), 4.31 (1, d), 7.33–7.97 (18, m), 8.13–8.41 (3, m), 10.8–11.11 (3, m). Anal. ($C_{72}H_{88}N_{13}O_{22}\cdot 4H_2O$) C, H; N: calcd, 11.70; found, 10.94.

(20) This compound as obtained in 70% yield from **9** (1 mmol) and PBD-Cl (1 mmol) following the procedure described for **8**. It was purified as the free base by chromatography.

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