# The Synthesis and Biochemical Pharmacology of Enantiomerically Pure Methylated Oxotremorine Derivatives

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Previous pharmacological studies of methylated oxotremorine derivatives bearing substituents at the 3-, 4-, and 5-positions of the pyrrolidinone ring have been conducted using racemic mixtures, and not with optically active compounds. The synthesis and radioligand binding data of optically active, methylated oxotremorine derivatives at the 3- and 4-positions are described. There are significant pharmacological differences between the 3- and 4-position derivatives. The 4-position enantiomers have weak, approximately equal affinity and antagonist-like profiles, whereas the 3-position enantiomers have significantly different affinities and partial agonist-like profiles.

Senile Dementia of the Alzheimer's Type (SDAT) is a neurodegenerative disease that results in progressive dementia and memory impairment. Autopsied brain tissue from SDAT patients has shown a consistent and significant decrease in cholinergic markers (among others), such as choline acetyltransferase and acetylcholine esterase, most notably in the nucleus basalis of Meynert, cerebral cortex, and hippocampus.<sup>1</sup> It has been suggested that the degeneration occurs presynaptically, while the postsynaptic muscarinic receptors remain intact.<sup>2</sup> These observations, in part, form the basis for the cholinergic hypothesis of age-related memory loss.<sup>3</sup> A series of drug discovery projects<sup>4</sup> have been initiated in our geriatric program to find cholinergic agents to ameliorate the cognitive symptoms and to possibly retard the behavioral sequelae<sup>5</sup> of this degenerative disease.

The introduction of a substitutent into a neurotransmitter mimic may decrease the affinity of the compound for its receptor by introducing steric hindrance between it and the receptor binding domain. Rather than a disadvantage, this effect may be useful as a possible method to construct molecules with the optimal degree of partial agonism and possibly with measurable pharmacological attributes such as receptor subtype or functional selectivity. The addition of a methyl group to a series of cholinergic agonists has been shown to dramatically decrease in vivo and in vitro cholinergic agonist activity. One such investigation was the synthesis and pharmacological studies of oxotremorine (Figure 1).<sup>6</sup> The systematic addition of a methyl group to oxotremorine produced compounds with pronounced pharmacological differences. These results provided evidence for the amount of steric tolerance that was available in oxotremorine for lipophilic substitutents within the ligand binding domain.

One drawback to the original work was that the pharmacological studies of the methylated compounds at the 3- and 4-position of the pyrrolidinone ring were conducted using racemic mixtures and not pure enantiomers. The reported data provided a detailed account of the general trend in activity for the compounds. However, the current program goal was to search for targets with selective pharmacological attributes, and the comparison of new optically active targets with racemic standards was not satisfactory. As a result, synthetic strategies to the optically active forms of the 3- and 4-methylated deriv-







Figure 2. Targeted structures. One of the substituents  $R_a$ ,  $R_b$ ,  $R_c$ ,  $R_d$  is  $CH_3$ , the remaining are H.

Scheme I



atives of oxotremorine and its congeners were sought. This report describes the syntheses of the target molecules (Figure 2) using either chiral auxiliary methodology or chiral starting materials derived from natural sources. The preliminary biochemical pharmacology of these target structures is also presented.

### Chemistry

Simple optical resolution of racemic targets I would have been an attractive approach since equal amounts of the two enantiomers were needed for the study. However, the success of this methodology appeared doubtful since the optical center and the amine moiety were not in close proximity. Conceptually the synthesis of the four different methylated derivatives could be achieved by the differential attack of diamine 2 with an enantiomeric pair of carbonyl differentiated succinates 1 (Scheme I). This type of approach was successfully used in the preparation of the hydroxylated oxotremorine derivatives from D- and L-malic acids.<sup>4b</sup> However, in this case it was more convenient to treat the synthesis of the the 3- and 4-methylated derivatives separately.

The 3-substituted pyrrolidinone derivatives 12ab and 13ab were prepared from the chiral auxiliary oxazolidi-

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Scheme II<sup>a</sup>



<sup>a</sup> (i) *n*-BuLi, CH<sub>3</sub>CH<sub>2</sub>COCl, THF, -78 °C; (ii) LDA, CH<sub>2</sub>=CHCH<sub>2</sub>-Br, THF, -78 °C; (iii) *n*-BuLi, PhCH<sub>2</sub>OH; (iv) O<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, -78 °C; (v) Zn, HOAc; (vi) NaBH<sub>3</sub>CN, HOAc, 24 or 25; (vii) MeOH, HCl.

nones 5 and 67 (Scheme II). Acylation of oxazolidinones 5 and 6 with propionyl chloride using *n*-butyllithium as base, followed by alkylation of the intermediates with allyl bromide using lithium diisopropylamide as base, afforded the corresponding methylated pentenoates 7 and 8. The absolute configuration and optical rotation of the pure enantiomers 7 and 8 have been previously determined.<sup>8</sup> Treatment of 7 and 8 with a THF solution of benzyl alcohol and 1.5 equiv of *n*-butyllithium produced benzyl esters 9ab. Ozonolysis of 9ab in methylene chloride followed by a reductive workup with zinc and acetic acid gave aldehydes 10ab, which were used directly after plug filtration through silica gel. Reductive amination of 10ab with diamines 24 and 25 in the presence of sodium cyanoborohydride in a mixture of acetic acid and methanol gave initially the ring opened diamine 11ab which ring closed to the target methylated oxotremorine derivative 13ab and 14ab, respectively.

The transformation of 5 and 6 into 13ab and 14ab was straightforward with overall isolated yields ranging from 30 to 40%. Some comments on the sequence of steps are required. Removal of the chiral auxiliary and formation of the benzyl ester prior to the reductive amination was necessary. Diamines 24 and 25 were found to react with the oxazolidinone moiety as well as the aldehyde functionality to produce a series of byproducts. Removal of formaldehyde, a byproduct in the ozonolysis reaction, by plug filtration through silica gel was important. If the formaldehyde was not removed, the major product in the reductive amination was the N-methyl derivative of the ring opened compound 11ab. The side product 12ab had an  $R_f$  value similar to the product which complicated the product isolation and purification.

The racemic methylated compounds could be obtained by mixing an equal amount of the two enantiomers or by direct synthesis. The direct synthesis proved quite simple <sup>8</sup> (Scheme III). Alkylation of dimethyl methylmalonate 15 with allyl bromide using sodium methoxide as base, followed by saponification and decarboxylation, afforded 2-methyl-4-pentenoic acid 16. Esterification with benzyl alcohol gives the racemic form of 9ab, compound 17. Conversion of 17 into the racemic targets 18 and 19 paralleled the chemistry used to prepare the optically active materials. Scheme III<sup>a</sup>



<sup>a</sup> (i) NaOMe, CH<sub>2</sub>==CHCH<sub>2</sub>Br; (ii) NaOH, EtOH, H<sub>2</sub>O; (iii) 180 °C; (iv) PhCH<sub>2</sub>OH, TsOH; (v) O<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 78 °C; (vi) Zn, HOAc; (vii) NaBH<sub>3</sub>CN, HOAc, 25 or 26; (viii) MeOH, HCl.

Scheme IV<sup>a</sup>



 $^{\rm a}$  (i) (CF\_3CO)\_2O, CH\_2Cl\_2; (ii) HNR\_2, (CH\_2O)\_n, CuCl, HOAc, dioxane; (iii) HCl, EtOH, heat or NaBH\_4, EtOH.

Scheme V<sup>a</sup>



<sup>a</sup> (i) Dihydropyran, HCl; (ii) LAH, THF; (iii) TsCl, Et<sub>3</sub>N; (iv) NaCN, EtOH; (v) MeOH, HCl; (vi) TMSI, EtOH; (vii) propargylamine, Na<sub>2</sub>CO<sub>3</sub>, EtOH; (viii) HNR<sub>2</sub>, CH<sub>2</sub>O, CuCl, HOAc, dioxane, heat.

The diamine intermediates 24 and 25 were readily prepared in multigram scale<sup>9</sup> (Scheme IV). Treatment of propargylamine 20 with trifluoroacetic anhydride afforded trifluoroacetamide 21 which was reacted with either dimethylamine or pyrrolidine and paraformaldehyde in a mixture of acetic acid and dioxane to give the aminoacetamide 22 and 23. Removal of the trifluoroacetamide group from 22 and 23 was achieved using either sodium borohydride in methanol, a nonacidic method, or ethanolic hydrogen chloride to afford the diamines 24 and 25.

The synthesis of 4-methylated oxotremorine derivatives 34cd and 35cd used (R)- and (S)-4-methylbutyrolactones 31cd as the key intermediates where the optical center was introduced from natural sources (Scheme V). The (S)-enantiomer 31d was obtained commercially, and the (R)-enantiomer was readily prepared from (S)-3-hydroxy-2-methylpropionate 26.<sup>10</sup> The four-step sequence for the conversion of 26 into 31d was somewhat lengthy but proceeded in high yield. Protection of the hydroxyl group in 26 as the tetrahydropyranyl ether 27 followed by reduction of the ester group with lithium aluminum hydride afforded the monoprotected diol 28. Conversion of the alcohol in 28 to the methanesulfonate 29 with methanesulfonyl chloride and triethylamine and displacenment of the methanesulfonate with cyanide yielded nitrile 30. Treatment of 30 with methanolic hydrogen

compd	amine	config	$[\alpha]^{26}$ D, <sup>a</sup> deg	form <sup>c</sup>	method	yield, %	mp, °C
13a	Pyr	(S)-3	-19 (c = 1.33)	FB	Α	49	oil
	Pyr	(S)-3	-7 (c = 1.025)	S			90-91
18	Pyr	(±)-3		FB			oil
	Pyr	(±)-3		S			oil
13b	Pyr	(R)-3	+20 (c = 1.560)	FB	Α	54	oil
	Pyr	(R)-3	+7 (c = 1.36)	S			88-89
34c	Pyr	(S)-4	+9 (c = 1.46)	FB	В	75	oil
	Pyr	<b>(S)-4</b>	+3 (c = 1.0)	S			116-118
34d	Pyr	( <i>R</i> )-4	-9 (c = 1.03)	FB	В	85	oil
	Pyr	( <i>R</i> )-4	-3 (c = 1.0)	S			111-112
14a	NMe <sub>2</sub>	(S)-3	-21 (c = 0.989)	FB	Α		oil
19	NMe <sub>2</sub>	(±)-3		FB	С		oil
14b	NMe <sub>2</sub>	(R)-3	+22 (c = 0.980)	FB	Α		oil
35c	$NMe_2$	(S)-4	+9 (c = 1.62)	FB	В	67	oil
	NMe <sub>2</sub>	(S)-4	+4 (c = 0.97)	S			151-152
35d	$NMe_2$	(R)-4	-8 (c = 1.1)	FB	В	62	oil
	$NMe_2$	(R)-4	-3 (c = 1.0)	S			153-154
36c	NMe <sub>3</sub>	<b>(S)-4</b>	+3 (c = 1.0)	S			148149
36d	NMe <sub>3</sub>	(R)-4	-2 (c = 1.0)	S			146-147

<sup>a</sup> Optical rotations are carried out in methylene chloride for the free bases and in methanol for the amine salts. <sup>b</sup> Pyr = pyrrolidinyl. <sup>c</sup> S = salt, FB = free base.

chloride afforded 31d in 54% isolated yield which was significantly higher than the reported procedure where potassium hydroxide followed by acidification<sup>11</sup> was used.

Treatment of butyrolactones 31cd with trimethylsilyl iodide in ethanol<sup>11</sup> followed by the addition of triethyl orthoformate afforded the iodo esters 32cd. Reaction of iodo esters 32cd with propargylamine in ethanol containing sodium carbonate as base gave, upon cyclization, the propargylpyrrolidinones 33cd. The 4-methylated oxotremorine derivatives 34cd and 35cd were then prepared by reaction of 33cd with paraformaldehyde and either pyrrolidine or dimethylamine in a mixture of acetic acid and dioxane containing a catalytic amount (5%) of cuprous chloride. Treatment of 35cd with methyl iodide produced the corresponding methiodides 36cd.

### Pharmacology

Displacement of [<sup>3</sup>H]quinuclidinyl benzilate ([<sup>3</sup>H]QNB) in muscarinic receptor binding assays has been used extensively as a rapid and efficient screen for test compounds that interact at cholinergic receptors.<sup>12</sup> Little information regarding the potential intrinsic activity or pharmacological selectivity of the compound for muscarinic receptor subtypes is obtained in this assay. Meaningful estimates of intrinsic activity and pharmacological selectivity can nevertheless be determined from the apparent equilibrium dissociation constants ( $K_i$ ) for test compounds.<sup>13,19</sup> This information can be obtained using a series of assays that employ tritiated agonists as well as antagonists to label receptor populations in several different tissues.

A method for using the relative affinities ( $K_i$  values) of test compounds to displace [<sup>3</sup>H]-N-methylscopolamine ([<sup>3</sup>H]NMS, antagonist) and [<sup>3</sup>H]oxotremorine-M ([<sup>3</sup>H]-Oxo-M, agonist) as an index of their efficacy at cortical muscarinic receptors (NMS/OXO-M ratio) has been described.<sup>13</sup> Alternatively, [<sup>3</sup>H]quinuclidinyl benzilate ([<sup>3</sup>H]QNB, antagonist) can be used to label both the highand low-affinity states<sup>14</sup> and [<sup>3</sup>H]-cis-[(methyltrimethylammonio)methyl]-1,3-dioxolane ([<sup>3</sup>H]CD, agonist) to label the high affinity agonist state of the muscarinic receptor in competition assays.<sup>15</sup> The experimental conditions were chosen to maximize the difference between agonists and antagonists, and the ratio of  $K_i$  values for the two assays provides a useful index of intrinsic activity. Muscarinic antagonists display similar affinity in both binding assays ( $K_i$  ratio is ~ 1.0). Full muscarinic agonists displace [<sup>3</sup>H]CD from the muscarinic receptor with high affinity relative to their displacement of [<sup>3</sup>H]QNB. Thus, full agonists such as carbachol exhibit large ratios ( $K_i$  ratios ~ 500-2000). Compounds that display partial muscarinic agonist activity, such as oxotremorine, in functional assays have intermediate ratios ( $K_i$  ratio ~ 50-500). The ratio of  $K_i$  values determined in the two assays correlates well with the ability of agonists to stimulate cortical phosphatidylinositol turnover.<sup>14</sup>

To evaluate pharmacological selectivity of these analogs, displacement of [3H] pirenzepine ([3H]PZ) from rat cortical receptors was used in conjunction with displacement of [<sup>3</sup>H]QNB from rat cardiac muscarinic receptors. Pirenzepine (PZ) is an antagonist that selectively labels a subpopulation of muscarinic receptors  $(M_1)$  in central and peripheral tissues relative to the classical antagonist QNB. Approximately 80% of rat cortical receptors labeled by  $[^{3}H]QNB$  bind PZ with high affinity (M<sub>1</sub>) while the remaining receptors bind PZ with low affinity  $(M_2)$ .<sup>16</sup> Using a low concentration of  $[^{3}H]PZ$ , the M<sub>1</sub> subpopulation of muscarinic receptors can be preferentially labeled. Rat cardiac receptors are essentially of the M<sub>2</sub> subtype with low affinity for PZ.<sup>17</sup> With this nearly homogeneous population, the  $M_2$  subtype of receptors can be separately labeled using rat cardiac tissue and [<sup>3</sup>H]QNB.

Guanine nucleotides shift  $M_2$  muscarinic receptor affinity for agonists to a single low affinity state.<sup>18</sup> Displacement of [<sup>3</sup>H]QNB from rat cardiac  $M_2$  was performed in the absence and presence of 10  $\mu$ M guanosine 5'-( $\beta$ , $\gamma$ imidotriphosphate) (Gpp(NH)p) to assess binding of compounds to all affinity states and to the low-affinity state, respectively, of the muscarinic receptors.<sup>19</sup>

Muscarinic receptor  $(M_1)$  selectivity was estimated from the apparent  $K_i$  ratio of  $K_i$  QNB (heart) and  $K_i$  PZ (cortex), and potential  $M_2$  agonist activity was determined in heart tissue from the apparent  $K_i$  ratio of  $K_i$  QNB + Gpp(NH)p and  $K_i$  QNB – without Gpp(NH)p. The results for the title compounds and a series of six standard ligands for the muscarinic receptor ranging from full agonist-like

Table II. Nonspecific Agonist Binding Properties of the Methylated Oxotremorine Derivatives and Standards

			$K_{ m i}, \mu { m M}$	ratio [3H]QNB/	
compd	R <sub>n</sub>	$NR_2$	[ <sup>3</sup> H]QNB	[ <sup>3</sup> H]CD	[ <sup>3</sup> H]CD <sup>b</sup>
13 <sup>e</sup>	R <sub>a</sub>	Pyr	$2.9 \pm 0.67$	$0.055 \pm 0.010$	51
18 <sup>e</sup>	$R_{a/b}$	Pyr	$0.71 \pm 0.03$	$0.015 \pm 0.001$	48
13be	Rb	Pyr	$0.43 \pm 0.13$	$0.023 \pm 8.9$	19
$34c^e$	$R_{c}$	Pyr	$4.7 \pm 0.66$	$2.5 \pm 0.29$	1.9
$34d^e$	$R_d$	Pyr	$2.3 \pm 2.2$	$1.5 \pm 0.11$	1.6
14 <b>a</b>	R <sub>a</sub>	$NMe_2$	>100	$0.63 \pm 0.15$	
19	$R_{a/b}$	$NMe_2$	$22 \pm 3.6$	$0.26 \pm 0.010$	85
14b	Rb	$NMe_2$	$8.9 \pm 1.1$	$0.071 \pm 0.005$	125
$35c^{e}$	$R_{c}$	$NMe_2$	>1000	>10	
35de	$R_d$	$\mathbf{NMe}_2$	150	>10	
36c	$R_{c}$	$\mathbf{NMe}_2$	>100	$0.98 \pm 0.21$	
36d	$R_d$	$NMe_2$	>100	$0.45 \pm 0.14$	
37, carbachol			$5.0 \pm 0.32$	$0.004 \pm 0.001$	1220
38, Oxo-M		$0.91 \pm 0.27$	$0.00078 \pm 0.0001$	1200	
<b>39</b> , Oxo-2		$2.9 \pm 0.04$	$0.0063 \pm 0.0004$	460	
40, oxotremorine		$0.06 \pm 0.01$	$0.0005 \pm 0.0001$	120	
41, Oxo-Pip			$0.12 \pm 0.05$	$0.056 \pm 0.011$	2.2
<b>42</b> , BM-5			$0.08 \pm 0.02$	$0.004 \pm 0.001$	19.5

<sup>a</sup> Mean of 2–7 determinations. <sup>b</sup> The [<sup>3</sup>H]QNB data is from rat cortex. <sup>c</sup> From one determination or a mean of two determinations. <sup>d</sup> The [<sup>3</sup>H]QNB data is from rat heart. <sup>e</sup> Identical results (2–4 determinations) were obtained in parallel assays with the free base of the compound.

(carbachol, Oxo-M) to muscarinic antagonist-like (Oxo-Pip) from the five *in vitro* assays are presented in Tables II and III.

In general, the data for both the 3-methyl- and the 4-methyl-substituted pyrrolidinone derivatives parallel the previously described observations for the racemic compounds. The 3-methyl derivatives retain agonist-like activity, albeit weaker in both affinity and efficacy, relative to oxotremorine, and the 4-methyl derivatives possess antagonist-like effects similar to Oxo-Pip<sup>20</sup> but with significantly weaker affinity. In addition, the dimethylamine derivatives, when compared to the corresponding pyrrolidino derivatives, have weaker affinities (potency) for the receptors but have greater agonist-like effects (efficacy). The major advantage to these data are the subtle differences that are observed between the optical isomers.

#### Discussion

Our drug discovery program over the past several years has searched for substituted oxotremorine derivatives as therapeutic drug leads for the treatment of SDAT. The cholinergic hypothesis of memory deficits implies that the desired drug candidate should be an agonist-like compound which is selective for the disease-affected receptors, presumably postsynaptic and M1, while other muscarnic receptors in the periphery and brain, presumably M2, would be unaffected by the agonist-like compound with little or no side effects. The systematic efforts of Ringdahl<sup>e</sup> to pharmacologically characterize the effect of methyl substitution about the muscarinic agonist oxotremorine provided a starting point for this current work. Substitution at the 3- and 4-position on the pyrrolidinone ring of oxotremorine became the major focus and unfortunately, in the origional studies only racemic material substituted at these positions was characterized. To provide a reference point for future work the synthesis and pharmacological characterization of optically pure 3- and 4-methylpyrrolidinone ring substituted oxotremorine derivatives was undertaken. A ligand-based biochemical profile of the optically active forms of the methylated derivatives, when compared to carbachol and the unsubstituted oxotremorine derivatives, provides some insight into the type of target compound that should be selected as a potential therapeutic agent.





The series of six muscarinic agents that were selected as standards ranged from full agoinist-like compounds (carbachol, Oxo-M) to the reported antagonist-like compound, Oxo-Pip<sup>20</sup> (Figure 3, Tables II and III). In addition, three of the standards (oxotremorine, Oxo-2 and Oxo-M) are the nonmethylated references of the title compounds. The full muscarinic agonists (carbachol, Oxo-M), having [<sup>3</sup>H]QNB/[<sup>3</sup>H]CD ratios of ca. 1200, elicited strong guanine nucleotide shifts (G-shifts of 63 and 23, respectively), suggesting strong agonist-like effects in the heart. which are presumably  $M_2$  mediated. The partial agonists oxotremorine and Oxo-2, having [3H]QNB/[3H]CD ratios of 120 and 420, produced equally large G-shifts (32 and 23) in the heart, suggesting strong  $M_2$  agonist-like effects. The muscarinic antagonist Oxo-Pip, having a predicted antagonist [3H]QNB/[3H]CD ratio of 2.2, produced only a weak shift (5.0) in the concentration-response curve with the addition of the stable guanine nucleotide analog.

The oxotremorine related standard BM-5<sup>21</sup> has been characterized as both a weak partial  $M_1$  agonist and as a  $M_2$  antagonist. Hypothetically, this profile defines a putative prototype SDAT therapeutic agent. In the current series of biochemical assays, BM-5 produced a weak partial agonist profile, ([<sup>3</sup>H]QNB/[<sup>3</sup>H]CD ratio of 20) and it produced a weaker G-shift (2.5) than the antagonist Oxo-Pip (5.0). The current data provide evidence complementary to the data reported for BM-5 as a mixed  $M_1$  partial agonist and  $M_2$  antagonist.

The apparent  $K_i$  ratio of  $K_i$  [<sup>3</sup>H]QNB (heart) to  $K_i$  [<sup>3</sup>H]-PZ (cortex) does not provide a direct measure of a compound's  $M_1$  selectivity or agonist-like effects. This ratio, when used in comparison to the ratios of other test compounds, provides the relative ranking for interaction at  $M_1$  and  $M_2$  receptors. The smaller the ratio the greater the  $M_2$  interaction and the larger the ratio the greater the  $M_1$  effects. Oxotremorine and the oxotremorine-like

Table III. Biochemical Data for M1 and M2 Selectivity of Methylated Oxotremorine Derivatives and Standards

compd	R <sub>n</sub>	$NR_2$	[ <sup>3</sup> H]PZ K <sub>i</sub> , µM rat <sup>c</sup>	[ <sup>3</sup> H]QNB K <sub>i</sub> , µM heart rat <sup>e</sup>	[ <sup>3</sup> H]QNB + GppNHp K <sub>i</sub> , μM heart	ratio [³H]QNB + GppNHp/ [³H]QNB	ratio [³H]QNB + GppNHp/[³H]PZ	ratio [ <sup>3</sup> H]QNB/ [ <sup>3</sup> H]PZ <sup>d</sup>
13 <b>a</b> e	Ra	Pyr	$2.2 \pm 0.17$	0.11 🛳 0.1	$3.3 \pm 1.7$	32.	1.5	0.05
18 <sup>e</sup>	$R_{a/b}$	Pyr	$0.29 \pm 0.14$	0.08 🛳 0.003	$1.1 \pm 0.07$	15.	3.9	0.26
13b <sup>e</sup>	$\mathbf{R}_{\mathbf{b}}$	Рут	0.25 单 0.06	0.09 🌰 0.05	$0.43 \pm 0.17$	4.9	1.8	0.36
34c <sup>e</sup>	$\mathbf{R}_{\mathbf{c}}$	Рут	$1.3 \pm 0.33$	$13. \pm 3.7$	$27. \pm 2.3$	2.0	21.	11.
34d°	$R_d$	Рут	0.51 🌰 0.1	3.8 🌒 1.0	$6.0 \pm 1.3$	1.6	12	12.
1 <b>4a</b>	$R_a$	$NMe_2$	$>1078 \pm 15$	$21 \pm 6.1$	30.	1.4.	0.4	0.27
19	$R_{a/b}$	$NMe_2$	$212 \pm 1.0$	1.3 🏚 0.53	$13 \pm 2.5$	10.	1.1	0.11
14b	Rb	$NMe_2$	$6.8 \pm 0.49$	$0.24 \pm 0.04$	9.3 🌨 3.4	38	1.4	0.04
35c <sup>e</sup>	$R_{c}$	$NMe_2$	>100	>100	>100	>100		
35d°	$R_d$	$NMe_2$	15	>100	>100	>100		
36c	$\mathbf{R}_{c}$	$NMe_2$	$>109.1 \pm 0.75$	$21 \pm 8.5$	$52 \pm 9.0$	2.4	5.7	2.3
36d	Rd	NMe <sub>2</sub>	$>108.3 \pm 0.41$	$1.9 \pm 0.62$	>100	0.23		
<b>37</b> , carbachol 5.8.		5.8.	2.3	160	69	28	0.39	
38, Oxo-M 1.2 ● 0.09		0.06 单 0.02	1.5  extstyle 0.02	25	1.3	0.05		
<b>39</b> , Oxo-2 6.8    0.69		$0.07 \pm 0.02$	$1.6 \pm 0.01$	23.	0.2	0.01		
40, oxotremorine $0.12 \pm 0.02$		$0.005 \pm 0.002$	$0.16 \pm 0.02$	32	1.3	0.04		
41, Oxo-Pip		0.13 🌨 0.03	$0.07 \pm 0.03$	0.35 🛳 0.11	5.0	2.7	0.54	
<b>42, BM-</b> 5		$0.04 \pm 0.01$	0.07	0.18	2.5	5.1	1.8	

<sup>a</sup> Mean of 2–7 determinations. <sup>b</sup> The [<sup>3</sup>H]QNB data is from rat cortex. <sup>c</sup> From one determination or a mean of two determinations. <sup>d</sup> The [<sup>3</sup>H]QNB data is from rat heart. <sup>e</sup> Identical results (2–4 determinations) were obtained in parallel assays with the free base of the compound.

agonists (Oxo-M and Oxo-2) have relatively low  $[^{3}H]QNB/$ [<sup>3</sup>H]PZ ratios which suggest a preference for heart muscarinic receptors and therefore  $M_2$  selectivity. These data are supported by the large G-shift values for these compounds. The antagonist Oxo-Pip has a 10-50-fold weaker preference for the heart or M<sub>2</sub> receptors and, therefore, should be more  $M_1$  selective. The increased preference for  $M_1$  receptors is further supported by the weak G-shift ratio (5.0) or  $M_2$  agonist-like effects. Oxo-Pip, however, is a functional antagonist (too little agonist character) and lies outside the scope of our interest. Taken together, compounds with full agonist-like effects have stronger preference for  $M_2$  receptors. In a series of previously reported compounds, as the agonist-like effects decrease (partial agonism) the preference for  $M_2$  receptors decreases faster than the preference for  $M_1$  receptors.<sup>22</sup>

The weak partial agonist/antagonist BM-5, which was characterized pharmacologically using functional assays, can serve as a prototype for the SDAT therapeutic agent. The compound is a weak partial agonist ([<sup>3</sup>H]QNB/[<sup>3</sup>H]-CD ratio = 20), it produced little if any G-shift, suggesting  $M_2$  antagonist-like profile, and relative to other oxotremorine-like compounds has a preference (selectivity) for  $M_1$ -like receptors.

The methylated derivatives, with no exception, produced weaker effects (higher  $K_i$  values) in all five receptor binding assays (Tables II and III) relative to their unsubstituted congeners. Steric size has a greater effect on the high affinity agonist binding site labeled by [<sup>3</sup>H]CD than the binding sites labeled by [<sup>3</sup>H]QNB. This trend is expressed in the lower ratios ([<sup>3</sup>H]QNB/[<sup>3</sup>H]CD) for the 3-methyl derivatives (13a,13b, 18, 14a, 14b, 19) and more significantly for the 4-methyl derivatives (34c, 34d, 35c, 35d, 36c, 36d). These agonist/antagonist profiles were predicted in the original racemic study; the 3-methylated derivatives were muscarinic agonists, albeit weaker than oxotremorine, and the 4-methylated derivatives were functional antagonists.<sup>6</sup>

The effect of the amine moiety is also parallel to that observed in the literature<sup>23</sup> for oxotremorine, Oxo-2, and Oxo-M in guinea pig ileum studies. The dimethylamino analog Oxo-2 had about one-fifth the potency of oxotremorine, both as a stimulant of the ileum and as a tremorogenic agent. This reduction in potency was entirely the result of the loss of affinity (consistent throughout), at least in the ileum, because Oxo-2 had 6-fold greater intrinsic efficacy than oxotremorine (increased [<sup>3</sup>H]QNB/ [<sup>3</sup>H]CD ratio).

A comparison of oxotremorine and enantiomers 13a and 13b may provide some insight into to obtaining muscarinic agonists which are less  $M_2$  selective (or more  $M_1$  selective). The addition of an (R)-methyl group to oxotremorine (13b) produced a 2-fold decrease in affinity for the receptors labeled by  $[^{3}H]PZ$  in the cortex  $(M_{1})$ , whereas the addition of an (S)-methyl group (13a) produced a significantly larger 20-fold decrease in affinity. With cardiac muscarinic receptors ( $[^{3}H]QNB$  heart), the addition of either an (R)or an (S)-methyl group (13a or 13b) gave an equal 20-fold reduction in affinity. The preference of 13b for pirenzepine labeled receptors  $(M_1)$  in the cortex is also expressed as the decrease in agonist-like effects in the heart  $(M_2)$ . The effect of the stable guanine nucleotide, Gpp(NH)p, on 13a (G-shift of 32) is equal and parallel to that for oxotremorine (G-shift of 32). The G-shift for 13b (4.9) is significantly weaker than for oxotremorine (32) and is similar to that observed for BM-5 (2.5). These data suggest that 13b, like BM-5, has significantly weaker agonist-like effects than 13a and oxotremorine in the heart  $(M_2)$  while maintaining a similar affinity for pirenzipine-labeled receptors  $(M_1)$  in the brain.

In summary, 13b has a biochemical profile similar to BM-5 and is significantly different from its enantiomer 13a and oxotremorine. Only after the optically pure (and not racemic) compounds were prepared and tested biochemically were the subtle differences in subtype selectivity and reduced affinity discovered for the enantiomeric compounds.

## **Experimental Section**

All melting points were determined using a Mel-Temp apparatus and are uncorrected. All NMR spectra were measured in CDCl<sub>3</sub> or CD<sub>3</sub>OD using a General Electric QE 300 spectrometer. The coupling constants are recorded in hertz (Hz), and the chemical shifts are reported in parts per million  $\delta$  (ppm) downfield from tetramethylsilane (TMS) which is used as an internal standard. IR spectra were recorded on a Nicolet 20 SXB FT-IR spectrophotometer. Low-resolution mass spectra (MS-LR) were recorded on a Finnigan MAT-90 mass spectrometer and highresolution mass spectra (MS-HR) were recorded on either a Finnigan MAT-90 for electron impact (EI) or a VG ZAB-SE mass spectrometer for fast atom bombardment (FAB). Specific rotations ( $[\alpha]^{26}_{D'}$  deg) were recorded on a Perkin-Elmer 241 polarimeter with the sample concentration in grams per milliliter of solution. Thin-layer chromatography was conducted using silica gel (Analtech) or aluminum oxide (Analtech) plates. For flash column chromatography, Kieselgel 60 (230–400 mesh ASTM, E. M. Science), for preparative HPLC Waters Prep-500 HPLC system with normal-phase silica gel cartridge and for gravity column chromatography alumina deactivated with 4.5% H<sub>2</sub>O was used. Celite (Johns Mansville) was used as a filter aid and Magnesol (FMC Corp), hydrous magnesium silicate, was used for plug filtration. (S)-Methyl L- $\beta$ -hydroxyisobutyrate and (S)- $\beta$ -Methyl- $\gamma$ -butyrolactone were purchased from Fluka Chem. Co.

**Benzyl (R)-2-Methyl-4-oxabutanoate (10b).** A stream of  $O_3$  was bubbled through a solution of benzyl (R)-2-methyl-4pentenoate **9b**<sup>7</sup> (3.32 g,16.3 mmol) in MeOH (150 mL) which was cooled to -78 °C until a blue color persisted. The excess ozone was removed by first purging with oxygen and then with argon. Zinc dust (2.47 g) and acetic acid (3 ml) were added, and the mixture was allowed to warm to room temperature with stirring for 1 h. The resulting precipitate was removed by filtration, and the solvent was evaporated *in vacuo*. The residue was triturated with ether, and the filtrate was evaporated to dryness. The residue was purified by plug filtration (SiO<sub>2</sub>; eluent, ether) to give 3.10 g (92%) of 10b as a colorless oil which was used in the next step directly without further purification.

**Benzyl** (S)-2-Methyl-4-oxabutanoate (10a). The analogous reaction of 9a (4.22 g, 20.7 mmol) gave a 97% yield of 10a as a colorless oil.

(R)-3-Methyl-1-[4-(1-pyrrolidinyl)-2-butynyl]-2-pyrroli**dinone (13b).** Benzyl (R)-2-methyl-4-oxobutanoate 10b (3.10 g)15.0 mmol) was added to a mixture of of 4-(1-pyrrolidinyl)-2butynyl-1-amine dihydrochloride 24 (5.02 g, 23.8 mmol), sodium acetate (4.17 g, 50.9 mmol), and acetic acid 4 (mL) in MeOH (50 mL) at room temperature. After stirring for 15 min, sodium cyanoborohydride (395 mg, 6.29 mmol) was added in several portions and then stirred for 3 h. The reaction was quenched by adding sodium carbonate, and the mixture was concentrated in vacuo. The residue was partitioned between water and CH<sub>2</sub>- $Cl_2$ , and the aqueous layer was extracted with  $CH_2Cl_2$ . The combined organic phase was dried over MgSO<sub>4</sub> and evaporated to dryness. The residue was purified by column chromatography (Al<sub>2</sub>O<sub>3</sub>, 300 g; eluent, 33-50% v/v EtOAc/hexanes) gave 1.78 g (54%) of 13b as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.12 (m, 2 H), 3.39 (m, 4 H), 2.59 (m, 4 H), 2.47 (m, 1 H), 2.26 (m, 1 H), 1.81 (m, 4 H), 1.60 (m, 1 H), 1.20 (d, J = 7.1 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) & 176.7, 79.8, 77.9; 52.7, 44.4, 43.3, 36.6, 32.4, 26.9, 23.7, 16.2; IR (neat) 1693 cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O) C, H, N

The sesquioxalate salt of 13b was prepared by the addition of oxalic acid (1.5 equiv) to a solution of 13b (0.653 g, 2.97 mmol) in MeOH (5 mL) at room temperature. After stirring for 15 min, the solvent was evaporated *in vacuo* to dryness. The white solid was recrystallized from MeOH / EtOAc to give the sesquioxalate salt of 13b as white crystals: mp 88–89 °C; <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  4.18 (m, 2 H), 4.13 (m, 2 H), 3.45 (m, 4 H), 3.31 (hept, J = 1.7 Hz, 2 H), 2.48 (m, 1 H), 2.32 (m, 1 H), 2.10 (m, 4 H), 1.67 (m, 1 H), 1.16 (d, J = 7.1 Hz, 3 H); IR (KBr) 1724, 1683, 1646 cm<sup>-1</sup>. Anal. (C<sub>16</sub>H<sub>23</sub>N<sub>2</sub>O<sub>7</sub>) C, H, N.

(S)-3-Methyl-1-[4-(1-pyrrolidinyl)-2-butynyl]-2-pyrrolidinone (13a). The analogous reaction of 10a (4.02 g, 19.5 mmol) gave a 49% yield of 13a as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.12 (m, 2 H), 3.39 (m, 4 H), 2.59 (m, 4 H), 2.46 (m, 1 H), 2.26 (m, 1 H), 1.81 (m, 4 H), 1.64 (m, 1 H), 1.20 (d, J = 7.2 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  177, 79.9, 78.0, 52.7, 44.5, 43.3, 36.6, 32.4, 27.0, 23.8, 16.2; IR (neat) 1694 cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O) C, H, N.

The sesquioxalate salt of 13a was prepared by the addition of oxalic acid (1.5 equiv) to a solution of 13a (1.42 g, 6.45 mmol) in MeOH (5 mL) at room temperature. After the mixture was stirred for 15 min, the solvent was evaporated *in vacuo to* dryness. The white solid was recrystallized from MeOH/EtOAc to give the sesquioxalate salt of 13a as white crystals: mp 90–91 °C; <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  4.18 (m, 2 H), 4.13 (m, 2 H), 3.44 (m, 4 H), 3.31 (hept, J = 1.6 Hz, 2 H), 2.49 (m, 1 H), 2.32 (m, 1 H), 2.10 (m, 4

H), 1.68 (m, 1 H), 1.16 (d, J = 7.1 Hz, 3 H); IR (KBr) 1724, 1688, 1638 cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O) C, H, N.

(S)-1-[(1-(Dimethylamino)-2-butyn-4-yl]-3-methyl-2-pyrrolidinone (14a). The analogous reaction of 10a gave 14a as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.21 (d, J = 7.1 Hz, 3 H), 1.64 (m, 1 H), 2.23–2.32 (m, 1 H), 2.28 (s, 6 H), 2.47 (m, 1 H), 3.24 (m, 2 H), 3.37–3.44 (m, 2 H), 4.14 (d, J = 1.3 Hz, 2 H);IR (neat) 1694 cm<sup>-1</sup>. Anal. (C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O) C, H, N.

(*R*)-1-[(1-(**Dimethylamino**)-2-butyn-4-yl]-3-methyl-2-pyrrolidinone (14b). The analogous reaction of 10a gave 14b as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.21 (d, *J* = 7.1 Hz, 3 H), 1.64 (m, 1 H), 2.23–2.32 (m, 1 H), 2.28 (s, 6 H), 2.47 (m, 1 H), 3.24 (m, 2 H), 3.37–3.44 (m, 2 H), 4.14 (d, *J* = 1.3 Hz, 2 H); IR (neat) 1694 cm<sup>-1</sup>. Anal. (C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O) C, H, N.

**Benzyl 2-Methyl-4-pentenoate** [( $\pm$ )-16]. Oxalyl chloride (48.2 mL, 55 mmol) was added dropwise to a solution of 2-methyl-4-pentenoic acid<sup>8</sup> and 0.3 mL of DMF in CH<sub>2</sub>Cl<sub>2</sub> (150 mL) which was stirred at 0 °C. The mixture was stirred at room temperature for 3 h and then concentrated *in vacuo* to dryness to give 2-methyl-4-pentenoyl chloride (28.5 g, 97%) as a yellow oil.

A solution of 2-methyl-4-pentenoyl chloride 28.5 g in CH2Cl2 (50 mL) was added dropwise to a solution of benzyl alcohol (23.8g, 0.22 mol) and pyridine (18.7 mL, 0.23 mol) in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) which was stirred at 0 °C. The reaction was stirred at room temperature for 4 h and washed with cold hydrochloric acid (150 mL, 2 N) and saturated aqueous NaHCO<sub>3</sub> (100 mL). The CH<sub>2</sub>-Cl<sub>2</sub> solution was dried (MgSO<sub>4</sub>) and concentrated *in vacuo* to dryness to give the crude 16 (42.1 g) as a yellow oil. Purification of the crude oil by Kugulrohr distillation (70–75 °C, 0.1 mmHg) afforded 16 (35.2g, 80%) as a colorless oil: IR (thin film) 1735 cm<sup>-1</sup> (C=O); NMR (CDCl<sub>3</sub>)  $\delta$  1.18 (d, J = 7 Hz, 3 H), 2.20 (m, 1 H), 2.44 (m, 1 H), 2.58 (m, 1 H), 5.01 (m, 2 H), 5.11 (s, 2 H), 5.73 (m, 1 H), 7.25–7.37 (m, 5 H); MS (CI-LR) MH<sup>+</sup> at *m/z* 205. Anal. (C<sub>13</sub>H<sub>20</sub>O<sub>2</sub> 0.1H<sub>2</sub>O) C, H, N.

Benzyl 2-Methyl-4-oxabutanoate  $[(\pm)-17]$ . The analogous reaction of 9a (4.22 g, 20.7 mmol) gave a 97% yield of 17 as a colorless oil.

3-Methyl-1-(1-pyrrolidinyl-2-butyn-4-yl)-2-pyrrolidinone [( $\pm$ )-18]. The analogus reaction of 10a gave 18 as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.20 (d, J = 7.2 Hz, 3H), 1.65 (m, 1H), 1.18 (m, 1 H), 2.26 (m, 1 H), 2.47 (m, 1 H), 2.59 (m, 4 H), 3.39 (m, 4 H), 4.12 (m, 2 H).

The sesquioxalate salt of 18 as colorless crystals: <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  1.16 (d, J = 6.9 Hz, 3 H), 1.68 (m, 1 H), 2.10 (m, 4 H), 2.32 (m, 1 H), 2.50 (m, 1 H), 3.31 (hept, J = 1.7 Hz, 2 H), 3.42 (m, 4 H), 4.13 (t, J = 2.0 Hz, 2 H), 4.18 (t, J = 1.8 Hz, 2 H).

1-[1-(Dimethylamino)-2-butyn-4-yl]-3-methyl-2-pyrrolidinone [(±)-19]. The analogous reaction of 10b (2.4g, 14.7 mmol) gave a 60% yield of 19 as a colorless oil: IR (thin film) 1692 cm<sup>-1</sup> (C=O); NMR (CDCl<sub>3</sub>) δ 1.21 (d, J = 7 Hz, 3 H), 1.65 (m, 1 H), 2.28 (s,6H), 2.47 (m, 1 H), 3.24 (t, J = 1 Hz, 2 H), 3.41 (m, 3 H), 4.14 (br s, 2 H); MS (LR-CI) MH m/z 195. Anal. (C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O) C, H, N.

*N*-(2-Propynyl)-2,2,2-trifluoroacetamide (21). A solution of trifluoroacetic anhydride (63 g, 0.3 mol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added dropwise to a solution of propargylamine 20 (15.0 g, 0.27 mol) in CH<sub>2</sub>Cl<sub>2</sub> (200 mL) which was stirred at 0 °C for 1 h followed by the addition of water (100 mL). The mixture was washed with 1 N HCl (150 mL) and saturated aqueous NaHCO<sub>3</sub> (150 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo* to dryness. The residue was distilled under vacuum to give 32.1 g of 21 as a colorless oil: bp 40–45 °C (0.2 mmHg); IR (KBr) 1713 cm<sup>-1</sup>; NMR (CDCl<sub>3</sub>)  $\delta$  2.35 (t, J = 7 Hz, 1 H), 4.15–4.18 (m, 2 H), 6.6 (br s, 1 H); MS (EI-LR) MH<sup>+</sup> m/z 151. Anal. (C<sub>5</sub>H<sub>4</sub>F<sub>3</sub>NO) C, H, N.

**N-[1-(Dimethylamino)-2-butyn-4-yl]-2,2,2-trifluoroacetamide Hydrochloride (22).** The analogous reaction of **23** gave **22** as colorless crystals: mp 112–114 °C; IR (KBr) 1715, 1731 cm<sup>-1</sup>; NMR (DMSO- $d_6$ )  $\delta$  2.75 (s, 6 H), 4.07 (d, J = 1.8 Hz, 2 H), 4.14 (d, J = 5.2Hz, 2 H), and 10.16 (br s, 1 H); MS (EI-LR) MH<sup>+</sup> at m/z 208. Anal. (C<sub>8</sub>H<sub>10</sub>F<sub>3</sub>N<sub>2</sub>O·HCl·0.25H<sub>2</sub>O) C, H, N.

2,2,2-Trifluoro-N-(1-pyrrolidin-2-butyn-4-yl)acetamide Hydrochloride (23). A mixture of 21 (50.0 g, 0.33 mol), pyrrolidine (47.1 g, 0.66 mol), paraformaldehyde (25g, 0.83mol), acetic acid (50 mL), cuprous chloride (0.5 g, 6 mmol), and dioxane (500 mL) was refluxed for 20 min. The reaction mixture was concentrated in vacuo and made basic to pH 10–11 with concentrated ammonium hydroxide. The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic extracts were dried over Na<sub>2</sub>-SO<sub>4</sub>, filtered through a pad of Celite, and concentrated *in vacuo* to dryness. The residue was purified by vacuum distillation (110–120 °C, 0.3 mmHg) to give 58.1 g (75%) of 23 as a yellow oil which was used directly in the next step. A sample of the oil was dissolved in 1 N methanolic hydrogen chloride. The resulting solid was collected and recrystallized from MeOH/ether to give the hydrochloride salt of 23 as colorless crystals: mp 125–127°C; IR (KBr) 1731 cm<sup>-1</sup>; NMR (DMSO-d<sub>0</sub>)  $\delta$  1.94 (br s, 4 H), 3.3 (br s, 2 H), 4.11 (s, 2 H), and 4.13 (s, 2 H); MS (EI-LR) MH<sup>+</sup> at m/z 233. Anal. (C<sub>10</sub>H<sub>13</sub>F<sub>3</sub>N<sub>2</sub>O·HCl) C, H, N.

N,N-Dimethyl-2-butyn-1,4-diyldiamine Dihydrochloride (24). The analogous reaction of 25 gave 24 as colorless crystals: mp 181–183 °C; Anal. ( $C_6H_{12}N_2$ ·2HCl) C, H, N.

4-(1-Pyrrolidinyl)-2-butyn-1-ylamine Dihydrochloride (25). A solution of 23 (50.0 g, 214 mmol) and 4 N hydrochloric acid (270 mL) was refluxed for 2 h. Concentrated hydrochloric acid (70 mL) was added, and the mixture was refluxed overnight. The reaction was concentrated *in vacuo*, and the residue was washed with CH<sub>2</sub>Cl<sub>2</sub>. The aqueous layer was basified with 10 N NaOH and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo* to give 28 g of a dark oil. The oil was treated with 450 mL of 1 N methanolic hydrogen chloride and concentrated *in vacuo*. The resulting solid was recrystallized from MeOH/ether to give 31.2 g (52%) of 25 as hygoscopic off-white crystals: mp 192–193 °C; NMR (DMSO-d<sub>6</sub>)  $\delta$  1.97 (br s, 4 H), 3.29–3.39 (m, 4 H) 3.43 (m, 1 H), 3.8 (s, 2 H) 4.2 (s, 2 H). Anal. (C<sub>8</sub>H<sub>14</sub>N<sub>2</sub>2HCl) C, H, N.

(S)-Methyl 3-(Tetrahydro-2-pyranyloxy)-2-methylpropionate (27). A solution of (S)-(+)-methyl L- $\beta$ -hydroxyisobutyrate 26 (10.93 g, 92.5 mmol), 3,4-dihydro-2H-pyran (9.34 g, 111 mmol), and p-toluenesulfonic acid monohydrate (0.22 g, 1.16 mmol) in ether (150 mL) was stirred at room temperature overnight. The reaction mixture was washed with aqueous NaHCO<sub>3</sub> and dried over MgSO<sub>4</sub>. The solvent was removed *in* vacuo, and the residue was purified by distillation to give 17.73 g (95%) of 27 as a colorless oil: bp 63-66 °C (0.35-0.4 mmHg); [ $\alpha$ ]<sup>26</sup><sub>D</sub> = +16° (c = 0.852, ether) [lit.<sup>10</sup> [ $\alpha$ ]<sup>21</sup><sub>D</sub> = -16.3° (c = 1.39, ether)];<sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.61(m, 1 H), 3.94-3.41 (m, 4 H), 3.70 (s, 3 H), 2.77 (m, 1 H), 1.81-1.49 (m, 7 H), 1.20 (d, J = 7.1 Hz, 1.5 H), 1.19 (d, J = 7.1 Hz, 1.5 H); IR (neat) 1741 cm<sup>-1</sup>.

(R)-3-(Tetrahydro-2-pyranyloxy)-2-methyl-1-propanol (28). A solution of lithium aluminum hydride (70 mL, 1 N, 70 mmol) in ether was slowly added via syringe pump to a solution of 27 (17.7 g, 87.6 mmol) in dry ether (50 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 4 h. The excess lithium aluminum hydride was decomposed by the successive addition of water (1.5 mL), 15% NaOH (4 mL), and water (9 mL) to the stirred ice-cooled mixture. After stirring for 1.5 h, the mixture was filtered and washed with ether. The combined filtrate and washings were dried over K<sub>2</sub>CO<sub>3</sub>. After concentration the residue was distilled to give 13.6 g (89%) of 28 as acolorless oil: bp 87-89 °C (0.9 mmHg);  $[\alpha]^{26}_{D} = +1^{\circ} (c$ = 1.885, ether) [lit.<sup>10</sup>  $[\alpha]^{21}_{D}$  = -1.2° (c = 1.471, ether)]; <sup>1</sup>H NMR  $(CDCl_3) \delta 4.59 (m, 1 H), 3.86 (m, 2 H), 3.70-3.50 (m, 4.5 H), 3.35$ (dd, J = 7.4, 9.5 Hz, 0.5 H), 2.34 (br s, 1 H), 2.05 (m, 1 H), 1.76(m, 2 H), 1.55 (m, 4 H), 0.91 (d, J = 7.0 Hz, 1.5 H) and 0.90 (d, J = 7.0 Hz, 1.5 H); IR (neat) 3430, 2942, 1032 cm<sup>-1</sup>.

(S)-3-(Tetrahydro-2-pyranyloxy)-2-methylpropyl p-Toluenesulfonate (29). In one portion p-TsCl (19.2 g, 101 mmol) was added to a solution of 28 (13.5 g, 77.6 mmol) in pyridine (120 mL) at 0 °C. The reaction mixture was kept in a refrigerator overnight and then poured into ice-water. The aqueous solution was extracted with ether, and the combined extract was washed with aqueous CuSO<sub>4</sub>, NaHCO<sub>8</sub>, and brine. The ether solution was dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give 24.1 g (95%) of 29 as a pale-yellow oil which was employed in the next step without further purification.

(R)-4-(Tetrahydro-2-pyranyloxy)-3-methylbutanenitrile (30). A solution of 29 (39.9 g, 0.121 mol) and NaCN (10 g, 0.204 mol) in DMSO (120 mL) was stirred for 2 days at room temperature. It was then diluted with water and extracted three times with 200 mL of petroleum ether (30-70 °C). The combined petroleum ether extract was dried over MgSO<sub>4</sub> and concentrated in vacuo to dryness. The residue was purified by HPLC (eluant, 1:4 v/v EtOAc/hexanes) to give 15.4 g (70%) of **30** as a colorless oil:  $[\alpha]^{26}_{D} = +24^{\circ}$  (c = 1.875, ether) [lit.<sup>10</sup>  $[\alpha]^{21.5}_{D} = -27.8^{\circ}$  (c =1.41, ether)]; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.53 (m, 1 H), 3.83 (m, 1 H), 3.76 (dd, J = 5.0, 9.8 Hz, 0.5 H), 3.55 (m, 1.5 H), 3.38 (dd, J = 4.8, 9.9 Hz, 0.5 H), 3.21 (dd, J = 8.3, 9.7 Hz, 0.5 H), 2.49 (m, 2 H), 2.17 (m, 1 H), 1.71 (m, 2 H), 1.55 (m, 4 H), 1.11 (d, J = 6.8 Hz, 1.5 H), and 1.10 (d, J = 6.8 Hz, 1.5 H); IR (neat) 2942, 2246, 1455, 1035 cm<sup>-1</sup>.

(R)-(+)- $\beta$ -Methyl- $\gamma$ -butyrolactone (31c). A solution of 30 (6.80 g, 37.1 mmol) in concentrated HCl (5 mL) and MeOH (50 mL) was heated to gentle reflux for 7 h. The mixture was concentrated *in vacuo* and then diluted with water (50 mL). The aqueous solution was neutralized with NaHCO<sub>3</sub>, stirred at room temperature for 1 h, and extracted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL). The CH<sub>2</sub>Cl<sub>2</sub> solution was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to dryness. The residue was purified by HPLC (eluant, 1:4 EtOAc/hexanes) to give 1.69 g (46%) of 31c as a colorless oil:  $[\alpha]^{26}_{D} = +24^{\circ}$  (c = 1.235, MeOH) [lit.<sup>10</sup>  $[\alpha]^{21}_{D} = -24.96^{\circ}$  (c = 1.771, MeOH)]; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.42 (dd, J = 7.3, 8.8 Hz, 1 H), 3.88 (dd, J = 6.4, 8.8 Hz, 1 H), 2.65 (m, 2 H), 2.14 (m, 1 H), 1.17 (d, J = 6.6 Hz, 3 H); IR (neat) 2378, 2243, 1774 cm<sup>-1</sup>. Anal. (C<sub>6</sub>H<sub>8</sub>O<sub>2</sub>) C, H, N.

Ethyl (R)-4-Iodo-3-methylbutyrate (32c). Iodotrimethylsilane (6.04 g, 30.2 mmol) was slowly added to a solution of 31c (1.5 g, 15. mmol) in EtOH (30 mL) at -20 °C. After stirring at -20 °C for 30 min, triethyl orthoformate (2.5 g, 15. mmol) was added, and the resulting solution was brought to gentle reflux overnight. The solution was concentrated *in vacuo*, and the residue was purified by column chromatography (SiO<sub>2</sub> eluant, 1:20 v/v EtOAc/hexanes) to give 3.09 g (79%) of 32c as a pale yellow oil:  $[\alpha]^{28}_{365} = +6^{\circ}$  (c = 1.98, MeOH); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.15 (q, J = 7.2 Hz, 2 H), 3.27 (m, 2 H), 2.46 (dd, J = 6.5, 15.7 Hz, 1 H), 2.23 (dd, J = 7.2, 15.7 Hz, 1 H), 2.03 (m, 1 H), 1.27 (t, J = 7.1 Hz, 3 H), 1.06 (d, J = 6.6 Hz, 3 H); IR (neat) 1733 cm<sup>-1</sup>. Anal. (C<sub>7</sub>H<sub>13</sub>IO<sub>2</sub>) C, H, I.

Ethyl (S)-4-Iodo-3-methylbutyrate (32d)<sup>3</sup>. Analogous reaction of iodotrimethylsilane (44. g, 0.22 mol) with (S)-β-methylγ-butyrolactone(11.2 g, 0.110 mol) gave an 88% yield of product as a pale yellow oil:  $[\alpha]^{28}_{385} = -6^{\circ}$  (c = 1.44, MeOH); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.15 (q, J = 7.1 Hz, 2 H), 3.27 (m, 2 H), 2.46 (dd, J = 6.5, 15.7 Hz, 1 H), 2.23 (dd, J = 7.2 Hz, 15.7 Hz, 1 H), 2.03 (m, 1 H), 1.27 (t, J = 7.2 Hz, 3 H), 1.06 (d, J = 6.6 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  172.0, 60.5, 41.1, 31.8, 20.5, 15.9, 14.2; IR (neat) 1733 cm<sup>-1</sup>. Anal. (C<sub>7</sub>H<sub>13</sub>IO<sub>2</sub>) C, H, N.

(R)-4-Methyl-1-(2-propynyl)-2-pyrrolidinone (33c). A solution of 32c (4.81 g, 18.6 mmol) in EtOH (20 mL) was slowly added over 6.5 h to a solution of propargylamine (6.14 g, 112 mmol) and Na<sub>2</sub>CO<sub>3</sub> (1.98 g, 18.6 mmol) in EtOH (10 mL) at 90 °C. The reaction mixture was cooled and concentrated in vacuo to dryness. Water (70 mL) was added to the residue and extracted three times with CH<sub>2</sub>Cl<sub>2</sub> (80 mL). The extract was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to dryness. The residue was purified by column chromatography (SiO<sub>2</sub>,eluent, 1:2 to 1:1 v/v EtOAc/hexanes) to give 2.40 g (94%) of 33c as a pale yellow oil:  $[\alpha]^{26}_{D} = -5^{\circ}(c = 0.7, CH_2Cl_2); {}^{1}H NMR (CDCl_3) \delta 4.10 (apparent$ t, J = 2.2 Hz, 2 H), 3.60 (dd, J = 7.7, 9.5 Hz, 1 H), 3.06 (dd, J= 6.0, 9.5 Hz, 1 H), 2.55 (m, 2 H), 2.22 (t, J = 2.5 Hz, 1 H), 2.03  $(dd, J = 6.3, 16.0 Hz, 1 H), 1.15 (d, J = 6.6 Hz, 3 H); {}^{13}C NMR$ (CDCl<sub>3</sub>) § 174.1, 77.7, 72.1, 53.6, 39.1, 31.8, 26.2, 19.8; IR (neat) 2120, 1688cm-1; MS (HREI) calcd for C8H11NO 137.0841, found 137.0846. Anal. (C<sub>8</sub>H<sub>11</sub>NO) H, N; C: calcd, 70.04; found, 67.21.

(S)-4-Methyl-1-(2-propynyl)-2-pyrrolidinone (33d). The analogous reaction of 32d (1.89 g, 7.33 mmol) with propargylamine (1.21 g, 22 mmol) and Na<sub>2</sub>CO<sub>3</sub> (0.389 g, 3.67 mmol) gave a 71% yield of product 33d as a pale yellow oil:  $[\alpha]^{26}_{D} = +6^{\circ}$  (c = 2.44, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.10 (apparent t, J = 2.2 Hz, 2 H), 3.60 (dd, J = 7.7, 9.5 Hz, 1 H), 3.06 (dd, J = 6.0, 9.5 Hz, 1 H), 2.59 (m, 2 H), 2.22 (t, J = 2.6 Hz, 1 H), 2.03 (dd, J = 6.3, 16.0 Hz, 1 H), 1.14 (d, J = 6.6 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  174.0, 77.6, 72.0, 53.5, 39.0, 31.7, 26.1, 19.7; IR (neat) 2119, 1689, 1626 cm<sup>-1</sup>; MS (HR-EI) calcd for C<sub>8</sub>H<sub>11</sub>NO 137.0841, found 137.0481. Anal. (C<sub>8</sub>H<sub>11</sub>NO) H, N; C: calcd, 70.04; found, 69.44.

(S)-4-Methyl-1-(1-pyrrolidinyl-2-butyn-4-yl)-2-pyrrolidinone (34d) (General Procedure). A mixture of 33d (0.584 g, 4.26 mmol), paraformaldehyde (0.34 g, 11.3 mmol), pyrrolidine (0.61 g, 8.5 mmol), CuCl (50 mg), and acetic acid (2 mL) in 30 mL of p-dioxane was stirred at room temperature for 15 min and then refluxed for 1.5 h. The reaction mixture was concentrated in vacuo to dryness. The residue was purified by column chromatography (Al<sub>2</sub>O<sub>3</sub> eluant, 1:2 to 1:1 v/v EtOAc/hexanes) to give 0.80 g (85%) of **34d** as a yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.11 (t, J = 2.0 Hz, 2 H), 3.59 (dd, J = 7.8, 9.5 Hz, 1 H), 3.40 (t, J = 2 Hz, 2 H), 3.04 (dd, J = 5.9, 9.5 Hz, 1 H), 2.59 (m, 4 H), 2.50 (m, 2 H), 2.02 (dd, J = 6.3, 16. Hz, 1 H), 1.81 (m, 4 H), 1.14 (d, J = 6.7 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  173.9, 79.8, 77.9, 53.7, 52.7, 43.3, 39.2, 32.1, 26.2, 23.7, 19.8; IR (neat) 1694 (C=O) cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O) C, H, N.

The sequioxalate salt of 34d was prepared by the addition of oxalic acid (1.5 equiv) to a solution of 34d (0.80 g, 3.63 mmol) in MeOH (10 mL) at room temperature. After stirring for 15 min, the solution was concentrated *in vacuo* to dryness. The resulting white solid was recrystallized from MeOH/EtOAc to give the sesquioxalate salt of 34d as white crystals: <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta 4.18$  (s, 2 H), 4.13 (t, J = 1.7 Hz, 2 H), 3.65 (dd, J = 7.8, 9.5 Hz, 1 H), 3.42 (br s, 4 H), 3.31 (hept, J = 1.6 Hz, 2 H), 3.10 (dd, J = 5.5, 15.6 Hz, 1 H), 1.13 (d, J = 6.6 Hz, 3 H); <sup>13</sup>C NMR (CD<sub>3</sub>OD)  $\delta 177.0$ , 165.3, 84.6, 74.2, 55.3, 54.5, 44.4, 39.9, 32.8, 27.6, 24.4, 19.8; IR (KBr) 1721 (C=O) cm<sup>-1</sup>. Anal. (C<sub>16</sub>H<sub>23</sub>N<sub>2</sub>O<sub>7</sub>) C, H, N.

(*R*)-4-Methyl-1-(1-pyrrolidinyl-2-butyn-4-yl)-2-pyrrolidinone (34c). The analogous reaction of 34d gave the product 34c as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.11 (t, J = 2.0 Hz, 2 H), 3.59 (dd, J = 7.8, 9.5 Hz, 1 H), 3.40 (t, J = 2.1 Hz, 2 H), 3.04 (dd, J = 6.0, 9.5 Hz, 1 H), 2.60 (m, 4 H), 2.50 (m, 2 H), 2.03 (dd, J = 6.3, 16.1 Hz, 1 H), 1.81 (m, 4 H), 1.14 (d, J = 6.7 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  174.0, 79.7, 78.0, 53.7, 52.7, 43.2, 39.2, 32.1, 26.2, 23.7, 19.8; IR (neat) 1692 (C=O) cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O) C, H, N.

The sesquioxalate salt of **34c** as white crystals: mp 116–118 °C; <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  4.14 (s, 2 H), 4.01 (t, J = 1.8 Hz, 2 H), 3.58 (dd, J = 7.7, 9.4 Hz, 1 H), 3.36 (br s, 4 H), 3.03 (dd, J = 5.9, 9.4 Hz, 1 H), 2.53 (m, 2 H), 2.10 (m, 4 H), 2.02 (dd, J = 5.5, 15.6 Hz, 1 H), 1.15 (d, J = 6.5 Hz, 3 H); <sup>13</sup>C NMR (CD<sub>3</sub>OD)  $\delta$  177.0, 165.3, 84.6, 74.2, 55.3, 54.5, 44.4, 39.9, 32.8, 27.6, 24.4, 19.8; IR (KBr) 1721, 1681 cm<sup>-1</sup>. Anal. (C<sub>16</sub>H<sub>23</sub>N<sub>2</sub>O<sub>7</sub>) C, H, N.

(S)-1-[1-(Dimethylamino)-2-butyn-4-yl]-4-methyl-2-pyrrolidinone (35d). The analogous reaction of 34d gave the product 35d as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.13 (t, J = 1.8 Hz, 2 H), 3.60 (dd, J = 7.7, 9.5 Hz, 1 H), 3.24 (t, J = 2.0 Hz, 2 H), 3.05 (dd, J = 5.9, 9.5 Hz, 1 H), 2.52 (m, 2 H), 2.28 (s, 6 H), 2.03 (dd, J = 6.3, 16.0 Hz, 1 H), 1.14 (d, J = 6.7 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  173.9, 79.0, 78.8, 53.7, 48.0, 44.2, 39.2, 32.1, 26.2, 19.8; IR (neat) 1692 cm<sup>-1</sup>. Anal. (C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O) C, H, N.

The ethanedioate salt of **35d** was prepared by the treatment of **35d** (1.36 g, 7.0 mmol) with oxalic acid (1 equiv) in MeOH (15 mL). Recrystallization of the resulting solid from MeOH/EtOAc gave the ethanedioate salt of **35d** as white crystals: mp 153–154°C; <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  4.10 (t, J = 1.9 Hz, 2 H), 3.97 (t, J = 2.2 Hz, 2 H), 3.56 (dd, J = 7.7, 9.5 Hz, 1 H), 3.21 (hept, J = 1.6 Hz, 2 H), 3.00 (dd, J = 2.9, 6.6 Hz, 1 H), 2.82 (s, 6 H), 2.43 (m, 2 H), 1.92 (dd, J = 5.5, 15.5 Hz, 1 H), 1.04 (d, J = 6.6 Hz, 3 H); <sup>13C</sup> NMR (CD<sub>3</sub>OD)  $\delta$  176.9, 166.5, 85.8, 73.5, 55.3, 47.8, 42.8, 39.9, 32.8, 27.6, 19.8; IR (KBr) 1702 cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub>) C, H, N.

**N-[(S)-4-(4-Methyl-2-oxo-1-pyrrolidinyl)-2-butynyl]**-**N,N,N-trimethylaminium Iodide (36d).** MeI (0.1 mL, 1.61 mmol) was added to a solution of **35d** (126 mg, 0.65 mmol) in ether (0.5 mL) at room temperature. The mixture was allowed to stand overnight. A yellow solid formed, and the solvent was evaporated *in vacuo* to leave a solid residue. The residue was recrystallized from CH<sub>3</sub>CN to give **36d** as a pale-yellow crystal: mp 146-147 °C; <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  1.09 (d, J = 6.3 Hz, 3 H), 1.93 (m, 1 H), 2.45 (m, 2 H), 3.03 (dd, J = 5.8, 9.2 Hz, 1 H), 3.18 (s, 9 H), 3.57 (dd, J = 7.8, 9.3 Hz, 1 H), 4.15 (apparent d, J = 1.8 Hz, 2 H), 4.31 (t, J = 2.1 Hz, 2 H); IR (KBr) 2234, 1704, 1677 cm<sup>-1</sup>. Anal. (C<sub>12</sub>H<sub>21</sub>IN<sub>2</sub>O) C, H, N.

(*R*)-4-Methyl-1-[1-(dimethylamino)-2-butyn-4-yl]-2-pyrrolidinone (35c). The analogous reaction of 34d gave the product 35c as a pale yellow oil: <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  4.13 (s, 2 H), 3.60 (dd, J = 7.6, 9.5 Hz, 1 H), 3.24 (t, J = 2.0 Hz, 2 H), 3.06 (dd, J = 5.9, 9.5 Hz, 1 H), 2.52 (m, 2 H), 2.28 (s, 6 H), 2.04 (m, 1 H), 1.14 (d, J = 6.6 Hz, 3 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)  $\delta$  173.9, 79.0, 78.7, 53.7, 47.9, 44.2, 39.2, 32.0, 26.2, 19.8; IR (neat) 1694 cm<sup>-1</sup>. Anal. (C<sub>11</sub>H<sub>18</sub>N<sub>2</sub>O) C, H, N.

The ethanedioate salt of 35c as white crystals: mp 151–152 °C; <sup>1</sup>H NMR (CD<sub>3</sub>OD)  $\delta$  4.19 (s, 2 H), 4.07 (t, J = 2.2 Hz, 2 H), 3.66 (dd, J = 7.7, 9.5 Hz, 1 H), 3.31 (hept, J = 1.6 Hz, 2 H), 3.10 (dd, J = 5.9, 9.5 Hz, 1 H), 2.92 (s, 6 H), 2.52 (m, 2 H), 2.01 (m, 1 H), 1.13 (d, J = 6.6 Hz, 3 H); IR (KBr) 1700 cm<sup>-1</sup>. Anal. (C<sub>13</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub>) C, H, N.

**N-[(R)-4-(4-Methyl-2-oxo-1-pyrrolidinyl)-2-butynyl]**-**N,N,N-trimethyl-1-ammonium Iodide (36c).** The analogous reaction of **35c** (127 mg, 0.65 mmol) with MeI (0.1 ml, 1.61 mmol) gave **36c** (recrystallized from CH<sub>3</sub>CN) as yellow crystals: mp 148-149 °C; <sup>1</sup>H NMR (CD<sub>3</sub>CN)  $\delta$ 1.10 (d, J = 6.5 Hz, 3 H), 2.45 (m, 2 H), 3.01 (dd, J = 5.9, 9.2 Hz, 1 H), 3.15 (s, 9 H), 3.57 (dd, J = 7.8, 9.3 Hz, 1 H), 4.15 (s, 2 H), 4.26 (apparent d, J = 1.5 Hz, 2 H); IR (KBr) 2234, 1704, 1677 cm<sup>-1</sup>. Anal. (C<sub>12</sub>H<sub>21</sub>IN<sub>2</sub>O) C, H, N.

Muscarinic Receptor Binding Assays. The receptor binding assays were adapted from published procedures.<sup>14-17</sup> The cerebral cortex from male Wistar rats (200–250-g body weight) was homogenized in 50 volumes (wet wt/vol) of ice-cold 10 mM (8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.9 mM KH<sub>2</sub>PO<sub>4</sub>) sodium-potassium phosphate buffer (NaKPB), pH 7.4. The resulting homogenate was then diluted 1:3000, 1:300, or 1:400 (original wet wt/vol) with ice-cold NaKPB for use in the [<sup>3</sup>H]QNB, [<sup>3</sup>H]CD, and [<sup>3</sup>H]PZ assays, respectively. The final protein content per 2.0 mL of incubation mixture was ~ 0.1 mg, 0.75 and 0.55 mg, respectively.

The heart from male Wistar rats (200-250-g body weight) was placed in ice-cold 10 mM (8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.9 mM KH<sub>2</sub>PO<sub>4</sub>) sodium-potassium phosphate buffer (NaKPB), pH 7.4, and gently squeezed to flush blood from the ventricles. Excess buffer was blotted from the heart which was weighed and placed into 50 volumes (wet wt/vol) of ice-cold buffer where it was minced with a scissor. The mince was then homogenized using a Polytron, setting 8, with PT-10 saw-tooth generator for 60 s. The homogenate was then centrifuged at 48000g for 15 min at 4 °C. The supernatant was discarded and the pellet resuspended in 50 volumes (wet wt/vol) of fresh buffer using the Polytron as above. The homogenate was then filtered through two layers of gauze and diluted to a final tissue concentration of 0.15–0.2 mg/2.0 mL in the assay tubes.

Binding assays were initiated by adding 1.80 mL of the appropriate tissue homogenate to tubes containing 100  $\mu$ L of tritiated ligand,  $50 \,\mu L$  of unlabeled compound (final concentration  $1 \times 10^{-3}$  to  $1 \times 10^{-12}$  M) and 50  $\mu$ L of buffer or atropine (final concentration =  $10 \,\mu$ M) to define nonspecific binding. For [<sup>3</sup>H]-QNB binding to cardiac membranes, Gpp(NH)p was added to the diluted homogenate to give a final concentration of 10  $\mu$ M. The concentrations of radioligand were [3H]QNB (30.0 Ci/mmol) 250 pM, [3H]CD (55.5 Ci/mmol) 1 nM, and [3H]PZ (84.1 Ci/ mmol) 0.1 nM. Samples were incubated at 25 °C for 120 min ([<sup>3</sup>H]QNB and [<sup>3</sup>H]CD) or 60 min ([<sup>3</sup>H]PZ). [<sup>3</sup>H]CD and [<sup>3</sup>H]-PZ have been shown to adhere readily to both glass and plastic surfaces. To eliminate this problem (and the chance for introducing artifacts into the results), stock vials, pipett tips, and all glass tubes were routinely treated with Prosil-28, a siliconizing agent, and oven-dried prior to use in an assay. Additionally, the GF/B glass fiber filters were presoaked in an aqueous polyethylenimine (PEI) solution (0.1%, pH 7.0) prior to use. All points of the inhibition curve (including total and nonspecific binding) were always measured on single PEI treated filter strips to minimize filter-to-filter variability. The [3H]CD and [3H]PZ solutions were prepared fresh in buffer just prior to use in the assay to avoid decomposition. The incubations were terminated by rapid filtration (Brandel 24 sample cell harvester), and tubes were rinsed twice with 5 mL of ice-cold NaKPB buffer. Receptor-bound radioligand was determined by liquid scintillation spectrometry. The data were analyzed by nonlinear regression (LIGAND) and apparent  $K_i$  values determined by fitting a one-site competitive model to the competition data.<sup>24</sup>

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