The urinary bladder strips were used for evaluation of antimuscarinic and muscarinic activity and the ileal preparations for studies of muscarinic activity (see Pharmacological Results and Discussion). Carbachol (carbamylcholine chloride) was used as the standard agonist. Concentration–response curves to agonists were generated either by the cumulative dose–response technique (bladder strip) or by the addition of single agonist concentrations (ileal preparations). In the latter case, the preparations were washed and allowed to rest between each concentration of agonist. EC_{50} values were graphically determined.

In studies of antagonism, a control concentration–response curve to carbachol was generated by cumulative addition of carbachol to the bladder strip (i.e., stepwise increase of the agonist concentration until the maximal contractile response was reached), followed by washing out and a resting period of at least 15 min prior to addition of a fixed concentration of the test compound (antagonist) to the organ bath. After 60 min of incubation, a second cumulative concentration–response curve to carbachol was generated. Responses were expressed as percent of the maximal response to carbachol. EC_{50} values for carbachol in the absence (control) and presence of antagonist were graphically derived and dose ratios (r) were calculated.

Dissociation constants, K_B, for the antagonists were then calculated by $K_B = [A]/r - 1$, where [A] is the concentration of the test compound.[34]

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Registry No. *cis*-1 free base, 64168-64-5; *trans*-1 HCl, 64168-67-8; *trans*-1 free base, 64168-65-6; **3**, 41353-91-7; **4**, 139689-94-4; **5**, 139689-95-5; **6**, 139757-87-2; **7**·HCl, 139689-96-6; **7** free base, 139689-97-7; **8**·HCl, 139689-98-8; **8** free base, 139689-99-9; **9**, 54954-73-3; **10** free base, 139690-00-9; **10**·HCl, 139690-01-0; **11** free base, 139690-02-1; **11**·HCl, 139690-03-2; **12**, 139690-04-3; **13**, 139757-88-3; **14**·HCl, 139690-05-4; **14** free base, 139690-06-5; **15**·HCl, 139690-07-6; **15** free base, 139690-08-7; **16**·HCl, 60171-85-9; **16** free base, 60211-59-8; **17**·HCl, 139690-09-8; **17** free base, 60394-35-6; 3-bromoprop-1-ene, 106-95-6; 3-hydroxy-3-(hydroxymethyl)-1-azabicyclo[2.2.2]octane, 61573-79-3.

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Synthesis and in Vitro Characterization of Novel Amino Terminally Modified Oxotremorine Derivatives for Brain Muscarinic Receptors

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A series of novel 2-substituted acetylenic pyrrolidines and piperidines related to oxotremorine (1) were prepared and evaluated in vitro as muscarinic cholinergic agents at brain M_1 and M_2 receptors. One analogue, 3-(2-oxo-1-pyrrolidinyl)-1-[2(*R*)-pyrrolidinyl]-1-propyne hydrogen oxalate (**6a**), was found to be a partial agonist producing a PI hydrolysis response at cortical M_1 receptors approximately 3-fold larger than that produced by 1. The intrinsic activity profile of **6a** at brain muscarinic receptors is similar to those of azetidine oxo analogue 2 and dimethylamino oxo analogue (3). All three compounds are partial M_1 agonists and full M_2 agonists; however, the profile of **6a** in binding studies is significantly different. While 2 and 3 exhibit large M_2 selectivities ranging between 8-fold to several hundred-fold, the binding profile of **6a** shows almost no subtype selectivity.

Introduction

Alzheimer's disease (AD) is a central nervous system (CNS) neurodegenerative disorder which leads to dramatic personality changes as well as a profound dementia in individuals who are afflicted. Although AD affects a number of brain neurochemical systems, a pronounced and consistent deficiency in cholinergic markers in the neocortex and hippocampus has led to the cholinergic hypothesis of AD-related memory loss.[1-3] Decreases in markers of cholinergic activity such as choline acetyltransferase (ChAT), high affinity choline uptake, and acetylcholine esterase activity have been found to occur in AD brains.[4,5] These decreases in presynaptic cholinergic markers, particularly that of ChAT, have been correlated with the severity of dementia. It is this cortical/hippocampal cholinergic dysfunction attributed to presynaptic cholinergic neuron loss which is considered to be a major factor responsible for the memory loss that characterizes AD.[6-8] It has been suggested that muscarinic cholinergic receptors, the majority of which are postulated to reside postsynaptically to the degenerating cholinergic neurons,

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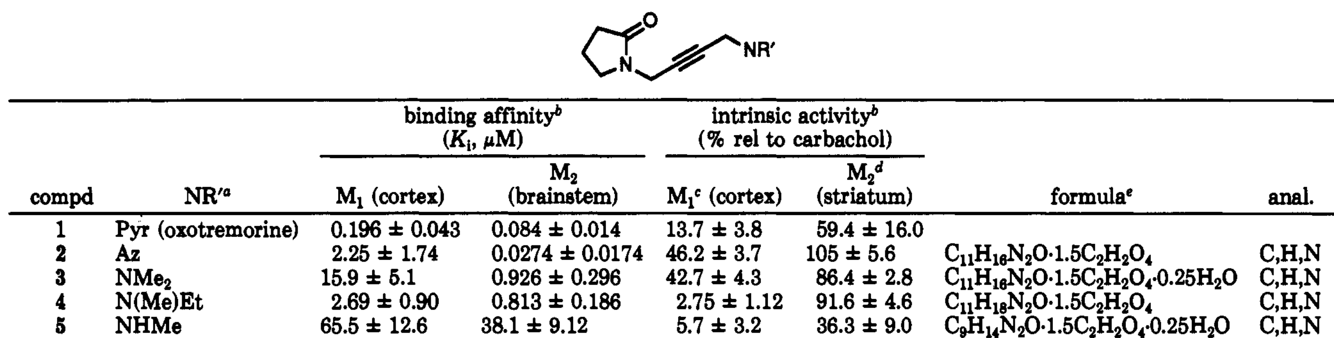
Table I. Biologic Data for Classic Amino Terminus Modifications of 1



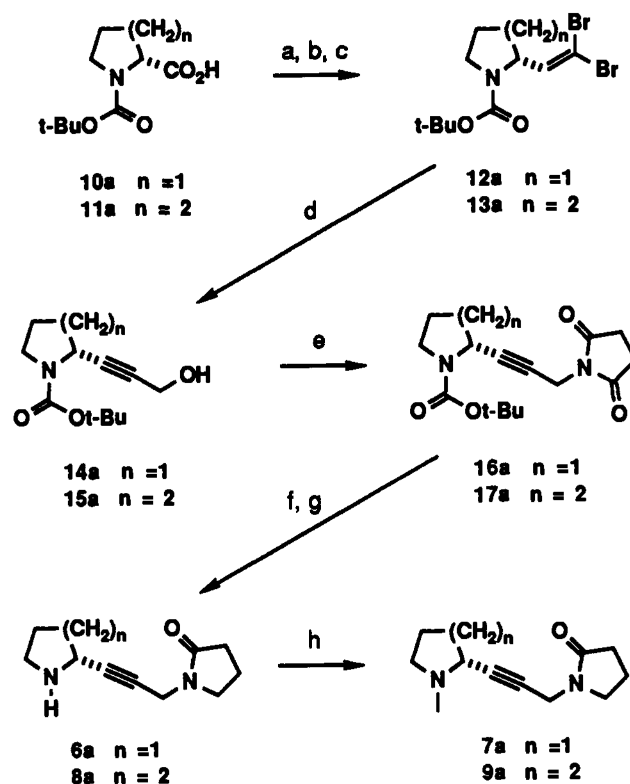
compd	NR'[a]	binding affinity[b] (K_i, μM) M$_1$ (cortex)	binding affinity[b] (K_i, μM) M$_2$ (brainstem)	intrinsic activity[b] (% rel to carbachol) M$_1$[c] (cortex)	intrinsic activity[b] (% rel to carbachol) M$_2$[d] (striatum)	formula[e]	anal.
1	Pyr (oxotremorine)	0.196 ± 0.043	0.084 ± 0.014	13.7 ± 3.8	59.4 ± 16.0		
2	Az	2.25 ± 1.74	0.0274 ± 0.0174	46.2 ± 3.7	105 ± 5.6	$C_{11}H_{16}N_2O{\cdot}1.5C_2H_2O_4$	C,H,N
3	NMe$_2$	15.9 ± 5.1	0.926 ± 0.296	42.7 ± 4.3	86.4 ± 2.8	$C_{11}H_{16}N_2O{\cdot}1.5C_2H_2O_4{\cdot}0.25H_2O$	C,H,N
4	N(Me)Et	2.69 ± 0.90	0.813 ± 0.186	2.75 ± 1.12	91.6 ± 4.6	$C_{11}H_{18}N_2O{\cdot}1.5C_2H_2O_4$	C,H,N
5	NHMe	65.5 ± 12.6	38.1 ± 9.12	5.7 ± 3.2	36.3 ± 9.0	$C_9H_{14}N_2O{\cdot}1.5C_2H_2O_4{\cdot}0.25H_2O$	C,H,N

[a] Pyr = pyrrolidinyl, Az = azetidinyl. [b] The standard deviation of the mean for three or more determinations. [c] Test compound concentration 1 mM. [d] Test compound concentration 100 μM. [e] Partial salts and/or hydrates are correlated with the integral of a broad peak of exchangeable protons in the ^{1}H NMR spectra.

may be generally preserved in Alzheimer's patients.[9,10] Accordingly, in recent years there has been an intense interest concentrating on the development of centrally active muscarinic agonists capable of pharmacologically enhancing cortical/hippocampal cholinergic tone via activation of the postsynaptic cortical muscarinic receptors. It is hypothesized that agents which activate the appropriate subpopulation of brain muscarinic receptors might ameliorate some of the symptoms of memory loss associated with AD without producing adverse side effects.

Although molecular biological studies have thus far discovered at least five genetic products which code for distinct muscarinic receptors in human brain,[11] it is still believed that the pirenzepine-sensitive M$_1$ site,[12] located postsynaptically to the degenerating cholinergic neuron, and coupled to phosphatidylinositol (PI) hydrolysis,[13,14] is that which is most clearly associated with cognitive function.[15-17] Muscarinic receptors exhibiting a lower affinity for the antagonist pirenzepine are pharmacologically defined as being of the M$_2$ subtype and have been shown to modulate the intracellular production of cyclic adenosine monophosphate (cAMP) in several brain regions.[18-20] Nonselective pharmacologic stimulation of

Scheme I[a]



[a] Reagents: (a) BH_3Me_2S, THF; (b) pyridine·SO_3, DMSO; (c) Ph_3P, CBr_4, CH_2Cl_2; (d) n-BuLi, THF, −78 °C, then $(CH_2O)_n$, −78 °C → room temperature; (e) succinimide, Ph_3P, DEAD, THF; (f) $NaBH_4$, MeOH; (g) Et_3SiH, CF_3CO_2H, $CHCl_3$; (h) 37% aqueous CH_2O, 88% HCO_2H, reflux.

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brain muscarinic receptors has been associated with a number of undesirable effects including hypothermia and tremor.[21,22] Therefore, a strategy based upon muscarinic agonist replacement therapy as a palliative treatment for the symptoms of memory loss associated with AD requires the development of ligands which can effectively stimulate PI hydrolysis in the cortex and hippocampus and possess low agonist efficacy or antagonist effects at brain M_2 receptors.[2]

Oxotremorine (1) has long been known to act as a central nervous system (CNS) muscarinic agonist. Many of the effects associated with the pharmacologic activation of CNS M_2 receptors have been defined based upon the properties of 1.[23,24] Although M_2 agonist effects are thought to be an undesirable property of any muscarinic agonist ligand to be used in Alzheimer's therapy, the ability of this compound to readily penetrate the blood-brain barrier, combined with its long duration of action, makes 1 and attractive structure to consider for chemical modification. Many derivatives of 1 have been synthesized over the past several decades, however, the majority of these analogues have been designed and evaluated for their M_2 antagonist profiles.[25] More recently one analogue, *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)acetamide (BM-5) has been reported to possess agonist properties at some muscarinic receptors while acting as an antagonist at others.[26,27] In the process of chemically modifying acetylenic structures related to 1, we hoped to discover ways of enhancing the compound's CNS M_1 agonist effects while simultaneously diminishing those at both central and peripheral M_2 receptors. Reported herein are the synthesis and in vitro characterization of a series of amino terminally modified oxotremorine derivatives one of which has been found to possess a significantly more favorable M_1/M_2 agonist profile relative to 1.

Chemistry

Classic amino terminally modified analogues 2–4 (Table I) were prepared via cuprous chloride catalyzed Mannich reactions of *N*-propargyl-2-pyrrolidinone with the appropriate secondary amine[24,28,29] while compound 5 was prepared by alkylation of *N*-(4-bromo-2-butynyl)-2-pyrrolidone[30] with methylamine. The differentially constrained new oxotremorine derivatives 6–9 were prepared as outlined in Scheme I[31] and described in detail in the Experimental Section. Briefly, *N*-*tert*-butyloxycarbonyl-protected (*R*)- and (*S*)-proline (10) and pipecolinic acids (11) were converted to the dibromovinyl intermediates 12 and 13 via standard reduction, oxidation, and Wittig protocols. Formation of the acetylide anion according to Corey's procedure[32] and quenching with paraformaldehyde gave the hydroxymethyl derivatives 14 and 15. Previously, we have shown that the stereochemical integrity of the chiral center of the hydroxymethylene intermediates remains intact under these conditions.[31] Mitsunobu reaction of 14 and 15 with succinimide gave the imides 16 and 17, which were converted to the lactam amines 6 and 8 via a two-step reduction procedure which concomitantly removed the amino protecting group. N-Methylation of amines 6 and 8 under Eschweiler-Clark conditions afforded the tertiary amine derivatives 7 and 9.

Pharmacology

Compounds were evaluated for their binding affinities to M_1 and M_2 receptors in rat brain. Pirenzepine selectively binds to muscarinic receptors of the M_1 subtype,[12] therefore, displacement of [^{3}H]pirenzepine was used to measure the affinity of the analogues to cortical M_1 receptor sites. Since pirenzepine binds to a majority of muscarinic receptors in the rat brain stem with lower affinity,[33] this tissue was utilized as a source of M_2 receptors. Displacement of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) from this region of the rat brain was used to assess the binding affinity of the analogues to M_2 receptors. The binding results of the test compounds have been normalized to their equilibrium dissociation constants (K_i).

Ligand intrinsic activities were determined by measuring the magnitude of the second messenger response attributed to each receptor subtype and expressing that response as a percentage of that produced by the agonist standard carbachol (CARB). For the M_1 receptor, PI hydrolysis stimulated by CARB at a concentration of 1 mM in rat cortex was defined as a full agonist response (100%). Similarly, a 100% agonist response at M_2 receptors was defined as the magnitude of the inhibitory response in cAMP production caused by 100 μM CARB in a preparation of rat striatum in which 10 μM forskolin had been used to stimulate an elevation in cAMP production. All agonist responses were determined to be muscarinic in nature in that the activities of the test compounds were blocked by the antagonist atropine.[34]

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Detailed protocols of both the binding and intrinsic activity assays are included in the Experimental Section.

Results and Discussion

Table I shows the subtype intrinsic activities and the binding affinities of several classic amino terminally modified derivatives of 1. Immediately apparent is the fact that M_1-mediated agonist effects in the cortex are much more difficult to produce with these nonquaternary ammonium ligands than is the functional response from M_2 receptors in the striatum. These intrinsic efficacy differences have been linked to variations in the coupling efficiency of the receptor subtypes in different tissues.[35] In the cerebral cortex, the M_1 receptor-mediated PI response appears to lack an effective receptor reserve while the striatal M_2 receptors which modulate intracellular cAMP production have been shown to possess a modest reserve.[34]

As previously reported by Fisher,[36] we found 1 to be a weak partial agonist at M_1 receptors stimulating a PI response only 14% that of CARB. Activity at M_2 was was much more robust with the inhibition of cAMP production being about 60% that of the quaternary agonist standard. Binding affinities to the M_1 and M_2 receptor subtypes were nearly equivalent with K_i's in the range of 0.1 μM.

Upon probing the effects related to variations in the molecular size of the amino terminus of 1, it was found that modifications in this region of the molecule had dramatic effects on both the muscarinic receptor subtype binding affinity as well as intrinsic activity of the ligand. The in vitro profiles of analogues **2–5** serve to illustrate that the structure–activity relationships (SAR) of compounds in this series are very sensitive to even small changes in the size and lipophilicity of the amino terminus. While most of these modifications had relatively little effect on the maximal M_2 agonist response, the compounds possess markedly different abilities to stimulate cortical M_1-mediated PI hydrolysis. Azetidine oxo analogue **2** and dimethylamino oxo analogue **3** at a concentration of 1 mM are able to elicit a PI response which is approximately one-half that of CARB. Both of these compounds contain amino substituents which are slightly smaller than the pyrrolidine ring found in 1. In contrast to these results are the M_1 intrinsic activities of **4** and **5**. Compound **4** which sterically is slightly larger than **2** or **3** is devoid of M_1 agonist effects. Similarly, analogue **5** which as a secondary amine is smaller in the amino region than **2** or **3** is also more hydrophilic than either of these molecules, and it may be this feature which leads to its inactivity as a M_1 agonist. As mentioned above, responses at M_2 receptors were found to be much more readily achieved with analogues in this series. In addition to the robust M_2 agonist responses of **1–3**, the agonist activity of **4** exemplifies that comparatively larger amine substituents are well tolerated by this receptor subtype. However, the diminished M_2 intrinsic activity of **5** indicates that there is still a requirement for ligands to have a minimal degree of hydrophobicity associated with the amino terminus. From the intrinsic activity data in Table I we tentatively concluded that in order to produce M_1 agonist effects with compounds in this series, modifications of the amino terminus had to meet very strict steric as well as lipophilic requirements while the specifications to satisfy agonist activity at M_2 receptors were more flexible.

With regard to the subtype binding affinity selectivity of these compounds, the profiles of **2** and **3** are opposite to that which is postulated to be therapeutically desirable. That is, the enhanced M_1 agonist efficacy of these ligands was accompanied by an increase in the binding selectivity of the compounds in favor of the M_2 subtype.

Thus, within this limited set of analogues there were large differences in the M_1 intrinsic activities which could be directly attributed to small modifications in the molecular size and lipophilicity of the amino termini. To accommodate the rather exacting steric and hydrophobic requirements associated with the cortical PI response and at the same time attempt to alter the binding selectivity in favor of the M_1 subtype, we chose to incorporate an element of asymmetry into the amino region of this series of compounds. Each of the muscarinic receptor subtypes has an agonist ligand binding domain which is chiral. Therefore, by synthesizing chiral molecules in enantiomerically pure form which meet the pharmacophore requirements of a muscarinic agonist, the probability of preparing a ligand with the desired subtype selectivity and efficacy is enhanced.[37] A similar strategy has been employed with regard to modifications in the amido region of 1 to produce BM-5 as well as several conformationally constrained derivatives of this molecule which have been reported more recently.[38,39]

Specifically, the differentially constrained analogues **6–9** were targeted for several reasons. Due to the steric restrictions associated with M_1 agonist activity encountered with the classic analogues **1–5**, we felt that incorporating chiral substituents larger than the dimethylamino or azetidine moieties would be detrimental. The obvious position for the introduction of asymmetry then became the propargylic carbon linking the amine and acetylene moieties. Ringdahl and Jenden previously described the pharmacology of an oxotremorine derivative in which a methyl group had been inserted at this position and reported the compound to behave as a weak antagonist.[40] Therefore, rather than synthesize the azetidine or dimethylamino derivatives in which a methyl has been substituted at the propargylic carbon, we chose to radically change the accessible local low-energy rotational conformations of the substituents surrounding the amine moiety by incorporating the asymmetric carbon and the basic nitrogen in a ring. In so doing we hoped that such a modification might yield a muscarinic agonist with the desired M_1 subtype selectivity. Finally, it was envisioned

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Table II. Biologic Data for Differentially Constrained Analogues of 1

compd	R′	binding affinity[a] (K_i, μM) M₁ (cortex)	binding affinity[a] (K_i, μM) M₂ (brainstem)	intrinsic activity[a] (% rel to carbachol) M₁[b] (cortex)	intrinsic activity[a] (% rel to carbachol) M₂[c] (striatum)	formula[d]	anal.
6a		3.07 ± 1.18	2.46 ± 0.53	43.0 ± 4.7	87.2 ± 1.9	$C_{11}H_{16}N_2O{\cdot}1.5C_2H_2O_4{\cdot}0.75H_2O$	C,H,N
6b		40.5 ± 7.8	5.64 ± 1.41	11.1	55.5 ± 11.9	$C_{11}H_{16}N_2O{\cdot}1.25C_2H_2O_4{\cdot}0.25H_2O$	C,H,N
7a		5.46 ± 0.13	5.86 ± 0.98	0	45.2 ± 1.3	$C_{12}H_{18}N_2O{\cdot}1.5C_2H_2O_4{\cdot}0.50H_2O$	C,H,N
7b		10.5 ± 0.8	10.8 ± 4.2	0	28.2	$C_{12}H_{18}N_2O{\cdot}1.0C_2H_2O_4$	C,H,N
8a		3.03 ± 1.57	0.636 ± 0.048	23.7 ± 7.5	101.6 ± 4.6	$C_{12}H_{18}N_2O{\cdot}1.25C_2H_2O_4$	C,H,N
8b		27.6 ± 0.5	15.8 ± 3.7	4.46 ± 2.50	59.5 ± 7.1	$C_{12}H_{18}N_2O{\cdot}1.0C_2H_2O_4{\cdot}0.250H_2O$	C,H,N
9a		1.20 ± 0.26	3.66 ± 0.33	0	9.1	$C_{13}H_{20}N_2O{\cdot}1.0C_2H_2O_4{\cdot}0.60H_2O$	C,H,N
9b		5.38 ± 0.66	7.06 ± 2.20	0	0	$C_{13}H_{20}N_2O{\cdot}1.5C_2H_2O_4$	C,H,N

[a] A mean of two determinations or the mean with the standard error of the mean for three or more determinations. [b] Test compound concentration 1 mM. [c] Test compound concentration 100 μM. [d] Partial salts and/or hydrates are correlated with the integral of a broad peak of exchangeable protons in the ¹H NMR spectra.

that the target compounds could be prepared in optically pure form via a general method starting from the readily available *d*- and *l*-proline and pipecolinic acids.

Table II contains the in vitro profiles of the novel differentially constrained derivatives of 1. Within this series, several observations concerning the SAR of the subtype binding affinities and intrinsic activities are apparent. First, the hypothesis regarding the interaction of an asymmetric ligand with the receptor subtypes was validated. The (*R*)-enantiomers **6a–9a** consistently yielded compounds with more potent binding affinities as well as larger intrinsic activity responses than did the corresponding (*S*)-enantiomers **6b–9b**.

Within the (*R*)-enantiomeric series, secondary amines **6a** and **8a** produced greater agonist responses at cortical M_1 receptors compared to the corresponding tertiary amines **7a** and **9a**. The size of the amine ring was also a contributing factor to the M_1 agonist activity. The substituted pyrrolidine derivative **6a** elicits agonist activity which is nearly twice the magnitude of that produced by the substituted piperidine analogue **8a**. In comparison, agonist activity throughout the series at the M_2 subtype is not as sensitive to these structural modifications. Although the same trends in intrinsic activity are observed with respect to the enantiomers, secondary amine methylation, and amine ring size, many of the compounds still retain M_2 agonist properties.

Further examination of the data in Table II suggests that the incorporation of chirality into this series of acetylenic derivatives related to 1 has had a beneficial effect upon the agonist subtype selectivity of one of the ligands. The pyrrolidinyl-substituted derivative **6a** has an intrinsic activity profile comparable to the classic agonist standards **2** and **3**. The property which distinguishes **6a** from those of the classic series is that in muscarinic binding it does not discriminate for M_2 receptors with such high selectivity. From the binding data in Table II, one can calculate that the subtype selectivity of **6a** is maximally only 2-fold in favor of the M_2 subtype. This is in contrast to the binding selectivities of **2** and **3** both of which from the K_i data in Table II are estimated to have affinities for M_2 receptors which are between 8-fold and several hundred-fold more potent than for the M_1 subtype. Thus, in the search for therapeutically useful muscarinic agonists to ameliorate the symptoms of memory loss associated with AD that have a lower potential for producing adverse M_2-related side effects, the overall in vitro pharmacologic profile of **6a** represents a significant improvement over those of the classic amino terminally modified derivatives **2** and **3**.

In summary, we have synthesized in enantiomerically pure form, a novel series of analogues related to 1 in which an asymmetric center has been introduced at the propargylic carbon adjacent to the amine moiety. Further-

more, the rotational freedom about this carbon–nitrogen bond has been restricted by incorporating it into a five or six-membered ring. Pharmacologic characterization of this series of compounds at brain muscarinic receptors has resulted in the identification of the (*R*)-substituted pyrrolidine **6a**. Like 1, this molecule has intrinsic agonist activity at both M_1 and M_2 receptors, however, it produces a more robust agonist response at cortical M_1 receptors relative to 1. When compared with 2 and 3, other acetylenic derivatives related to 1 which have muscarinic intrinsic activities similar to **6a**, the new analogue was found to possess a more favorable M_1/M_2 binding profile in that it is much less selective for M_2 receptors.

Experimental Section

Proton magnetic resonance spectra were obtained on a Nicolet QE-300 (300 MHz) and a General Electric GN-300 (300 MHz) instrument. Chemical shifts are reported as δ values (ppm) relative to Me_4Si as an internal standard unless otherwise indicated. Mass spectra were obtained with Hewlett Packard HP5965 spectrometer. Elemental analyses and the above determinations were performed by the Analytical Research Department, Abbott Laboratories.

Thin-layer chromatography (TLC) was carried out by using E. Merck precoated silica gel F-254 plates (thickness 0.25 mm). Flash chromatography was carried out using Merck silica gel 60, 200–400 mesh.

Melting points are uncorrected and were determined on a Buchi melting point apparatus. Optical rotation data was obtained on a Perkin-Elmer Model 241 polarimeter. Protected prolines were purchased from Bachem (Torrance, CA) and pipecolinic acids from Aldrich (Milwaukee, WI). All reactions were performed under anhydrous conditions unless otherwise noted.

2(*R*)-(2,2-Dibromoethenyl)-*N*-(*tert*-butoxycarbonyl)pyrrolidine (12a). To a mixture of triphenylphosphine (13.0 g, 49.54 mmol), Zn dust (2.16 g, 33.0 mmol), and carbon tetrabromide (11.0 g, 33.0 mmol) was added dichloromethane (80 mL) at room temperature. After the mixture was stirred for 5 min, a solution of *N*-(*tert*-butoxycarbonyl)-(*R*)-prolinal[41] (3.29 g, 16.5 mmol) in dichloromethane (25 mL) was added. After stirring for 1 h, the reaction mixture was diluted with a mixture of EtOAc/hexane (1:1) and filtered through basic alumina (0.25 in. thick)/silica gel (0.5 in. thick, 40–60 μm). The filter cake was washed with a mixture of dichloromethane/EtOAc/hexane (1:1:1). The filtrate was concentrated, and the residue was taken up in ethyl acetate/hexane (1:1). The resulting precipitate was filtered off. After concentration of the filtate, the residual oil was subjected to flash chromatography using EtOAc/hexane (1:6.5 → 1:5) as the eluent. The pure solid product was isolated in 91% yield (5.31 g): TLC R_f = 0.35 (EtOAc/hexane = 1:4); $[\alpha]^{26}_D = -17.4°$ (*c* 1.15, MeOH); mp = 65–66 °C; MS (CI) *m/e* 354 $(M + H)^+$; ^1H NMR (DMSO-d_6, 70 °C) δ 6.57 (d, *J* = 8.1 Hz, 1 H), 4.26 (ddd, *J* = 7.9, 7.9, 4.9 Hz, 1 H), 3.30 (m, 2 H), 2.05–2.17 (m, 1 H), 1.72–1.92 (m, 2 H), 1.60–1.71 (m, 1 H), 1.40 (s, 9 H). Anal. ($C_{11}H_{17}Br_2NO_2$) C, H, N.

2(*S*)-(2,2-Dibromoethenyl)-*N*-(*tert*-butoxycarbonyl)pyrrolidine (12b): white solid; $[\alpha]^{26}_D = +17.4°$ (*c* 0.97, MeOH), mp = 67–68 °C.

3-Hydroxy-1-[2(*R*)-[*N*-(*tert*-butoxycarbonyl)pyrrolidinyl]]-1-propyne (14a). To a solution of **12a** (1.54 g, 4.34 mmol) in THF cooled to −78 °C was added a solution of *n*-BuLi in hexane (5.6 mL, 1.6 M, 8.92 mmol) dropwise over a period of 5 min. After stirring at −78 °C for 50 min, powdered paraformaldehyde was added. The reaction mixture was allowed to gradually warm up to ambient temperature over a period of 16 h, and then it was quenched with saturated aqueous $NaHCO_3$ solution. Extraction with EtOAc (3X), drying ($MgSO_4/Na_2SO_4$), concentration, and flash chromatography (EtOAc/hexane = 1:3 → 1:2) provided 486 mg (50%) of the required product as a clear oil: TLC R_f = 0.12 (EtOAc/hexane = 1:3); $[\alpha]^{26}_D = +137.1°$ (*c* 0.62, MeOH); MS (CI) *m/e* 226 $(M + H)^+$, ^1H NMR (CD_3OD) δ 4.49 (br s, 1 H), 4.17 (br s, 2 H), 3.34–3.45 (m, 1 H), 3.22–3.30 (m, 1 H), 1.85–2.15 (m, 4 H), 1.47 (s, 9 H).

3-Hydroxy-1-[2(*S*)-[*N*-(*tert*-butoxycarbonyl)pyrrolidinyl]]-1-propyne (14b): $[\alpha]^{23}_D = -139.0°$ (*c* 0.74, MeOH).

3-(2,5-Dioxo-1-pyrrolidinyl)-1-[2(*R*)-[*N*-(*tert*-butoxycarbonyl)pyrrolidinyl]]-1-propyne (16a). To a solution of **14a** (464 mg, 2.06 mmol), succinimide (306 mg, 3.09 mmol), and triphenylphosphine (1.08 g, 4.12 mmol) in THF cooled to 0 °C was added diethyl azodicarboxylate (718 mg, 4.12 mmol) dropwise. The reaction mixture was allowed to gradually warm to ambient temperature overnight. Concentration and flash chromatography (EtOAc/hexane = 1:1) provided 574 mg (91%) of the desired product as an amber viscous oil: TLC R_f = 0.21 (EtOAc/hexane = 1:1); $[\alpha]^{25}_D = +81.8°$ (*c* 0.62, MeOH); HRMS (CI) *m/e* calcd for $C_{16}H_{23}N_2O_4$ 307.1658 $(M + H)^+$, found 307.1661; ^1H NMR (CD_3OD) δ 4.40 (br m, 1 H), 4.22 (d, *J* = 1.8 Hz, 2 H), 3.38 (m, 1 H), 3.27 (m, 1 H), 2.70 (s, 4 H), 1.85–2.15 (m, 4 H), 1.46 (s, 9 H).

3-(2-Oxo-1-pyrrolidinyl)-1-(2(*R*)-pyrrolidinyl)-1-propyne Hydrogen Oxalate (6a). To a solution of **16a** (498 mg, 1.62 mmol) in MeOH (30 mL) cooled to −5 °C (ice and brine) was added $NaBH_4$ (492 mg, 13.0 mmol) in one portion. After stirring for 45 min, the reaction mixture was poured into a solution of saturated aqueous $NaHCO_3$ solution and extracted with chloroform (3 × 60 mL). The combined organic layers were dried (Na_2SO_4) and concentrated, and the residue was flash chromatographed (EtOAc/hexane = 9:1 → 0.1% MeOH in EtOAc), providing 277 mg (56%) of the desired product (oil) as a mixture of diastereomers at the hydroxy center: TLC R_f = 0.24 (EtOAc); HRMS (FAB) *m/e* calcd for $C_{16}H_{23}N_2O_3$ 291.1709 $(M - OH)^+$, found 291.1711. Anal. ($C_{16}H_{24}N_2O_4{\cdot}0.25H_2O$) C, H, N.

To a solution of 3-(5-hydroxy-2-oxo-1-pyrrolidinyl)-1-[2(*R*)-*N*-(*tert*-butoxycarbonyl)pyrrolidinyl]-1-propyne (163 mg, 0.53 mmol) and triethylsilane (154 mg, 133 mmol) in chloroform (2 mL) was added trifluoroacetic acid (TFA) (1.02 mL, 13.23 mmol) at 23 °C. After stirring for 2 h, the reaction mixture was concentrated to dryness, and the residue was taken up in chloroform and washed with saturated aqueous K_2CO_3. THe aqueous layer was extracted with chloroform (5×). The combined organic phase was dried (Na_2SO_4) and concentrated, and the resulting oil was flash chromatographed using 10% $MeOH/CHCl_3$ as the eluent to afford 75 mg (74%) of the desired product as an oil. Addition of ethereal oxalic acid to a solution of the product in ether/MeOH gave the oxalate salt after storing at −20 °C for a week: mp = 85–87 °C; TLC R_f = 0.4 ($CHCl_3/MeOH/NH_4OH$ = 9:1:0.1); $[\alpha]^{23}_D = +19.6°$ (*c* 0.28, MeOH); MS (CI) *m/e* 193 $(M + H)^+$; ^1H NMR (DMSO-d_6, 100 °C, 500 MHz) δ 4.31 ("tt", *J* = 7.2, 1.8 Hz, 1 H), 4.06 (s, *J* = 1.8 Hz, 2 H), 3.38 (t, *J* = 7.0 Hz, 2 H), 3.14–3.28 (m, 2 H), 2.24 (m, 3 H), 2.05–1.85 (m, 5 H). Anal. ($C_{11}H_{16}N_2O{\cdot}1.5C_2H_2O_4{\cdot}0.75H_2O$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(2(*S*)-pyrrolidinyl)-1-propyne hydrogen oxalate (6b): $[\alpha]^{23}_D = -18.2°$ (*c* 0.24, MeOH). Anal. ($C_{11}H_{16}N_2O{\cdot}1.25C_2H_2O_4{\cdot}0.25H_2O$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(*N*-methyl-2(*R*)-pyrrolidinyl)-1-propyne Hydrogen Oxalate (7a). A mixture of the free base of **6a** (181 mg, 0.94 mmol) and 10-fold excess each of 37% formalin and 88% formic acid was heated at reflux for 16 h. After cooling to room temperature, the reaction mixture was acidified with 6 N HCl. The aqueous phase was extracted with ether, and the phases were separated. The aqueous phase was basified with K_2CO_3 and extracted with $CHCl_3$ (3×). The combined organic phases were dried (Na_2SO_4) and concentrated under vacuum to give 153 mg (79%) of the desired product as an oil. Addition of oxalic acid to a solution of the product in ether/MeOH gave the oxalate salt as a viscous oil: TLC R_f = 0.40 (10% MeOH in $CHCl_3$); $[\alpha]^{23}_D = +42.7°$ (*c* 0.86, MeOH); MS (CI) *m/e* 207 $(M + H)^+$; ^1H NMR (DMSO-d_6, 95 °C) δ 4.06 (d, *J* = 1.6 Hz, 2 H), 3.62 (m, 1 H), 3.39 (t, *J* = 7.0 Hz, 2 H), 2.97 (m, 1 H), 2.69 (m, 1 H), 2.50 (s, 3 H), 2.22 (t, *J* = 8.1 Hz, 2 H), 2.15 (m, 1 H), 1.97 (m, 2 H), 1.85 (m, 3 H). Anal. ($C_{12}H_{18}N_2O{\cdot}1.5C_2H_2O_4{\cdot}0.5H_2O$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(*N*-methyl-2(*S*)-pyrrolidinyl)-1-propyne hydrogen oxalate (7b): $[\alpha]^{23}_D$ =

(41) Hamada, Y.; Shioro, T. New Methods and Reagents in Organic Synthesis. 29. A Practical Method for the Preparation of Optically Active N-Protected α-Amino Aldehydes and Peptide Aldehydes. *Chem. Pharm. Bull.* **1982**, *30*, 1921–1924.

−48.5° (c 0.88, MeOH). Anal. ($C_{12}H_{18}N_2O{\cdot}C_2H_2O_4$) C, H, N.

2(*R*)-1-(*tert*-Butoxycarbonyl)piperidinecarboxylic Acid (11a). (*R*)-Pipecolinic acid (2.95 g, 22.8 mmol) and *N,N*-diisopropylethylamine (4.8 mL, 27.4 mmol) were combined in 90 mL of 1,4-dioxane/H_2O (1:1) and cooled to 0 °C. To this solution was added di-*tert*-butyl dicarbonate (7.5 g, 34.2 mmol), and the mixture was allowed to stir at room temperature for 2 days. The reaction contents were then poured over saturated sodium bicarbonate solution and extracted with EtOAc (2×). The aqueous phase was next acidified with 10% citric acid and a small amount of 2 N HCl to pH = 3. The acidified solution was then extracted with EtOAc (4×). The organics were combined, washed with brine, dried (Na_2SO_4), and concentrated to leave a white solid (5.03 g) in 96% yield: TLC R_f = 0.36 ($CHCl_3$/MeOH/NH_4OH 10:4:1); $[\alpha]^{23}_D$ = +43.6° (c 0.96, MeOH); mp − 112–118 °C; MS (CI) *m/e* 230 (M + H)$^+$; ^{1}H NMR (DMSO-d_6, 100 °C) δ 4.58 (dd, *J* = 5.9, 2.4 Hz, 1 H), 3.81 (dm, 1 H) 2.93 (ddd, *J* = 12.8, 12.8, 2.8 Hz, 1 H), 2.07–2.04 (m, 1 H), 1.63–1.56 (m, 3 H), 1.38–1.22 (m, 2 H), 1.39 (s, 9 H). Anal. ($C_{11}H_{19}NO_4{\cdot}{}^3/_{10}H_2O$) C, H, N.

2(*S*)-1-(*tert*-Butoxycarbonyl)piperidinecarboxylic acid (11b): $[\alpha]^{23}_D$ = −43.4° (c 1.00, MeOH); mp = 114–118 °C.

2(*R*)-(2,2-Dibromomethenyl)-1-(*tert*-butoxycarbonyl)piperidine (13a). To a stirred solution of **11a** (4.88 g, 21.3 mmol) in 27 mL of THF at 0 °C was added borane–methyl sulfide complex (2.0 M in THF), 16.0 mL, 32 mmol) dropwise. After complete addition, the reaction was allowed to warm to room temperature and to stir overnight. Saturated $NaHCO_3$ solution was added, and the mixture was vigorously stirred for 30 min and then extracted with EtOAc (4×). The organics were combined, washed with brine, dried over Na_2SO_4, and concentrated. The crude product was purified by flash chromatography using (EtOAc/hexane 1:3 → 1:1 → 1:0) as the elutant. A white solid (3.94 g) was isolated in 86% yield: TLC R_f = 0.37 (10% MeOH in $CHCl_3$); $[\alpha]^{23}_D$ = +38.7° (c 0.98, $CHCl_3$); mp = 81–83 °C; MS (CI) *m/e* 216 (M + H)$^+$; ^{1}H NMR (DMSO-d_6, 100 °C, 500 MHz) δ 4.21 (t, *J* = 5.5 Hz, 1 H), 4.06–4.02 (m, 1 H), 3.82–3.78 (m, 1 H), 3.51 (ddd, *J* = 10.6, 8.0, 5.8 Hz, 1 H), 3.44 (ddd, *J* = 10.5, 6.4, 5.3 Hz, 1 H), 2.76 (ddd, *J* = 13.5, 12.6, 3.1 Hz, 1 H), 1.76 (dm, 1 H), 1.57–1.46 (m, 3 H), 1.44–1.36 (m, 1 H), 1.39 (s, 9 H), 1.33–1.26 (m, 1 H). Anal. ($C_{11}H_{21}NO_3$) C, H, N.

The product alcohol (3.18 g, 14.8 mmol) and triethylamine (6.2 mL, 44.4 mmol) were dissolved in 18.5 mL of DMSO. To this solution was added pyridinium sulfur trioxide (7.1 g, 44.4 mmol) in 13 mL of DMSO dropwise. After complete addition the reaction was stirred for 10 min, and then the reaction contents were poured over ice and extracted with diethyl ether (4×). The organics were combined and washed in succession with 10% citric acid, water, and saturated Na_2CO_3 solution, and finally dried over Na_2SO_4. The solvent was evaporated, leaving 3.06 g of a clear oil. An analytical amount was subjected to flash chromatography using (Et_2O/hexane 1:12) as the eluant: TLC R_f = 0.23 (EtOAc/hexane 1:8); $[\alpha]^{23}_D$ = +36.8° (c 1.14, $CHCl_3$); HRMS (CI) *m/e* calcd for $C_{11}H_{20}NO_3$ 214.1443 (M + H)$^+$, found 214.1440; ^{1}H NMR (DMSO-d_6, 100 °C, 360 MHz) δ 9.54 (s, 1 H), 4.44 (dd, *J* = 6.0, 3.5 Hz, 1 H), 3.79–3.73 (m, 1 H), 2.98–2.88 (m, 2 H), 2.12–2.05 (m, 1 H), 1.67–1.27 (m, 3 H), 1.42 (s, 9 H), 1.26–1.22 (m, 1 H).

Triphenylphosphine (16.8 g, 64 mmol) and carbon tetrabromide (8.5 g, 25.6 mmol) were combined in 21 mL of CH_2Cl_2 and stirred at room temperature for 20 min. The product aldehyde (2.7 g, 12.8 mmol) was then added via cannula at a steady rate. After complete addition, the reaction was allowed to stir for 5 min, and then the entire reaction was poured into EtOAc/hexane (1:1). The slurry that resulted was filtered through $^1/_4$ in. basic aluminum oxide/$^1/_4$ in. silica gel via vacuum filtration. The filter cake was washed with ethyl acetate/hexane (1:1). The obtained filtrate was concentrated and subjected to flash chromatography (Et_2O/hexane 1:19). A white solid (3.97 g) was isolated in 84% yield: TLC R_f = 0.13 (Et_2O/hexane 1:15); $[\alpha]^{23}_D$ = −31.6° (c 0.97, $CHCl_3$); mp = 108–110 °C; MS (FAB) *m/e* 370 (M + H)$^+$; ^{1}H NMR (DMSO-d_6, 100 °C, 500 MHz) δ 6.93 (d, *J* = 8.1 Hz, 1 H), 4.77 (ddd, *J* = 8.1, 5.3, 5.0 Hz, 1 H), 3.83 (m, 1 H), 2.92 (ddd, *J* = 13.5, 12.3, 3.1 Hz, 1 H), 1.68–1.58 (m, 4 H), 1.50–1.43 (m, 1 H), 1.42 (s, 9 H), 1.40–1.34 (m, 1 H). Anal. ($C_{12}H_{19}Br_2NO_2$) C, H, N.

2(*S*)-(2,2-Dibromoethenyl)-1-(*tert*-butoxycarbonyl)piperidine (13b): $[\alpha]^{23}_D$ = +30.3° (c 1.00, $CHCl_3$); mp = 109–111 °C. Anal. ($C_{12}H_{19}Br_2NO_2$) C, H, N.

3-Hydroxy-1-[2(*R*)-[1-(*tert*-butoxycarbonyl)piperidinyl]]-1-propyne (15a). The title compound was prepared by the same procedure as **14a**. The crude product was subjected to flash chromatography using EtOAc/hexane (1:4 to 1:2) as the eluant. A light yellow oil was isolated in quantitative yield: TLC R_f = 0.47 (EtOAc/hexane 1:2); $[\alpha]^{23}_D$ = +112.0° (c 1.12, $CHCl_3$); HRMS (CI) *m/e* calcd for $C_{13}H_{22}NO_3$, 240.1600 (M + H)$^+$, found, 240.1601; ^{1}H NMR (DMSO-d_6, 100 °C) δ 5.00 (br s, 1 H), 4.80 (t, *J* = 5.8 Hz, 1 H), 4.11 (dd, *J* = 5.8, 1.6 Hz, 2 H), 3.81 (dm, 1 H), 2.97 (ddd, *J* = 13.0, 13.0, 2.6 Hz, 1 H), 1.74–1.58 (m, 4 H), 1.43 (s, 9 H), 1.58–1.30 (m, 2 H).

3-Hydroxy-1-[2(*S*)-[1-(*tert*-butoxycarbonyl)piperidinyl]]-1-propyne (15b): yellow oil; $[\alpha]^{23}_D$ = −120.4° (c 1.20, $CHCl_3$).

3-(2,5-Dioxo-1-pyrrolidinyl)-1-[2(*R*)-[1-(*tert*-butoxycarbonyl)piperidinyl]]-1-propyne (17a). The compound was prepared by the same procedure as **16a**. The crude product was purified by flash chromatography using (EtOAc/hexane 1:2 → 1:1) as the eluant. However, flash chromatography was not successful in removing the impurity diethyl hydrazinedicarboxylate. A yield of 70% was calculated on the basis of the molar ratio of impurity vs product as seen from ^{1}H NMR. An analytical amount of the above material was deprotected with TFA, flash chromatographed, reprotected with di-*tert*-butyl dicarbonate, and flash chromatographed once again to give the product as a pure white solid: TLC R_f = 0.31 (EtOAc/hexane 1:1), $[\alpha]^{23}_D$ = +91.4° (c 0.98, $CHCl_3$); mp = 108–110 °C; MS (CI) *m/e* 321 (M + H)$^+$; ^{1}H NMR (DMSO-d_6, 100 °C, 500 MHz) δ 4.92 (br s, 1 H), 4.18 (d, *J* = 1.8 Hz, 2 H), 3.77 (dm, 1 H), 2.89 (ddd, *J* = 13.0, 13.0, 2.6 Hz, 1 H), 2.67 (s, 4 H), 1.68–1.51 (m, 5 H), 1.40 (s, 9 H), 1.32–1.26 (m, 1 H). Anal. ($C_{17}H_{24}N_2O_4$) C, H, N.

3-(2,5-Dioxo-1-pyrrolidinyl)-1-[2(*S*)-[1-(*tert*-butoxycarbonyl)piperidinyl]]-1-propyne (17b): $[\alpha]^{23}_D$ = −93.1° (c 1.00, $CHCl_3$); mp = 109–111 °C. Anal. Calcd for ($C_{17}H_{24}N_2O_4$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(2(*R*)-piperidinyl)-1-propyne Hydrogen Oxalate (8a). The title compound was prepared from **17a** by using the same procedure as that described for **6a**. Lactol: The crude product was flash chromatographed using (EtOAc/hexane 2:1) as the eluant. A white solid was isolated in 56% yield: TLC R_f = 0.38 (EtOAc); mp = 104–106 °C. HRMS (CI) *m/e* calcd for $C_{17}H_{27}N_2O_4$, 323.1971 (M + H)$^+$, found, 323.1973; ^{1}H NMR (DMSO-d_6, 98 °C, 500 MHz) δ 5.75 (dd, *J* = 6.9, 2.4 Hz, 1 H), 5.19–5.17 (m, 1 H), 4.96 (br s, 1 H), 4.30–4.26 (ddd, *J* = 17.3, 2.0, 1.8 Hz, 1 H), 3.80–3.73 (m, 2 H), 2.93 (ddd, *J* = 12.9, 10.9, 2.1 Hz, 1 H), 2.40–2.35 (m, 1 H), 2.22–2.12 (m, 2 H), 1.79–1.72 (m, 1 H), 1.68–1.52 (m, 5 H), 1.41 (s, 9 H), 1.37–1.27 (m, 1 H). Anal. ($C_{17}H_{26}N_2O_4$) C, H, N. **8a**: $[\alpha]^{23}_D$ = +10.9° (c 0.96, MeOH); mp = 77.81 °C; MS (CI) *m/e* 207 (M + H)$^+$; ^{1}H NMR (DMSO-d_6, 100 °C, 500 MHz) δ 4.15–4.11 (m, 1 H), 4.08 (d, *J* = 1.8 Hz, 2 H), 3.41 (t, *J* = 7.0 Hz, 2 H), 3.26 (ddd, *J* = 12.5, 5.4, 4.8 Hz, 1 H), 2.93 (ddd, *J* = 12.7, 8.0, 4.4 Hz, 1 H), 2.23 (t, *J* = 8.0 Hz, 2 H), 2.00–1.92 (m, 3 H), 1.77–1.69 (m, 2 H), 1.68–1.62 (m, 2 H), 1.57–1.50 (m, 1 H). Anal. ($C_{12}H_{18}N_2O{\cdot}1.25C_2H_2O_4$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(2(*S*)-piperidinyl)-1-propyne hydrogen oxalate (8b): $[\alpha]^{23}_D$ = −9.6° (c 0.90, MeOH). Anal. ($C_{12}H_{18}N_2O{\cdot}C_2H_2O_4{\cdot}0.25H_2O$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(*N*-Methyl-2(*R*)-piperidinyl)-1-propyne Hydrogen Oxalate (9a). The salt was prepared by a procedure similar to that described for **7a**. A clear glass solid was obtained in 87% yield: $[\alpha]^{23}_D$ = +20.4° (c 0.94, MeOH); MS (FAB) *m/e* 221 (M + H)$^+$; ^{1}H NMR (DMSO-d_6, 100 °C, 500 MHz) δ 4.15 (s, 2 H), 3.74 (br s, 1 H), 3.48 (t, *J* = 7.0 Hz, 2 H), 2.81–2.79 (m, 1 H), 2.65–2.62 (m, 1 H), 2.49 (s, 3 H), 2.29 (t, *J* = 8.1 Hz, 2 H), 2.03 ("q", *J* = 7.4 Hz, 2 H), 1.91–1.87 (m, 1 H), 1.77–1.72 (m, 1 H), 1.67–1.62 (m, 3 H), 1.53–1.49 (m, 1 H). Anal. ($C_{13}H_{20}N_2O{\cdot}C_2H_2O_4{\cdot}0.6H_2O$) C, H, N.

3-(2-Oxo-1-pyrrolidinyl)-1-(*N*-methyl-2(*S*)-piperidinyl)-1-propyne hydrogen oxalate (9b): $[\alpha]^{23}_D$ = −27.1° (c 0.50, MeOH). Anal. ($C_{13}H_{20}N_2O{\cdot}1.5C_2H_2O_4$) C, H, N.

Muscarinic Receptor Binding Assays. The cortical M_1 receptor was identified with the binding of [^{3}H]pirenzepine in homogenates. Adult rat fronto-parietal cortex was dissected on an ice-cold block and homogenized (1:200, w/v) with a polytron in 50 mM sodium–potassium phosphate buffer (pH 7.4). Com-

petition between [^{3}H]pirenzepine (6 nM) and the unlabeled test compounds was measured in an assay volume of 1 mL of this buffer with 0.5 mg of tissue; after 1 h at 25 °C, the bound ligand was separated from free ligand with filtration over 0.1% polyethylenimine-treated Whatman GF/B filters. Atropine (10 μM) was used to determine the level of nonspecific binding.

The brainstem M_2 receptor was studied with [^{3}H]QNB binding. Adult rat pons–medulla was dissected free on an ice-cold block and homogenized (1:200, w/v) with a polytron in 50 mM sodium phosphate buffer, pH 7.4. The competition assay was conducted with 0.2 nM [^{3}H]QNB, 0.5 mg of tissue, and various concentrations of unlabeled ligand in an assay volume of 1 mL at 25 °C for 75 min. The bound [^{3}H]QNB was separated from free by filtration over Whatman GF/B filters. Atropine (10 μM) was used to determine the level of nonspecific binding.

For the binding studies the ALLFIT[42] program was used to provide the IC_{50} and the Hill slope for inhibition of [^{3}H]pirenzepine or [^{3}H]QNB. The K_i value for the unlabeled drug was obtained by correction of the IC_{50} for the presence of the radiolabeled antagonist using the method of Cheng and Prusoff.[43]

Muscarinic Receptor Intrinsic Activity Assays. Cortex or striatum, dissected from adult rat (male Sprague–Dawley) brain, was minced with a razor blade on an ice-cold surface, suspended in ice-cold Puck's D1 solution (138 μM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose, and 58.4 mM sucrose; osmotic strength adjusted to 340 mosM; pH 7.4), and filtered sequentially through two Nitex bags (210 μm and 130 μm pore). For the cAMP assays, the final suspension was washed twice with a physiological PBS solution (110 mM, NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, 25 mM Na_2HPO_4; osmotic strength adjusted to 340 mosM; pH 7.4). For the phosphoinositide assays, the final suspension was washed twice with oxygenated, modified Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2{\cdot}6H_2O$, 1.2 mM glucose). Washed dissociated tissue was suspended in PBS or Krebs–Henseleit buffer for distribution into tubes or culture wells (2–4 mg/mL final concentration). The final preparation is a mixture of dispersed cells and small fragments of brain tissue of about 100 μm diameter.

For phosphoinositide assays, resuspended dissociated cortical tissue was rejuvenated with three sequential 20-min incubations in oxygenated Krebs–Henseleit buffer (composition given above) at 37 °C under 95% O_2/5% CO_2, with the renewal of the buffer after each incubation; the final incubation was in the presence of 10 mM lithium ion. The rejuvenated cells were than labeled 60 min at 37 °C in a volume of 4 mL, under an atmosphere of 95% O_2/5% CO_2, with 60 μCi of [^{3}H]inositol (New England Nuclear, Boston, MA) per rat frontoparietal cortex (two sides; approximately 260 mg total original wet weight of tissue). For cAMP assays, dispersed cells were incubated 45 min with 20–40 μCi of [^{3}H]adenine (22 Ci/mmol; Amersham, Arlington Heights, IL) at 37 °C in a volume of 2 mL under ambient air.

For the phosphoinositide assays, the suspension of prelabeled dissociated tissue was diluted to approximately 15 mg/mL with Krebs–Henseleit buffer, containing 10 mM lithium ion, and aliquoted to 4-mL plastic tubes to a final concentration of 2–4 mg/mL. Antagonists when utilized were added, and 15 min later test compounds were added to a final volume of 300 μL of a 1 mM solution; the cells were further incubated under 95% O_2/5% CO_2 for 60 min. Reactions were terminated with the addition of 1 mL of chloroform/methanol (2:1). Chloroform (1 mL) and water (1 mL) were added to facilitate phase separation. The water contained approximately 2000 dpm of [^{14}C]inositol 1-phosphate (Amersham) as a "recovery standard". The samples were capped, vigorously mixed, and then centrifuged at 3000 rpm for 15 min to separate phases. The aqueous phases of the samples were transferred to new tubes and mixed with 2 mL of water. [^{3}H]-Inositol 1-phosphate was purified on Dowex columns as previously described.[44]

Dissociated adult rat striatal tissue was prepared by mechanical sieving, and the striatal minces were filtered sequentially through two Nitex filters (210 and 130 μm pore sizes) in ice-cold Puck's D1 solution (138 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2PO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose, and 58.4 mM sucrose; 340 mosM; pH 7.4). The tissue was pelleted and then washed and resuspended in ice-cold phosphate-buffered saline (100 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, and 25 mM Na_2HPO_4; 340 mOsm; pH 7.4).

Cyclic AMP levels in the tissue were assayed by a prelabeling method. ATP pools were labeled by incubating dissociated striatum with [^{3}H]adenine for 45 min at 37 °C. After removal of the excess label, the tissue was distributed into a 24-well culture dish. The tissue was incubated with isobutylmethylxanthine (1.5 mM) and antagonists (when present) for 30 min before the addition of 10 μM forskolin and 100 μM of the test compound; total assay volume was 400 μL. After 10 min the reaction was stopped with the addition of trichloroacetic acid, and the [^{3}H]cyclic AMP in the wells was determined with the use of ion-exchange columns, as described previously.[23]

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