Muscarinic Receptor Binding and Activation of Second Messengers by Substituted N-Methyl-N-[4-(1-azacycloalkyl)-2-butynyl]acetamides

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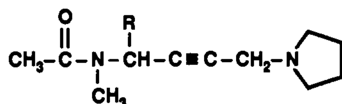
A series of substituted azacycloalkyl analogues of the muscarinic agonist UH 5 (N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]acetamide, **1a**) were synthesized and evaluated pharmacologically. These compounds were developed as intermediates for further derivatization leading to functionalized congeners of **1a**. The compounds were synthesized by using a Mannich-type condensation of N-acetyl-N-methylpropargylamine to various substituted saturated azaheterocycles. The compounds were screened at a single concentration in competitive binding assays in rat cerebral cortical membranes against either [^{3}H]N-methylscopolamine (at 100 μM) or [^{3}H]oxotremorine-M (at 1 μM) labels. Candidates were then selected for further evaluation of their effect on phosphoinositide (PI) turnover in membranes from A9L cells transfected with cDNA of either m_1-muscarinic cholinergic receptors (m_1AChRs) or m_3AChRs. The analogues were also tested for the inhibition of adenylate cyclase in NG108-15 cells expressing m_4AChRs. The azetidine analogue of **1a** had a K_i value of 12 nM for the inhibition of [^{3}H]oxotremorine-M binding in rat brain and had an agonist potency at m_1-, m_3-, and m_4AChRs comparable to **1a**. The substituted 5- and 6-member ring analogues generally had lower binding affinities and were less potent than **1a** in stimulating PI turnover. Several compounds were moderately effective in inhibiting cyclic AMP production in NG108-15 cells.

The muscarinic cholinergic receptors (mAChRs) have recently been classified into multiple subtypes based on both pharmacological and molecular characterizations.[1] Three pharmacologically defined subtypes, usually designated with a capital letter "M", have been characterized as follows: the M_1 receptor, abundant in brain tissue; the M_2 receptor, found predominantly in cardiac tissue; and the M_3 receptor, found mainly in glandular tissues.[2] Selective antagonists for M_1 (pirenzepine[3]), M_2 (methoctramine[4]), and M_3 (p-fluorohexahydrosiladifenidol[5]) subtypes have been identified. Molecular biological studies using DNA cloning techniques have defined five classes of mAChRs based on the differences in amino acid sequences and are usually designated with a lower case "m".[6] The m_1, m_2, and m_3 receptors correspond to their analogous numbered pharmacological class of receptor. Two new subtypes designated m_4 and m_5 have also been genetically defined. The functional properties of the cloned mAChRs have also been elucidated.[7] The m_1-, m_3-, and m_5AChRs preferentially stimulate phosphoinositide (PI) metabolism.[8] The m_2- and m_4AChRs are preferentially coupled through a pertussis toxin sensitive inhibitor G-protein to adenylate cyclase and cause a decrease in cAMP levels upon activation.[8,9]

The lack of selective agonists or antagonists for the genetically defined mAChR subtypes has prompted us to investigate potential new muscarinic agents based on the muscarinic agonist UH 5 (N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]acetamide, **1a**).[10] Selectivity can be induced in **1a** by the introduction of a methyl group alpha to the amide nitrogen to give the partial agonist BM 5 (**1b**). This agent has been described as a partial agonist



at postsynaptic mAChRs and an antagonist at presynaptic mAChRs.[11] A more recent study in mAChR-transfected cell lines has demonstrated that BM 5 is a partial agonist in cAMP-coupled subtypes (m_4AChR) and an antagonist in PI-coupled subtypes (m_1- and m_3AChRs).[12] Thus, it has been inferred that in certain brain areas presynaptic receptors are preferentially coupled to stimulation of phosphoinositide metabolism, and postsynaptic mAChRs are preferentially coupled to inhibition of cyclic AMP production.[12] A compound with a profile of receptor activation similar to BM 5 may stimulate cholinergic receptors both directly (postsynaptic) and indirectly (presynaptic). In addition, BM 5 has a tertiary nitrogen rather than a quaternary nitrogen (as found in many muscarinic agents) and should therefore penetrate the blood-brain barrier. This pharmacologic profile makes BM 5 and related analogues candidates for use as central muscarinic agonists.

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§ Supported by the Cystic Fibrosis Foundation/NIDDK Fellowship Program.

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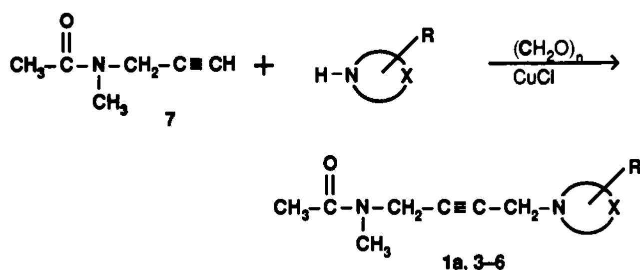
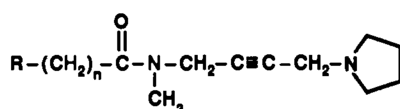
Scheme I



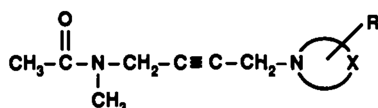
In an ongoing effort to develop muscarinic agonist and antagonist functionalized congeners[13] in our labs, **1a** was explored for potential sites that allow substitution without dramatically diminishing the binding affinity of the parent pharmacophore. In an earlier study, a series of congeners (**2**) was explored in which a spacer chain with a terminal functionalized amine was extended from the amide end of **1a**.[14] Longer chain derivatives (n = 6, 7) had compa-



2, n = 1–7, R = NH_2, NHBoc, NHAc, NHBzl

rable affinities to **1a** at both m_3AChRs and m_4AChRs. However, these compounds were antagonists and showed no selectivity for these two subtypes.

In this study, we explore the pyrrolidine end of the **1a** pharmacophore for sites of derivatization to lead to muscarinic agonist congeners with greater affinity and/or selectivity. The size of the heterocyclic ring was varied (**3–6**) and substituted with functional groups such as hydroxyl, alkyl, ester, and halo groups. This initial set of



3: X = $(CH_2)_4$

4: X = $(CH_2)_5$

5: X = $(CH_2)_2NH(CH_2)_2$

6: X = $(CH_2)_3$

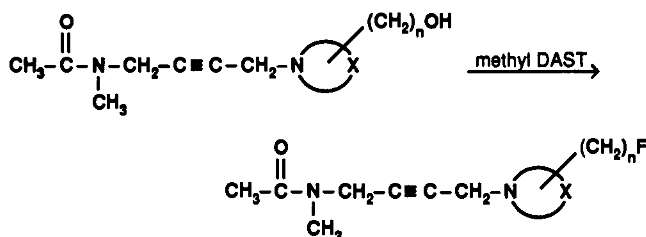
derivatives was designed with the intent of developing functionalized congeners having varying length spacer chains and prosthetic groups to be used as possible mAChR affinity probes or therapeutic agents.

Results

Chemistry. The structures of the N-methyl-N-[4-(1-azacycloalkyl)-2-butynyl]acetamide analogues synthesized are presented in Table I. Substituted and unsubstituted azacyclic rings are included, with ring sizes ranging from 4–6-member rings.

The substituted analogues and intermediates were constructed by using a Mannich-type condensation of N-acetylpropargylamine (**7**) with paraformaldehyde and the appropriate substituted nitrogen heterocycle (Scheme I). Attempted preparation of the piperazine analogue of **1a** directly from piperazine and **7** by this route gave several impurities. Thus, mono-*tert*-butyloxycarbonyl (Boc) protected piperazine was prepared and used in a Mannich condensation with **7** to give **5b**. Removal of the Boc group with trifluoroacetic acid gave **5a**, which then reacted with acetic anhydride to give **5c**. The acetoxymethyl derivative **3d** was prepared from **3b** by acetylation with acetic anhydride. The tosylate (**3i**), mesylate (**3j**), and benzoate (**3k**) derivatives of **3g** were prepared from **3g** by using standard methodologies. The fluorinated derivatives **3e**, **3f**, **3h**, and **4l** were prepared from the corresponding primary or secondary hydroxy-substituted compounds by reaction with dimethylaminosulfur trifluoride (Scheme II).

Scheme II



Pharmacology. The **1a** derivatives were initially screened at several concentrations in binding assays indicative of affinity for muscarinic receptors. To measure affinity for the agonist high affinity state of the receptor,[15] [^{3}H]oxotremorine-M ([^{3}H]Oxo-M, a muscarinic agonist) was used as the radioligand. Displacement of specific binding of [^{3}H]Oxo-M in rat forebrain membranes (mainly m_1AChRs) was determined for each compound at concentrations of 100 μM and 1 μM and is expressed as a percent of [^{3}H]Oxo-M binding in the absence of inhibitor (Table I). The following k_i values in rat brain were determined (in μM): **1a**, 0.0045 ± 0.0018; **3a**, 7.41 ± 4.2; **3b**, 2.24 ± 0.05; **3d**, 3.93 ± 1.8; **3e**, 1.70 ± 0.8; **3f**, 2.21 ± 1.5; **3i**, 1.64 ± 0.35; **3g**, 0.80; **4a**, 0.25; **6**, 0.041 ± 0.020. Inhibition of specific binding of [^{3}H]Oxo-M at a concentration of 1 μM in rat heart membranes was also measured as an indication of affinity at m_2AChRs.

Since we had observed previously[7] that muscarinic agonists in the butynamide class tend to be converted to antagonists upon functional derivatization, the new analogues were also screened in an assay indicative of binding to the antagonist site of the receptor. Thus, a separate assay was carried out for each compound at a single concentration in rat cerebral cortical membranes with [^{3}H]-N-methylscopolamine ([^{3}H]NMS) used as the radiolabel (Table I). While a concentration of 1 μM was adequate for most of the compounds in the [^{3}H]Oxo-M binding assay, a concentration of 100 μM was required for a relative comparison of affinities in [^{3}H]NMS binding.

The title compounds were then evaluated for biological effects on the second messenger systems that are activated by each muscarinic receptor subtype. The increase in phosphoinositide (PI) metabolism in m_3AChR-, and m_1AChR-transfected A9L cells was measured for each compound at a concentration of 100 μM and is reported as the percent increase relative to the full agonist, Oxo-M (Table II). In addition, the percent inhibition of cyclic AMP (cAMP) formation in NG108-15 cells which express exclusively m_4AChR was measured and is also reported

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Table I. Characterization and Muscarinic Receptor Affinities

$CH_3-C(=O)-N(CH_3)-CH_2-C{\equiv}C-CH_2-N$ (ring X, R)

no.	X	R	formula[a]	[^{3}H]NMS[b]	[^{3}H]Oxo-M[c]
1a (UH5)	$(CH_2)_4$	H		89.4 ± 1.1	94.7 ± 3.3
3a	$(CH_2)_4$	(*S*)-2-CH_2OH	$C_{12}H_{20}N_2O_2$[d]·oxalate·0.5H_2O	33.1 ± 1.5	25.0 ± 2.8
3b	$(CH_2)_4$	(*R*)-2-CH_2OH	$C_{12}H_{20}N_2O_2$·0.5oxalate·0.5H_2O	56.2 ± 1.4	39.7 ± 3.1
3c	$(CH_2)_4$	(*S*)-2-CH_2OCH_3	$C_{13}H_{22}N_2O_2$·oxalate·0.5H_2O	52.0 ± 0.3	—
3d	$(CH_2)_4$	(*R*)-2-CH_2OCOCH_3	$C_{14}H_{22}N_2O_3$·0.5oxalate	61.6 ± 3.2	42.1 ± 7.1
3e	$(CH_2)_4$	(*S*)-2-CH_2F	$C_{12}H_{19}N_2OF$·0.5oxalate·0.33H_2O[e]	53.8 ± 4.2	50.5 ± 5.0
3f	$(CH_2)_4$	(*R*)-2-CH_2F	$C_{12}H_{19}N_2OF$·0.5oxalate·0.33H_2O	67.2 ± 0.2	61.5 ± 6.3
3g	$(CH_2)_4$	(±)-3-OH	$C_{11}H_{18}N_2O_2$·oxalate·0.5H_2O	21.5 ± 3.5	39.9 ± 5.7
3h	$(CH_2)_4$	(±)-3-F	$C_{11}H_{17}N_2OF$·oxalate·1.5H_2O	29.0 ± 1.0	38.1 ± 3.3
3i	$(CH_2)_4$	(±)-3-OTs	$C_{18}H_{24}N_2O_4S$·0.5oxalate·0.5H_2O	51.6 ± 4.8	64.8 ± 11.6
3j	$(CH_2)_4$	(±)-3-OMs	$C_{12}H_{20}N_2O_4S$·0.5oxalate	20.2 ± 3.2	25.2 ± 6.2
3k	$(CH_2)_4$	(±)-3-$OCOC_6H_5$	$C_{18}H_{22}N_2O_3$·0.5oxalte	57.4 ± 1.4	39.1 ± 2.3
4a	$(CH_2)_5$	H	$C_{12}H_{20}N_2O$·0.5oxalate	83.4 ± 1.0	68.9 ± 9.0
4b	$(CH_2)_5$	(±)-2-CH_2OH	$C_{13}H_{22}N_2O_2$·0.5oxalate·0.5H_2O	31.2 ± 1.2	20.4 ± 2.4
4c	$(CH_2)_5$	(±)-2-CO_2Et	$C_{15}H_{24}N_2O_3$·0.5oxalate·0.5H_2O	5.8 ± 1.0	24.9 ± 2.6
4d	$(CH_2)_5$	(±)-3-OH	$C_{12}H_{20}N_2O_2$·0.5oxalate·0.6H_2O	21.7	9.1 ± 6.0
4e	$(CH_2)_5$	(±)-3-CH_2OH	$C_{14}H_{23}N_2O_5$·0.5oxalte·0.5H_2O	45.0 ± 4.7	22.4 ± 5.9
4f	$(CH_2)_5$	(±)-3-CO_2Et	$C_{15}H_{24}N_2O_3$·0.5oxalate·0.5H_2O	39.8 ± 2.2	31.2 ± 0.3
4g	$(CH_2)_5$	4-OH	$C_{12}H_{20}N_2O_2$·0.5oxalate·0.6H_2O	3.8 ± 3.2	9.2 ± 5.0
4h	$(CH_2)_5$	4-CH_2OH	$C_{13}H_{22}N_2O_2$·0.5oxalate·0.25H_2O	10.2 ± 1.8	14.7 ± 1.8
4i	$(CH_2)_5$	4-$(CH_2)_2OH$	$C_{14}H_{24}N_2O_2$·0.5oxalate·0.5H_2O	16.3 ± 2.2	11.0 ± 4.0
4j	$(CH_2)_5$	4-CO_2Et	$C_{15}H_{24}N_2O_3$·0.5oxalte·0.5H_2O	47.5 ± 1.3	32.1 ± 2.2
4k	$(CH_2)_5$	4-CO_2H	$C_{13}H_{20}N_2O_3$·1.5H_2O[f]	i	26.8 ± 8.3
4l	$(CH_2)_5$	4-F	$C_{12}H_{19}N_2OF$·0.5oxalate[g]	18.8 ± 3.4	—
5a	$(CH_2)_2NH(CH_2)_2$	H	$C_{11}H_{19}N_3O$·0.5oxalate[h]	51.2 ± 3.6	33.8 ± 8.1
5b	$(CH_2)_2NH(CH_2)_2$	4-Boc	$C_{16}H_{27}N_3O_3$·0.5oxalate	7.7 ± 0.4	10.5 ± 6.0
5c	$(CH_2)_2NH(CH_2)_2$	4-$COCH_3$	$C_{13}H_{21}N_3O_2$·0.5oxalate·0.5H_2O[i]	59.2 ± 3.0–	i
5d	$(CH_2)_2NH(CH_2)_2$	4-$(CH_2)_2OH$	$C_{13}H_{23}N_3O_2$·0.5oxalate·0.6H_2O	9.3	22.3 ± 3.3
6	$(CH_2)_3$	H	$C_{10}H_{16}N_2O$·0.5oxalate·0.75H_2O	38.0 ± 2.2	94.0 ± 0.9

[a] All compounds were analyzed for C, H, and N and were within ±0.4% of the theoretical value except for those formula marked with a footnote. [b] Each compound was tested at 100 μM in rat forebrain tissue. Data is reported as the percent displacement (± standard error) of bound [^{3}H]NMS and is the average of two to five trails. Data without standard errors were determined on one trial only. i = inactive (< 1%). [c] Each compound was tested at 1 μM in rat forebrain tissue. Data is reported as the percent displacement (±standard error) of bound [^{3}H]Oxo-M and is the average of two to five trails. i = inactive (<1%). — = Not determined. [d] See 14. [e] Anal. Calcd for $C_{12}H_{19}N_2OF$·0.5oxalate·0.33H_2O: N, 10.10. Found: N, 9.65. [f] Anal. Calcd for $C_{13}H_{20}N_2O_3$·1.5H_2O: H, 8.30. Found: H, 7.65. [g] High-resolution MS (CI/NH_3): Calcd for $C_{12}H_{19}H_2OF$ + H: 227.1560. Found: 227.1541. [h] High-resolution MS (CI/NH_3): Calcd for $C_{11}H_{19}N_3O$ + H: 210.1606. Found: 210.1612. [i] Anal. Calcd for $C_{13}H_{21}N_3O_2$·0.5oxalate·0.5H_2O: N, 13.76. Found: N, 13.22.

relative to Oxo-M (Table II).

In the series of 5-membered ring derivatives, all of the pyrrolidine substitutions led to analogues that were weaker in the displacement of either [^{3}H]Oxo-M or [^{3}H]NMS binding than the unsubstituted derivative, UH-5 (**1a**). The most potent substituted 5-membered ring derivatives against [^{3}H]Oxo-M binding in rat brain membranes (K_i values 1–2 μM) were compounds **3e** and **3f** (enantiomers of the 2-(fluoromethyl)pyrrolidine derivatives) and **3i** (the racemic 3-(oxytosyl)pyrrolidine), with ≥50% of the radioligand displaced at a concentration of 1 μM. In an effort to explain what structural features of **3i** led to greater potency in displacing [^{3}H]Oxo-M than the corresponding 3-hydroxypyrrolidine derivative **3g**, the mesylate **3j** and benzoate **3k** were prepared. Both **3j** and **3k** were less effective than the more bulky tosylate in displacing [^{3}H]Oxo-M. This suggests that steric features of **3g**, rather than electronic effects on the pyrrolidine ring carbons might interact favorably at the agonist binding site to increase affinity. Hypothetically, the tosylate might be bound at the receptor in a different orientation from the other members of the series. The sulfonyl group may act as the isosteric equivalent of the ester carbonyl of acetylcholine in the binding interaction. Further experiments to explore the enantiomeric pairs of **3g–k** are needed to determine if stereoisomeric differences play a role in their pharmacological activities.

There was a small degree of stereoselectivity (3.3-fold) in binding when comparisons were made between the *S* and *R* enantiomers of the 2-(hydroxymethyl)pyrrolidine analogues, **3a** and **3b**, respectively. The *R* enantiomer **3b** was also slightly more potent in displacing the binding of [^{3}H]NMS in rat brain at the dose tested. The corresponding (*S*)- and (*R*)-2-(fluoromethyl)pyrrolidine analogues, **3e** and **3f**, respectively, displayed no stereoselectivity in binding to [^{3}H]Oxo-M high affinity sites. Between the 2-methylene substituted *S* enantiomers, there was a slight preference (4.4-fold) for the fluoro analogue **3e** versus the corresponding hydroxy analogue **3a** at the agonist binding site. Interestingly, the *S* enantiomer of the 2-fluoromethyl analogues was a more potent agonist in inhibiting cAMP production in NG108-15 cells. There was no significant difference in affinity at [^{3}H]Oxo-M high affinity sites between the (*R*)-2-(hydroxymethyl)pyrrolidine analogue **3b** and its acetylated derivative **3d**.

In the series of 6-membered ring derivatives, only the unsubstituted piperidine derivative **4a** displaced more than 50% of the [^{3}H]Oxo-M binding at a concentration of 1 μM, but **4a** was much less potent than the pyrrolidine homologue **1a**. Substitution on the piperidine ring appeared to greatly diminish the affinity of the analogues for both agonist and antagonist states of the receptor. For example, compound **4g** (4-hydroxypiperidine analogue) displaced <10% of either [^{3}H]Oxo-M or [^{3}H]NMS at cerebral muscarinic receptors. Similarly, piperazine analogues, **5a–d** had greatly diminished affinity for both agonist and antagonist states of the receptor. The azetidine analogue **6** had an inhibition constant (K_i) of 41 nM versus [^{3}H]-

Table II. Second Messenger Effects

no.	R	PI turnover[a] A9L-m_1 cells	PI turnover[a] A9L-m_3 cells	cAMP inhibition[b] NG108-15 cells
1a	H	52.5	41.6	100.0 ± 0
3a	(*S*)-2-CH_2OH	5.1	4.7	i
3b	(*R*)-2-CH_2OH	2.6	4.8	i
3c	(*S*)-2-CH_2OCH_3	6.2	5.5 ± 0.6	15.4 ± 3.3
3d	(*R*)-2-CH_2OCOCH_3	i	2.6	i
3e	(*S*)-2-CH_2F	11.1 ± 0.1	10.6 ± 0.1	62.4 ± 7.0
3f	(*R*)-2-CH_2F	7.2 ± 2.8	10.4 ± 1.4	38.6 ± 8.0
3g	(±)-3-OH	17.1	7.4	22.5 ± 8.3
3h	(±)-3-F	11.3	7.9	8.9 ± 4.9
3i	(±)-3-OTs	23.1	23.1	83.6 ± 7.9
3j	(±)-3-OMs	10.4 ± 0.5	17.2 ± 0.4	38.5 ± 1.6
3k	(±)-3-$OCOC_6H_5$	1.4 ± 0.6	4.0 ± 1.2	i
4a	H	i	5.6	i
4b	(±)-2-CH_2OH	7.3	12.5	i
4c	(±)-2-CO_2Et	12.8	15.2	44.1 ± 6.1
4d	(±)-3-OH	i[c]	4.9	3.4 ± 1.7
4e	(±)-3-CH_2OH	i	2.8	i
4f	(±)-3-CO_2Et	6.6	12.9	23.6 ± 4.0
4g	4-OH	i	2.6	i
4h	4-CH_2OH	8.8	16.0	4.8 ± 3.1
4i	4-$(CH_2)_2OH$	i	i[c]	i
4j	4-CO_2Et	3.3	8.8	i
4k	4-CO_2H	i	5.2	17.5 ± 5.4
4l	4-F	—	—	—
5a	H	18.2 ± 0.5	12.0 ± 2.1	16.8 ± 4.3
5b	4-Boc	i	i[c]	i
5c	4-$COCH_3$	1.0 ± 0.3	3.1 ± 2.1	i
5d	4-$(CH_2)_2OH$	i	i	i
6	H	61.3	59.8	95.7 ± 4.3
oxotremorine		—	35.6	111.8 ± 13.1
carbachol		—	86.1	104.5 ± 7.4

[a] Each compound was tested at 100 μM. Data are reported as the percent increase in [^{3}H]*myo*-inositol (± range) relative to Oxo-M (100%) and is the average of two duplicate determinations. Data without range were determined on one duplicate determination. i = inactive (< background). [b] Each compound was tested at 100 μM. Data are reported as the percent inhibition (± standard error) of cyclic AMP relative to Oxo-M (100%) and is the average of two or three trials. Data without standard errors were determined on one trial only. i = inactive (< 2%). [c] Two duplicate determinations were made. — = not determined.

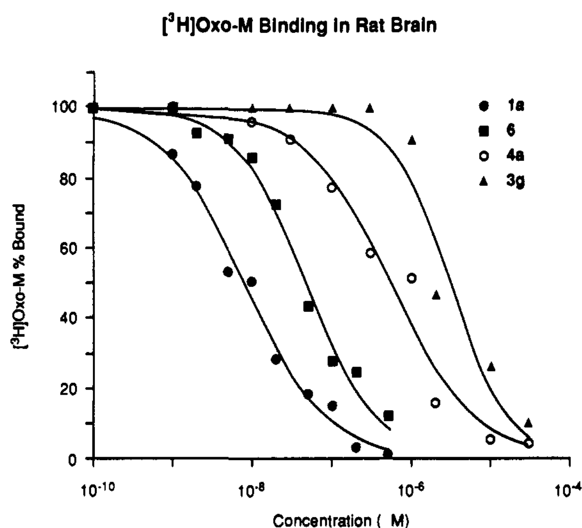

Figure 1. Representative inhibition curves for selected derivatives versus [^{3}H]oxotremorine-M in rat brain membranes. Binding was carried out for 20 min at 37 °C, in the presence of 2.7 nM [^{3}H]oxotremorine-M. Nonspecific binding was defined in the presence of 1 μM atropine. IC_{50} values were converted to K_i values by using the Cheng–Prusoff equation. The following K_i vaues (in μM) were determined, for one to three triplicate determinations: **1a**, 0.0045 ± 0.0018; **3a**, 7.41 ± 4.2; **3b**, 2.24 ± 0.05; **3d**, 3.93 ± 1.8; **3e**, 1.70 ± 0.8; **3f**, 2.21 ± 1.5; **3i**, 1.64 ± 0.35; **3g**, 0.80; **4a**, 0.25; **6**, 0.041 ± 0.020.

Oxo-M in rat brain, while that of the parent compound, UH 5 (**1a**) was 4.5 nM (Figure 1). Compound **6**, however, was more potent than **1a** in stimulating PI turnover at both m_1AChRs and m_3AChRs, while **4a** was nearly inactive in

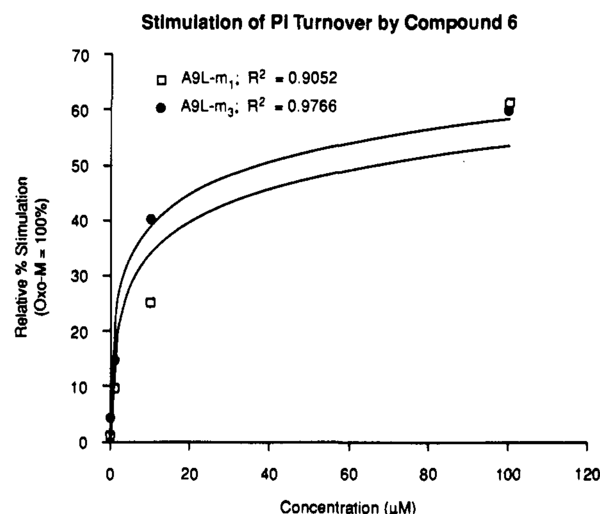

Figure 2. Stimulation of metabolism of phosphoinositides by compound **6** in A9L cells that express m_1 (open squares) or m_3 (closed circles) muscarinic receptors. Activity was assayed by using an ionic exchange method to extract ^{3}H-labeled inositol phosphates from sonicated cells, which had been exposed to [^{3}H]*myo*-inositol.

stimulating PI turnover (Figure 2). This is consistent with earlier studies of analogous derivatives of oxotremorine which report the azetidine analogue as a potent muscarinic agonist[16] and the piperidine analogue as a weak antagonist.[17]

(16) Ringdahl, B. In *Dynamics of Cholinergic Function*; Hanin, I., Eds.; Plenum: New York, 1983; pp 395–404.

In a comparison of potencies for [^{3}H]Oxo-M binding sites in the cerebral cortex (mainly m_1) versus heart (m_2) for several of the compounds, selectivity was not evident. Values for percent inhibition of the specific binding of [^{3}H]Oxo-M in rat heart membranes by the analogue at a single concentration of 1 μM were as follows: 39.7% (**4b**), 14.7% (**4c**), 27.2% (**4f**), 23.4% (**4h**), 15.4% (**4j**), and 94.2% (**6**).

Comparisons of K_1 values for muscarinic ligands in competing for agonist binding sites vs antagonist binding sites has been shown to be predictive of agonist potency.[18] Although the data in Table I are for a single concentration, a comparison of the ratios of [^{3}H]Oxo-M displacement potency vs [^{3}H]NMS displacement potency does suggest a predictive trend. Several of the compounds (e.g., **6**, **3i**, and **3j**) appear to have greater potency in competing for [^{3}H]Oxo-M binding sites vs [^{3}H]NMS binding sites in rat brain membranes. All of the compounds with >10% displacement of [^{3}H]NMS and an [^{3}H]Oxo-M/[^{3}H]NMS binding ratio > 0.9 appeared to be agonists at m_1- and m_3AChRs and elicited >7% stimulation of PI turnover. Outside of this criteria range, only 2 of 13 and 3 of 13 compounds had >7% stimulation of PI at m_1- and M_3AChRs, respectively. Likewise, within this same criteria, all compounds had >4% inhibition of cAMP, and outside of the criteria, only 2 of 13 had >4% inhibition of cAMP at m_4AChRs.

The unsubstituted azetidine analogue **6** at 100 μM stimulated approximately 60% of the PI turnover relative to Oxo-M. In full dose–response studies of PI turnover (Figure 2), compound **6** displayed EC_{50} values of 3 and 4 μM at m_1- and m_3AChRs, respectively. Of the 5-membered ring substituted analogues tested, compound **3g** was the most effective stimulator of PI turnover in A9L transfected cell lines. EC_{50} values for **3g** were found to be 50 μM and 40 μM at m_1- and m_3AChRs, respectively. Most of the other analogues were much weaker in the assay, with no selectivity between m_1 and m_3 receptors evident at the concentration evaluated (100 μM).

Many of the analogues tested demonstrated substantial activity in inhibiting the production of cAMP via m_4AChRs in NG108-15 cells. The azetidine analogue **6** was nearly as potent as **1a** in this assay, with an inhibition of 95.7% relative to Oxo-M at 100 μM. Other compounds (e.g., **3e**, **3i**, and **4c**) were agonists at m_4AChRs with an inhibition of cAMP accumulation of ≥40%. Compounds **3c**, **3f**, **3j**, **4f**, **4k**, and **5a** also demonstrated moderate agonist activity.

Comparison of cAMP inhibition potency to PI turnover stimulation potency indicates that several of the substituted analogues (e.g., **3e**, **3f**, **3c**, **3i**, **3j**, and **4c**), as well as the parent compound **1a**, are moderately selective for m_4AChRs. Conversely, compounds **4b**, **4h**, and **4j** have weak activity at m_1- and m_3AChRs and no activity at m_4AChRs.

Conclusions

Substitution of the pyrrolidine ring in series **3** generally decreased binding affinity. This result is similar to the findings of Ringdahl et al. for analogues of the closely related oxotremorine with substitutions on the pyrrolidine ring.[19] These compounds were weak to moderate agonists, at best, at both m_1- and m_3AChRs in stimulating PI turnover. The substituted piperidine compounds in series **4** were generally weak agonists or inactive at muscarinic receptors, as expected from the lack of effects on second messengers demonstrated by the corresponding parent analogue **4a**. It is interesting to note that the 2- and 3-ethylcarboxylate derivatives **4c** and **4f** had weak agonist effects at muscarinic receptors. A tempting postulate may involve an alternate binding mode to the receptor through the ester and piperidine nitrogen, similar to the postulate discussed above for the tosylate derivative **3i**. The piperazine analogue **5a** showed weak agonist effects in both PI turnover and cAMP inhibition assays, however the substituted derivatives **5b–d** were virtually inactive. The azetidine analogue **6** was the most potent analogue tested, having agonist potency at the receptors tested comparable to **1a**. These results are similar to those reported for analogues of oxotremorine. The piperidine and piperazine analogues of oxotremorine were previously reported to be weak muscarinic antagonists,[17] and the azetidine analogue was described as a potent muscarinic agonist.[16]

Building functionalized congeners from the piperidine or piperazine analogue of **1a** does not appear to be a viable design approach due to the substantial loss of activity of **4a** and **5a** relative to **1a**. For the pyrrolidine series, the data suggest that a hydroxy group near the tertiary amine of the *N*-methyl-*N*-(4-amino-2-butynyl)acetamide muscarinic pharmacophore generally decreases binding and activity at muscarinic receptors. Conversion of the hydroxyl to an ether (**3c**), fluorine (**3e** and **3f**), or sulfonyl ester (**3i** and **3j**) does improve muscarinic activity, suggesting that the pyrrolidine series of analogues (**3**) may be possible intermediates for further derivatization. Further studies toward developing functionalized congeners of **1a** would focus on the extension of longer alkyl chains from the pyrrolidine ring to position the terminal functional group at a more distal, noninterfering site away from the central pharmacophore.

Experimental Section

General. All ^{1}H NMR spectra were recorded with use of a Varian XL-300 FT-NMR spectrometer, and all values are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Chemical-ionization MS using ionized NH_3 gas were recorded with use of a Finnigan 1015D mass spectrometer modified with EXTREL electronics. Thin-layer chromatography (TLC) analyses were carried out with use of EM Kieselgel 60 F254, DC-Alufolien 200-μm plates and were visualized in an iodine chamber and/or with 1% ninhydrin in ethanol. Silica gel columns used MN-Kieselgel 60, 0.063–0.2 mm silica gel. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA. The optically pure secondary amine intermediates were commercially available (Aldrich Chemical Co.). The term in vacuo refers to a water aspirator (15–30 mmHg) rotary evaporator. Percent yields are rounded to the nearest whole number. An asterisk (*) for the ^{1}H NMR data indicates the larger integrated signal for the pair of signals which represent the same proton(s) of the amide tautomers.

***N*-Methyl-*N*-propargylacetamide (7).** Acetic anhydride (75 mL, 0.80 mol) was added dropwise to a stirring solution of *N*-methylpropargylamine (50 g, 0.72 mol) and triethylamine (120 mL, 0.86 mol) in acetonitrile (300 mL) and then stirred for 24 h at room temperature. All volatiles were removed in vacuo, the crude oil was redissolved in EtOAc (250 mL), and the organic layer was washed with saturated $NaHCO_3$ solution (2 × volume) and 3 N citric acid (2 × volume). The organic layer was dried over Na_2SO_4 and filtered, and the EtOAc removed in vacuo. The crude

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(19) (a) Resul, B.; Ringdahl, B.; Dahlbom, R. *Acta Pharm. Suec.* **1979**, *16*, 161. (b) Ringdahl, B.; Jenden, J. *Life Sci.* **1983**, *32*, 2401.

oil was vacuum distilled to give **7** (68.4 g, 66%) as a clear liquid: bp 55–62 °C (0.01 mmHg); ^{1}H NMR ($CDCl_3$) δ 2.04* and 2.09 (s, 3 H, Ac), 2.16* and 2.27 (t, J = 2.3 Hz, 3), 2.92 and 3.02* (s, 3 H, NMe), 3.97 and 4.15* (d, J = 2.3 Hz, 2 H, 1); MS (EI) m/e 111 (M^+), 96, 82, 68 (base), 54.

4-(Hydroxymethyl)piperidine. A solution of ethyl isonipecotate (5.0 g, 31.8 mmol) in THF (100 mL) was slowly added to a stirring suspension of $LiAlH_4$ (1.2 g, 31.8 mmol) in THF (250 mL) cooled to 0 °C. After stirring overnight the reaction was quenched by slow addition of $MgSO_4 \cdot 7H_2O$ until all evolution of gas ceased. The slurry was filtered and washed thoroughly with THF, evaporated in vacuo, and recrystallized from EtOAc to give 4-(hydroxymethyl)piperidine as a white waxy solid (3.3 g, 89.3%).

***tert*-Butyl 1-Piperazinecarboxylate.** A solution of di-*tert*-butyl dicarbonate (2.93 g, 12.77 mmol) in MeOH (25 mL) was slowly added to a stirring solution of piperazine (1.00 g, 11.61 mmol) in MeOH (50 mL) at 0 °C. The reaction was then heated to 50 °C for 1 h and cooled to room temperature, and the solvent removed in vacuo. The crude solid was redissolved in Et_2O (100 mL) with warming, and a white precipitate filtered off. The product was extracted from the mother liquor with 1 M citric acid solution (3 × 50 mL), and the aqueous layer was washed with EtOAc (3 × 50 mL), basified with Na_2CO_3 (pH 11), and extracted with EtOAc (3 × 50 mL). The organic layer was then dried over Na_2SO_4 and evaporated in vacuo, and the crude solid recrystallized from EtOAc/Et_2O (1/1) to give *tert*-butyl 1-piperazinecarboxylate as a waxy white solid (1.08 g, 50.0%), mp 45–46 °C.

General Mannich-type Condensation. Paraformaldehyde (2 equiv) and CuCl (0.05 equiv) were added to a stirring solution of the secondary amine (1 equiv) in dioxane. After 30 min, a solution of *N*-acetylpropargylamine (**7**, 0.95 equiv) was added and the pale green mixture was stirred at 25–50 °C until complete by TLC analysis (12–120 h). The mixture was then filtered, the solvent was evaporated in vacuo, and the crude oil was chromatographed on silica gel column (20–95% $CHCl_3$, 5–80% of 10/1 MeOH/NH_4OH eluent). Unless otherwise indicated, the purified products (**3–6**) were viscous, clear oils.

(*S*)-*N*-Methyl-*N*-[4-[1-[2-(hydroxymethyl)pyrrolidinyl]]-2-butynyl]acetamide (3a). Column eluent: $CHCl_3$/MeOH/NH_4OH, 90/10/1; yield, 68%; ^{1}H NMR ($CDCl_3$) δ 4.22* and 4.04 (s, 2 H), 3.65–3.40 (complex m, 4 H), 3.05* and 2.96 (s, 4 H (single hydrogen signal under the singlet at 3.05 ppm)), 2.81 (m, 1 H), 2.63 (m, 1 H), 2.44 (br s, 1 H), 2.14 and 2.09* (s, 3 H), 1.88 (m, 1 H), 1.75 (m, 3 H); MS (CI/NH_3) m/e 225 (MH^+), 193, 141, 124. The specific rotation of the monooxalate, hemihydrate of **3a** was found to be $[\alpha]^{24}{}_D$ (c = 0.3 in methanol) = −22°; and of the free base of **3b**, $[\alpha]^{24}{}_D$ (c = 0.6 in methanol) = +53.4°.

The corresponding methyl ether *S* isomer **3c** was prepared from (*S*)-(+)-2-(methoxymethyl)pyrrolidone (Aldrich Chemical Co., Milwaukee, WI) and characterized similarly and found to have a specific rotation (oxalate, hemihydrate): $[\alpha]^{24}{}_D$ (c = 0.2 in methanol) = −28°.

(*R*)-*N*-Methyl-*N*-[4-[1-[2-(acetoxymethyl)pyrrolidinyl]]-2-butynyl]acetamide (3d). Acetic anhydride (12.7 μL, 0.134 mmol), triethylamine (18.9 μL, 0.134 mmol), 4-(dimethylamino)pyridine (2.0 mg, 0.016 mmol), and **3b** (20.1 mg, 0.090 mmol) were stirred in acetonitrile (2 mL) until the reaction was complete by TLC analysis (~2 h). The volatiles were removed under a stream of nitrogen, and the crude product was chromatographed ($CHCl_3$/MeOH/NH_4OH, 80/20/20) on a silica gel column to give **3d** as a light yellow oil (23.0 mg, 96.2%): ^{1}H NMR ($CDCl_3$) δ 1.58–2.06 (br m, 4 H), 2.08–2.16 (m, 6 H), 2.67 (m, 1 H), 2.81 (m, 1 H), 2.98 and 3.08* (s, 3 H), 3.21 (m, 1 H), 3.62 and 3.71* (br s, 2 H), 4.06 and 4.24* (s, 2 H), 4.17 (m, 2 H); MS (CI/NH_3) m/e 267 (MH^+).

***N*-Methyl-*N*-[4-[1-[3-[(4-methylphenyl)sulfonyl]pyrrolidinyl]]-2-butynyl]acetamide (3i).** A solution *p*-toluenesulfonyl chloride (325.5 mg, 1.85 mmol), **3g** (194.4 mg, 0.92 mmol), and triethylamine (0.32 mL, 2.31 mmol) in 20 mL of dry CH_2Cl_2 was stirred over 4-Å molecular sieves at 50 °C for 48 h. The brown solution was then washed with saturated bicarbonate solution (2 × volume) and then extracted into 0.5 N HCl (2 × volume). The acid layer was washed with CH_2Cl_2 (2 × volume) and then basified with 1 N NaOH to pH 12. The product was extracted into ethyl acetate (3 × volume), dried over sodium sulfate, evaporated in vacuo, and chromatographed ($CHCl_3$/MeOH/NH_4OH, 90/10/1) to give **3i** (221.0 mg, 65.6%) as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.79 (d, J = 8.1 Hz, 2 H), 7.35 (d, J = 8.1 Hz, 2 H), 5.00 (br s, 1 H), 4.23* and 4.05 (s, 2 H), 3.45 (br s, 2 H), 3.06* and 2.96 (s, 3 H), 2.85 (br m, 3 H), 2.67 (br m, 1 H), 2.46 (s, 3 H), 2.14 and 2.10* (s, 3 H), 2.00 (br m, 2 H); MS (CI/NH_3) m/e 365 (MH^+), 349, 211, 193 (base).

***N*-Methyl-*N*-[4-[1-[3-[(methylsulfonyl)oxy]pyrrolidinyl]]-2-butynyl]acetamide (3j).** A solution methanesulfonyl chloride (24.3 μL, 0.31 mmol), **3g** (59.9 mg, 0.28 mmol), and triethylamine (47.6 μL, 0.34 mmol) in 5 mL of dry CH_2Cl_2 was stirred at 25 °C for 2 h. Analysis of the reaction by TLC showed the reaction was not yet complete so more methanesulfonyl chloride (12.1 μL, 0.15 mmol) and triethylamine (23.8 μL, 0.17 mmol) were added, and the reaction was stirred at 40 °C for an additional 2 h (complete by TLC analysis). The solvent was removed under a stream of nitrogen, the crude oil was redissolved in ethyl acetate (~5 mL), and the organic layer washed with saturated $NaHCO_3$ solution (3 × volume). The organic layer was removed under a stream of nitrogen, and the crude product was chromatographed on a silica gel column ($CHCl_3$/MeOH/NH_4OH, 90/10/1) to give 58.4 mg (71%) of **3j** as a light yellow oil: MS (CI/NH_3) m/e 289 (MH^+); ^{1}H NMR ($CDCl_3$) d 5.22 (br s, 1 H), 4.23* and 4.05 (s, 2 H), 3.48 (s, 2 H), 3.06* (associated tautomeric signal within multiplet at 2.97 ppm) (s, 3 H), 2.97 (m, 3 H), 2.62 (m, 1 H), 2.32 (m, 1 H), 2.14 and 2.09* (s, 4 H (single hydrogen signal under the singlet at 2.14 ppm)).

***N*-Methyl-*N*-[4-[1-[3-(benzoyloxy)pyrrolidinyl]]-2-butynyl]acetamide (3k).** Benzoyl chloride (21.4 μL, 0.184 mmol) was added to a solution of **3g** (35.2 mg, 0.167 mmol) and triethylamine (28.0 μL, 0.201 mmol) in CH_2Cl_2 and stirred for 2 h at 25 °C. The volatiles were removed under a stream of nitrogen, the crude oil was redissolved in EtOAc and washed with saturated $NaHCO_3$ (2 × volume). The organic layer was removed under a stream of nitrogen and the crude product was chromatographed on a silica gel column ($CHCl_3$/MeOH/NH_4OH, 90/10/1) to give 49.2 mg (94%) of **3k** as a clear oil: MS (CI/NH_3) m/e 315 (MH^+); ^{1}H NMR ($CDCl_3$) δ 8.05 (d, J = 7.6 Hz, 2 H), 7.56 (t, J = 7.6 Hz, 1 H), 7.44 (t, J = 7.6 Hz, 2 H), 5.44 (br s, 1 H), 4.23* and 4.04 (s, 2 H), 3.50 (s, 2 H), 3.07 (m, 1 H), 3.04* and 2.97 (s, 3 H), 2.93 (m, 2 H), 2.66 (m, 1 H), 2.39 (m, 1 H), 2.14 and 2.08* (s, 4 H (single hydrogen signal under the singlet at 2.08 ppm)).

General Procedure for Fluorination. A solution of (dimethylamino)sulfur trifluoride (methyl DAST, 1.5 equiv) in dry CH_2Cl_2 was cooled to −78 °C under a nitrogen atmosphere. To this was slowly added a cooled solution (−78 °C in CH_2Cl_2) of the alcohol (1 equiv) and the mixture was allowed to warm to room temperature overnight. More methyl DAST was added, at −78 °C, if TLC analysis indicated the presence of the starting alcohol. The reaction was quenched by slowly adding methanol at −20 °C, then it was warmed to room temperature and the solvents evaporated under a stream of nitrogen. The crude dark oil was then purified on a silica gel column ($CHCl_3$/MeOH/NH_4OH, 90/10/1).

(*S*)-*N*-Methyl-*N*-[4-[1-(3-fluoropyrrolidinyl)]-2-butynyl]acetamide (3e): MS (CI/NH_3) m/e 213 (MH^+); ^{1}H NMR ($CDCl_3$) δ 5.18 (br d, J = 55.2 Hz, 1 H), 4.23* and 4.05 (s, 2 H), 3.49 (s, 2 H), 3.06* and 2.97 (s, 3 H), 2.93 (m, 3 H), 2.64 (m, 1 H), 2.14 and 2.09* (s, 5 H (two hydrogen multiplet under the singlets at 2.14 and 2.09 ppm)). The corresponding *R* isomer **3f** was prepared and characterized similarly and found to have a specific rotation (hemioxalate, 0.33 hydrate): $[\alpha]^{24}{}_D$ (c = 0.2 in methanol) = +14°.

Inhibition of Radioligand Binding. Radioligands ([^{3}H]-Oxo-M and [^{3}H]NMS) were obtained from DuPont NEN Products (Boston, MA). Stock solutions of the unlabeled competing compound in the millimolar concentration range in dimethyl sulfoxide were prepared for binding assays. The solutions were diluted with 50 mM sodium phosphate buffer pH 7.4 as necessary and were stable to storage in the frozen state. For each assay, inhibition of binding by single concentrations of the drug was assessed in triplicate, in multiple or single experiments. Protein was determined by using the BCA (based on the complex with cuprous ions and bicinchoninic acid) protein assay reagents purchased from Pierce Chemical Co., Rockfield, IL.

Membranes from rat brain or heart were prepared as follows. Rats (Sprague-Dawley, either sex, 200 g) were lightly anesthetized

with ether and decapitated, and their brain (forebrain) and heart removed. Brain was homogenized in ice-cold 50 mM sodium phosphate buffer pH 7.4 (32 mL per gram of tissue) in a Polytron (3 × 30 s, 75% maximum), centrifuged at 40000*g* for 2 min, resuspended in the original volume of buffer and recentrifuged. The resulting pellet was resuspended at a concentration of 3 mg of protein per milliliter and stored in aliquots at -70 °C. Heart tissue was minced, homogenized in 145 mM NaCl, 10 mM Tris-HCl pH 7.4 in a Polytron (3 × 30 s, 75% maximum) and centrifuged at 40000*g* for 20 min. The resulting pellet was washed three times by resuspension in fresh buffer followed by centrifugation as above. The final pellet was resuspended at 6 mg of protein per milliliter and stored at -70 °C.

Binding of [^{3}H]NMS was performed by incubating 200-300 μg of membrane with 0.5 nM [^{3}H]NMS and the indicated compound in a total volume of 1 mL of phosphate-buffered saline. After 60 min at 37 °C, the mixture was rapidly filtered over glass fiber filters which were washed three times with ice-cold 0.9% NaCl and processed for scintillation counting. Binding of [^{3}H]Oxo-M was performed by incubating membrane with 2.7 nM [^{3}H]Oxo-M and the indicated compound in a total volume of 1 mL of buffer G (50 mM $NaPO_4$, 2 mM $MgCl_2$, pH 7.4) for 20 min at 37 °C. This mixture was then filtered over glass fiber filters that had been presoaked in 0.05% polyethylenimine. The filters were washed with ice-cold 0.9% NaCl, then processed for scintillation counting. Nonspecific binding was determined by coincubation with 1 μM atropine and was routinely subtracted from the total binding. The nonspecific binding amounted to less than 10% of total binding for [^{3}H]NMS and to 20-50% for [^{3}H]Oxo-M binding.

The K_i values were determined by computer analysis of displacement curves obtained by incubating membranes with various concentrations of either **1a** or **6** and with 2.7 nM [^{3}H]Oxo-M as described above. The data represent means ± SEM from three determinations each made in duplicate.

Stimulation of PI Turnover in A9L Cells. Transfected A9L fibroblast cells were obtained from Dr. Mark Brann (NIH) and were grown in DMEM supplemented with 10% newborn calf serum (Advanced Biotechnologies, Columbia, MD). Cells were plated into 24-well plates at 100 000 cells per well. After 24 h, they were labeled overnight with 2 μCi per well of [^{3}H]*myo*-inositol (American Radiolabeled Chemicals, Inc., St. Louis, MO; 15 Ci/mM). The cells were then rinsed twice with 10 mM LiCl in DMEM-Hepes and the cells were incubated in this solution for 5 min at 37 °C. The indicated compound was then added at a final concentration of 100 μM and the cells were incubated for another 30 min at 37 °C. The reaction was stopped by aspirating the solution and adding cold methanol to the cells. After the cells were transferred to a glass tube and sonicated briefly, chloroform and water were added to make a two-phase system. The upper aqueous phase was applied to 0.6-mL anion exchange columns (AG X8, Bio-Rad), and the columns were washed and total inositol phosphates were eluted with 1 M ammonium formate and 0.1 M formic acid.

Inhibition of PGE$_1$-Stimulated cAMP Levels in NG108-15 Cells. This assay was performed as previously reported.[14]

Isomeric Monomethyl Ether Derivatives of (*RS*)-9,10-Dihydroxyaporphine ("Isoapomorphine") as Possible Products of Metabolism by Catechol-*O*-methyltransferase

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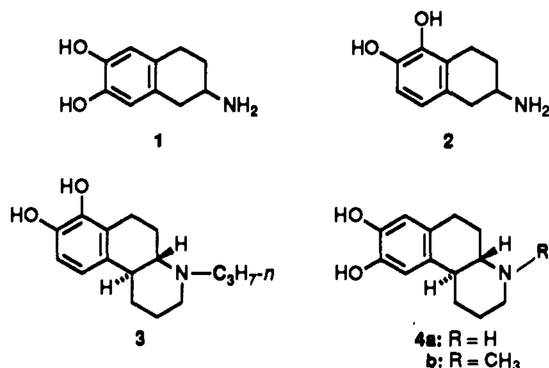
Received August 6, 1990

The isomeric monomethyl ether derivatives of (*RS*)-9,10-dihydroxyaporphine ("isoapomorphine") were synthesized unequivocally as possible metabolites in catechol-O-methyltransferase (COMT) mediated O-methylation reactions. In vitro incubation studies revealed that isoapomorphine is not a substrate for the COMT using experimental conditions under which apomorphine (10,11-dihydroxyaporphine) is converted in high yield into its 10-methyl ether, apocodeine. The in vivo dopaminergic inactivity of isoapomorphine (as compared with that of apomorphine) seems to be due to factors other than metabolic inactivation by COMT.

Rollema et al.[1] have reported that incubation of (*RS*)-6,7-dihydroxy-2-aminotetralin (**1**) with crude rat liver



catechol-*O*-methyltransferase (COMT) gave rise to the two isomeric monomethyl ethers in approximately equal amounts. When (*RS*)-5,6-dihydroxy-2-aminotetralin (**2**) was incubated under the same conditions, it was found to be a poor substrate. This difference in susceptibility to COMT has been invoked[2] to explain observed differences in central nervous system (CNS) effects of **1** and **2**.

We recently reported[3] similar studies with (*RS*)-7,8-dihydroxy- and (*RS*)-8,9-dihydroxy-4-*n*-propyl-1,2,3,4,4a,5,6,10b-octahydrobenzo[*f*]quinolines (**3** and **4a**) using porcine COMT. Only the 8,9-dihydroxy isomer **4a** was a substrate for the enzyme, and the sole detectable product of the reaction was the "meta"-methylated product **4b**. In the 2-aminotetralins (**1, 2**) and in angularly annulated octahydrobenzoquinolines (**3, 4b**) it is the β conformer[4] (**1, 4b** of the dopaminergic moiety which is a substrate for COMT, and the α-conformer[4] (**2, 3**) is re-

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