Annulated Heterocyclic Bioisosteres of Norarecoline. Synthesis and Molecular Pharmacology at Five Recombinant Human Muscarinic Acetylcholine Receptors

Hans Bräuner-Osborne, Bjarke Ebert, Mark R. Brann,† Erik Falch, and Povl Krogsgaard-Larsen*

PharmaBiotec Research Center, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, DK-2100 Copenhagen, Denmark, Molecular Neuropharmacology Section, Department of Psychiatry, College of Medicine, University of Vermont, Burlington, Vermont 05405, and Receptor Technologies Inc., Winooski, Vermont 05404

Received January 20, 1995®

A series of *O*-alkylated analogs of 5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepin-3-ol (THAO) were synthesized and characterized as ligands for muscarinic acetylcholine receptors (mAChRs). *O*-Methyl-THAO (**4a**), *O*-ethyl-THAO (**4b**), *O*-isopropyl-THAO (**4c**), and *O*-propargyl-THAO (**4d**) were shown to be potent inhibitors of the binding of tritiated quinuclidinyl benzilate (QNB), pirenzepine (PZ), and oxotremorine-M (Oxo-M) to tissue membrane preparations. In the [^{3}H]-Oxo-M binding assay, receptor affinities in the low nanomolar range were measured for **4a** (IC_{50} = 0.010 μM), **4b** (IC_{50} = 0.003 μM), **4c** (IC_{50} = 0.011 μM), and **4d** (IC_{50} = 0.0008 μM). Pharmacological effects (EC_{50} or K_i values) and intrinsic activities (per cent of maximal carbachol responses) were determined using five recombinant human mAChRs (m1–m5) and the functional assay, receptor selection and amplification technology (R-SAT). Compound **4c** antagonized carbachol-induced responses at m1, m3, and m5. With the exception of **4b**, which was an antagonist at m5, **4a,b,d** showed partial agonism at m1–m5 with very similar subtype selectivity (m2 > m4 > m1 ≥ m3 > m5). Agonist index values for **4a–d**, which were calculated from [^{3}H]QNB (brain) and [^{3}H]Oxo-M (brain) binding data, were shown to be predictive of pharmacologically determined intrinsic activities at m1–m5, the same rank order of intrinsic activity being observed at all five mAChRs (**4a** > **4d** > **4b** > **4c**). It is concluded that within this class of high-affinity mAChR (m1–m5) ligands, containing secondary amino groups, minor changes of the bioisosteric ester alkyl groups have marked effects on potency and, in particular, intrinsic activity.

Introduction

During the past decades, accumulating evidence has supported the "cholinergic hypothesis",[1] postulating that the documented deficit in central cholinergic transmission causes the learning and memory impairments seen in patients with Alzheimer's disease (AD) and senile dementia of the Alzheimer type (SDAT).[2–4] It has been shown that basal forebrain acetylcholine neurons degenerate in AD patients[5] and that the memory dysfunction can be mimicked to some extent by muscarinic acetylcholine receptor (mAChR) antagonists[1,5] or by destruction of the nucleus basalis of Meynert (part of basal forebrain).[6] The basal forebrain innervates the cerebral cortex and the hippocampus by cholinergic transmission.[5] In AD patients, loss of the presynaptic marker enzyme ChAT in the cortex and hippocampus correlates with the severity of the disease,[5,7] and this finding can be mimicked by lesions in the basal forebrain.[8]

In the mapping of pathophysiological changes in the brains of AD or SDAT patients, alterations of the densities of pharmacologically characterized (M_1 and M_2) and cloned (m1–m5) mAChRs have been extensively studied. Thus, basal forebrain lesions in experimental animals lead to loss of M_2 receptor sites but unaltered densities of postsynaptic M_1 sites,[8] an observation similar to findings in AD patients.[8,9] It still is uncertain which subtypes of the cloned mAChRs parallel the M_1 and M_2 receptor sites, but it has been shown by immunoprecipitation that the forebrain and the cerebral cortex as well as the hippocampus possess a majority of the m1 mAChR subtype.[10–12]

The cholinergic communication between the basal forebrain and the cortex/hippocampus decays as the disease progresses, and this leads to the growing memory and cognitive problems. It is therefore believed that a higher acetylcholine level would be beneficial for AD patients and that reduced side effects could be obtained by designing agonists acting at the postsynaptic M_1 receptor,[13,14] antagonists for the M_2 autoreceptor,[8] or, ideally, a drug combining these two pharmacological effects.[15] Partial agonists have been shown to have less predisposition to cause receptor desensitization,[16–18] making partial agonist at the M_1 receptor more interesting from a therapeutic point of view.

In order to define mAChR agonist pharmacophore(s) relevant to AD/SDAT and to design mAChR ligands of therapeutic interest in these diseases, we have previously described a different series of bicyclic muscarinic agonists and partial agonists bioisosterically derived from norarecoline (**1**) or arecoline as exemplified by 3-methoxy-5-methyl-6,7-dihydro-4*H*-thiopyrano[3,4-*d*]isoxazol-5-ium (*O*,5-dimethyl-DHTO, **2**)[19] and 3-methoxy-4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridinium (*O*-methyl-THPO, **3**)[20–22] halides (Scheme 1). In this paper we report the synthesis and pharmacological characteriza-

* Address correspondence to: Professor Povl Krogsgaard-Larsen, PharmaBiotec Research Center, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark. Phone: (+45) 35376777...247. Fax: (+45) 35372209.
† University of Vermont and Receptor Technologies Inc.
® Abstract published in *Advance ACS Abstracts*, May 15, 1995.

0022-2623/95/1838-2188$09.00/0

Table 1. Receptor Binding Data of Muscarinic Ligands Using Rat Heart and Brain Tissues

ligand	receptor binding, IC_{50} [μM] [^{3}H]QNB (brain)	[^{3}H]QNB (heart)	[^{3}H]PZ (brain)	[^{3}H]Oxo-M (brain)	M2/M1 index	agonist index
arecoline	28	1.7	2.1	0.016	0.10	280
oxotremorine	2.3	0.084	0.093	0.0012	0.11	310
O-methyl-THAO (**4a**)[a]	6.4	0.35	0.39	0.010	0.11	104
O-ethyl-THAO (**4b**)[a]	0.53	0.14	0.018	0.0034	0.97	25
O-propargyl-THAO (**4d**)	0.48	0.082	0.023	0.00075	0.45	104
O-isopropyl-THAO (**4c**)	0.37	0.36	0.013	0.011	3.5	5.4

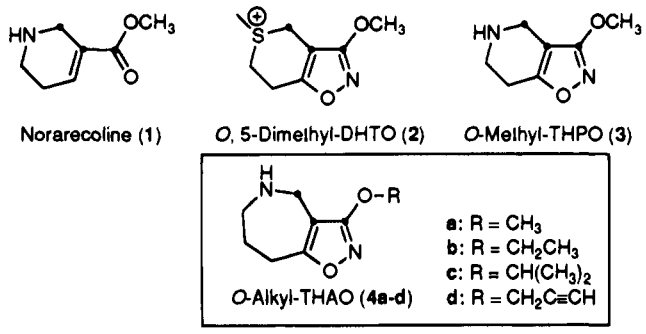
[a] From ref 37. Data represent the mean of three independent experiments, and SEMs were less than 10%.

Table 2. Pharmacological Parameters of Muscarinic Agonists and Antagonists at the Five Cloned Human Muscarinic Receptor Subtypes Determined by Receptor Selection and Amplification Technology (R-SAT)

ligand/receptor	EC_{50} [μM] (% of maximal carbachol response) m1	m2	m3	m4	m5	EC_{50}(m2)/EC_{50}(m1)
carbachol	4.6 ± 0.1 (100%)	0.074 ± 0.005 (100%)	1.8 ± 0.6 (100%)	0.075 ± 0.011 (100%)	0.36 ± 0.04 (100%)	0.016
arecoline[a]	3.2 ± 0.7 (86 ± 3%)	0.025 ± 0.001 (105 ± 0%)	0.34 ± 0.11 (66 ± 9%)	0.13 ± 0.05 (70 ± 3%)	0.60 ± 0.05 (77 ± 2%)	0.078
oxotremorine[a]	0.39 ± 0.13 (75 ± 10%)	0.019 ± 0.010 (105 ± 6%)	0.21 ± 0.06 (66 ± 5%)	0.033 ± 0.014 (102 ± 3%)	0.055 ± 0.001 (74 ± 2%)	0.049
O-methyl-THAO (**4a**)	0.46 ± 0.22 (74 ± 5%)	0.043 ± 0.005 (97 ± 9%)	0.62 ± 0.24 (72 ± 3%)	0.24 ± 0.06 (110 ± 8%)	4.7 ± 0.8 (60 ± 3%)	0.094
O-ethyl-THAO (**4b**)	0.095 ± 0.018 (42 ± 2%)	0.041 ± 0.014 (85 ± 6%)	0.50 ± 0.16 (34 ± 6%)	0.067 ± 0.014 (89 ± 5%)	2.1 ± 0.9[b]	0.43
O-propargyl-THAO (**4d**)	0.14 ± 0.02 (59 ± 9%)	0.0067 ± 0.0028 (86 ± 2%)	0.13 ± 0.02 (70 ± 8%)	0.030 ± 0.006 (105 ± 4%)	0.95 ± 0.40 (36 ± 5%)	0.048
O-isopropyl-THAO (**4c**)	0.17 ± 0.02[c]	nd	0.14 ± 0.04[d]	nd	0.11 ± 0.01[c]	

[a] From ref 27. [b] **4b** was an antagonist at m5. Full inhibition of 1 μM carbachol obtainable. K_i value calculated for IC_{50} by the Cheng–Prusoff equation.[44,47,48] [c] **4c** was an antagonist at m1 and m5. Full inhibition of carbachol not obtainable. K_i values calculated from Schild analysis.[30] Slope not significantly different from 1 ($p < 0.05$). [d] **4c** was an apparent noncompetitive antagonist at m3. Full inhibition of carbachol not obtainable. Schild analysis shows signs of insurmountable antagonism (depression of maximal response at higher antagonist doses). K_i values calculated as described by Kenakin.[49] nd, not determined. Data represent the mean (±SEM) of three or four experiments.

Scheme 1



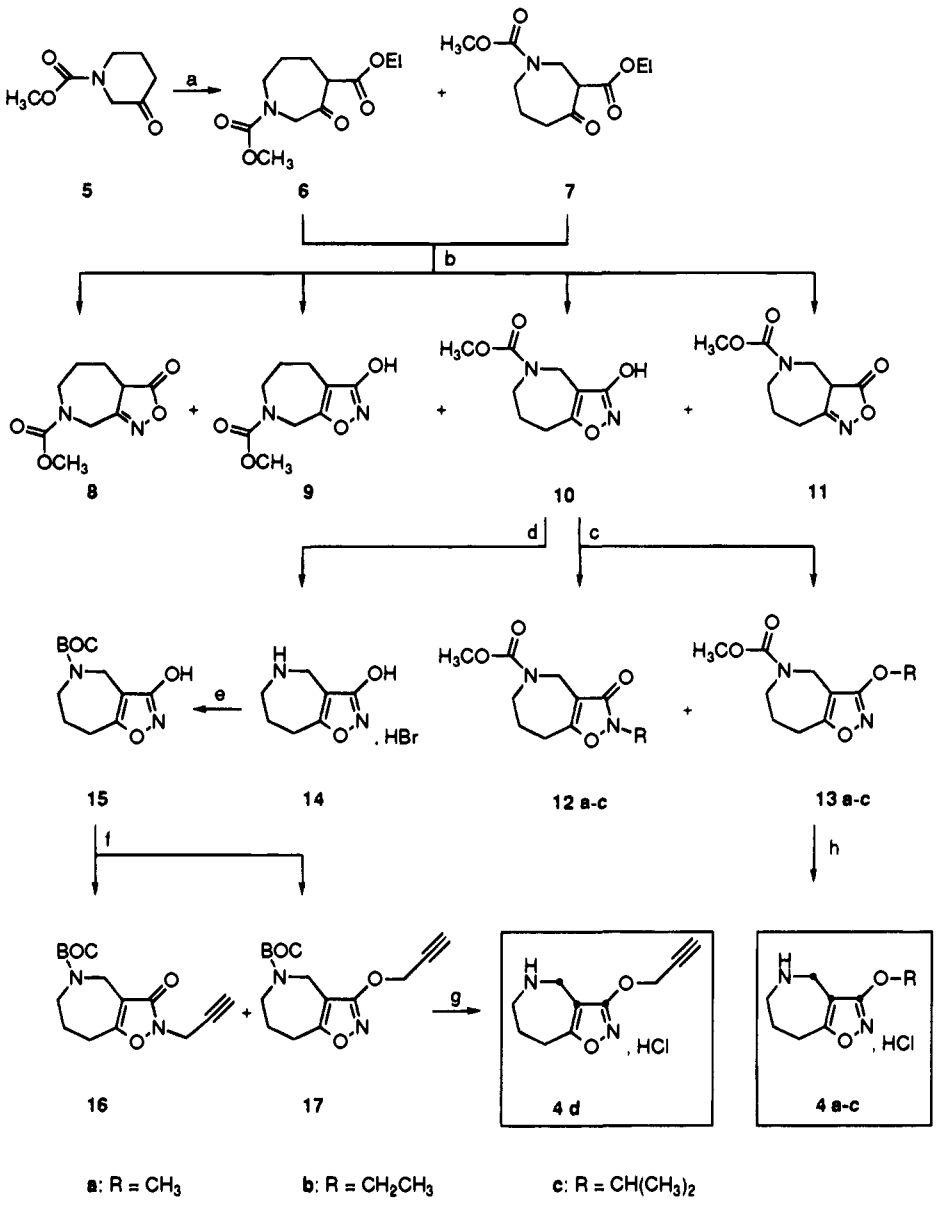
tion of a series of structurally related *O*-alkyl analogs (**4a–d**) of 5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepin-3-ol (THAO) in order to further elucidate the influence of the bioisosteric ester alkyl groups on the pharmacological profile of this class of mAChR ligands. The pharmacology of these compounds was studied in tissue binding experiments and on the five cloned human mAChRs using the functional assay, receptor selection and amplification technology (R-SAT).

Results

Chemistry. A mixture of the isomeric β-oxo esters **6** and **7** (Scheme 2) was synthesized by treatment of the piperidone ring of **5** with ethyl diazoacetate and boron trifluoride etherate as previously described.[23] Attempts to simplify a very time-consuming procedure for the separation of **6** and **7**, involving selective copper chelate formation of **7** and multiple chromatographic procedures,[23] failed. Consequently, the mixture of **6** and **7** was converted into a mixture of the 3-hydroxyisoxazoles, **9** and **10**, and the 2-isoxazolin-5-ones, **8** and **11**, using a published general method.[24] These two pairs of compounds were easily separated by flash chromatography, and subsequently, **9**[25] and **10**[25] were separated chromatographically. The 3-hydroxyisoxazole unit is isosteric with the carboxyl group,[25] and accordingly, alkylations of **10** gave chromatographically separable mixtures of the isomeric *N*-alkylated (**12a–c**) and *O*-alkylated (**13a–c**) products. Deprotection of **13a–c** gave **4a–c** containing hydrolysis-resistant 3-alkoxyisoxazole bioisosteric ester groups (Scheme 2). Deprotection of **10** gave **14** (THAO), which was reprotected to the BOC derivative, **15**. Alkylation of **15** by 2-propynyl bromide gave a separable mixture of **16** and **17**, the latter of which was deprotected to give the target compound, **4d**.

In Vitro Pharmacology. The compounds **4a–d** were studied in receptor binding assays using rat brain and rat heart membranes and tritiated quinuclidinyl benzilate ([^{3}H]QNB), pirenzepine ([^{3}H]PZ), and oxotremorine-M ([^{3}H]Oxo-M) as ligands. On the basis of these binding studies, M_2/M_1 index[22] and agonist index[26,22] values, predictive of M_1 selectivity and relative efficacy, respectively,[22,26] were calculated (Table 1). From these calculations, the compounds under study were predicted to exhibit a rank order of M_1 selectivity of **4c** > **4b** > **4d** > **4a** whereas the predicted order of relative efficacy turned out to be the reverse, namely **4a** > **4d** > **4b** > **4c**. The agonist index values for **4a**, **4b**, and **4d** were consistent with partial agonism, whereas that of **4c** indicated antagonism.

Scheme 2[a]



[a] Reagents: (a) $N_2CHCOOEt$, $BF_3{\cdot}O(CH_2CH_3)_2$; (b) NH_2OH; (c) N_2CH_2 (**a**), CH_3CH_2Br, K_2CO_3 (b), $(CH_3)_2CHBr$, K_2CO_3 (**c**); (d) HBr, HOAc; (e) $(BOC)_2O$, K_2CO_3; (f) $CH{\equiv}CCH_2Br$, K_2CO_3; (g) HCl, EtOAc; (h) NaOH, HCl.

The pharmacological effects of **4a**–**d** were studied using five cloned human mAChRs (m1–m5) and the functional assay, R-SAT.[27-29] R-SAT is based on the observation that phosphatidylinositol-coupled muscarinic receptors (m1, m3, and m5) induce agonist-dependent focus formation in NIH 3T3 cells. In fact, the dose–response relationships of focus formation and phospholipase C stimulation are identical. Unfortunately, the focus assay is labor-intensive and difficult to quantitate. In R-SAT we co-express a convenient marker enzyme (β-galactosidase) with a phosphatidylinositol-coupled receptor. Ligands stimulate proliferation of the cells that express the receptor (and thus the marker enzyme), and thus, ligand-induced effects are read by assay of the marker enzyme (β-galactosidase hydrolysis of *o*-nitrophenyl β-D-galactopyranoside).[27-29] Muscarinic receptors coupled to adenyl cyclase inhibition (m2 and m4) do not induce foci in NIH 3T3 cells when exposed to agonists and thus cannot be measured directly in R-SAT. In stead, we co-express the chimeric G-protein Gq-i5 with the receptor and marker enzyme. This chimeric G-protein allows receptors coupled to adenyl cyclase inhibition to couple to the phosphatidylinositol pathway. In this way m2 and m4 can mediate R-SAT responses.[27-29]

All four compounds were very potent muscarinic ligands, showing EC_{50} or K_i values in the low nanomolar range (Figure 1 and Table 2). At all five receptors, the order of intrinsic activity of the compounds (**4a** > **4d** > **4b** > **4c**) was identical with that predicted on the basis of agonist index values derived from binding studies (Table 1), and it was confirmed that **4a**, **4b**, and **4d** were partial agonists and **4c** an antagonist. The only exception was **4b**, which turned out to be a partial agonist at m1–m4 but an antagonist at m5 (Figure 1). The three partial agonists, **4a**, **4b**, and **4d**, all showed higher intrinsic activity at m2 and m4 than at m1, m3, and m5. This peculiarity of m2 and m4 effects is, at least partially, an inherent result of the R-SAT assay system,

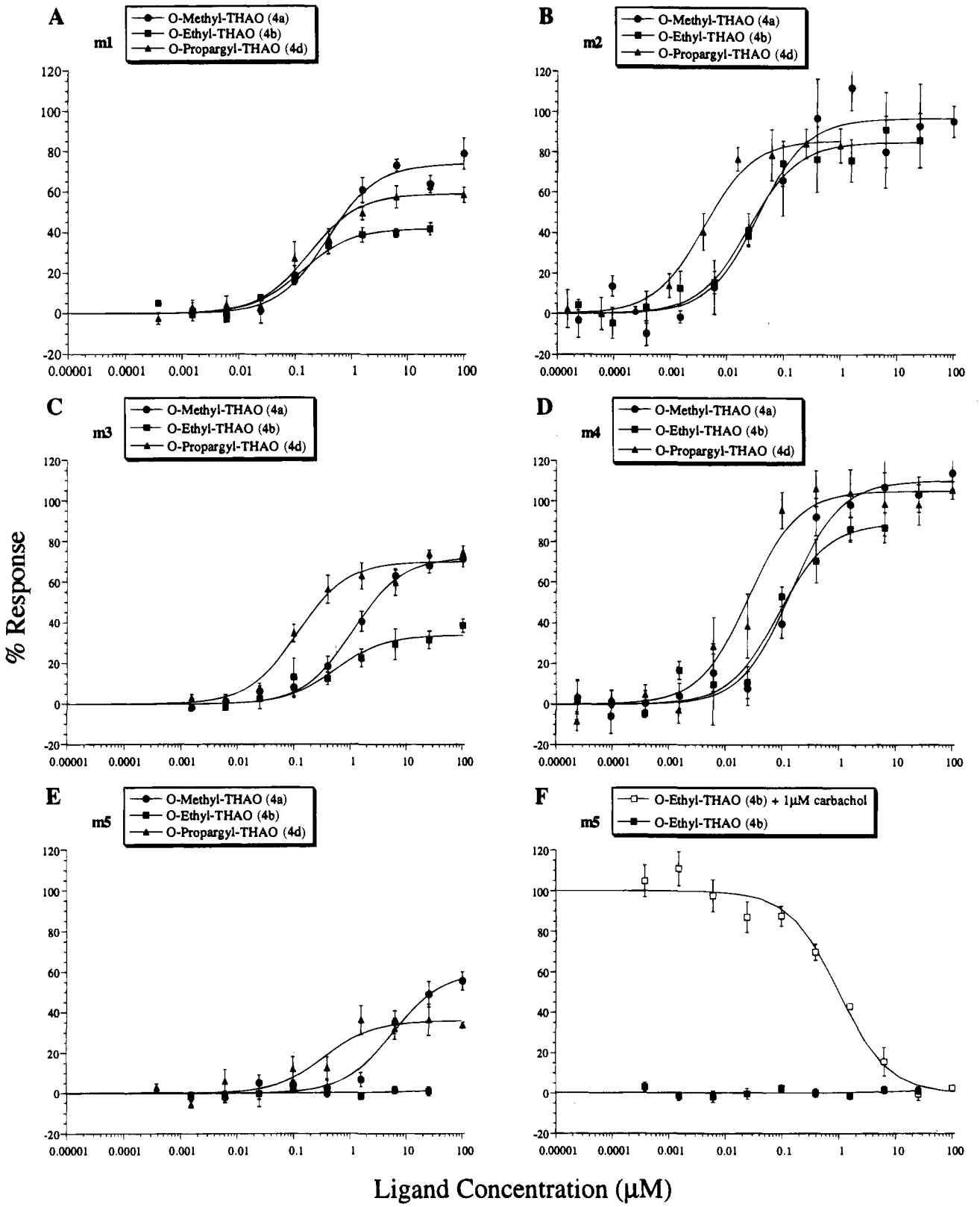


Figure 1. Pharmacological profile of *O*-methyl-THAO (**4a**), *O*-ethyl-THAO (**4b**), and *O*-propargyl-THAO (**4d**) using the cloned human mAChR's as determined by Receptor Selection and Amplification Technology (R-SAT): (A) m1, (B) m2, (C) m3, (D) m4, and (E, F) m5. % response indicates responses relative to maximal responses by the full agonist, carbachol. Each datapoint is the mean value ± SEM of a representative experiment performed in triplicate (m1, m3, and m5) or quadruplicate (m2 and m4).

which shows the same tendency for standard compounds (see Table 2).[27]

Generally, the three partial agonists **4a**, **4b**, and **4d** showed the same order of potency at all five receptor subtypes, **4d** being the most potent and **4a** the least potent compound. The only exception was **4b**, which showed higher potency at the m1 receptor than **4d**. Furthermore, **4a**, **4b**, and **4d** showed the same order of

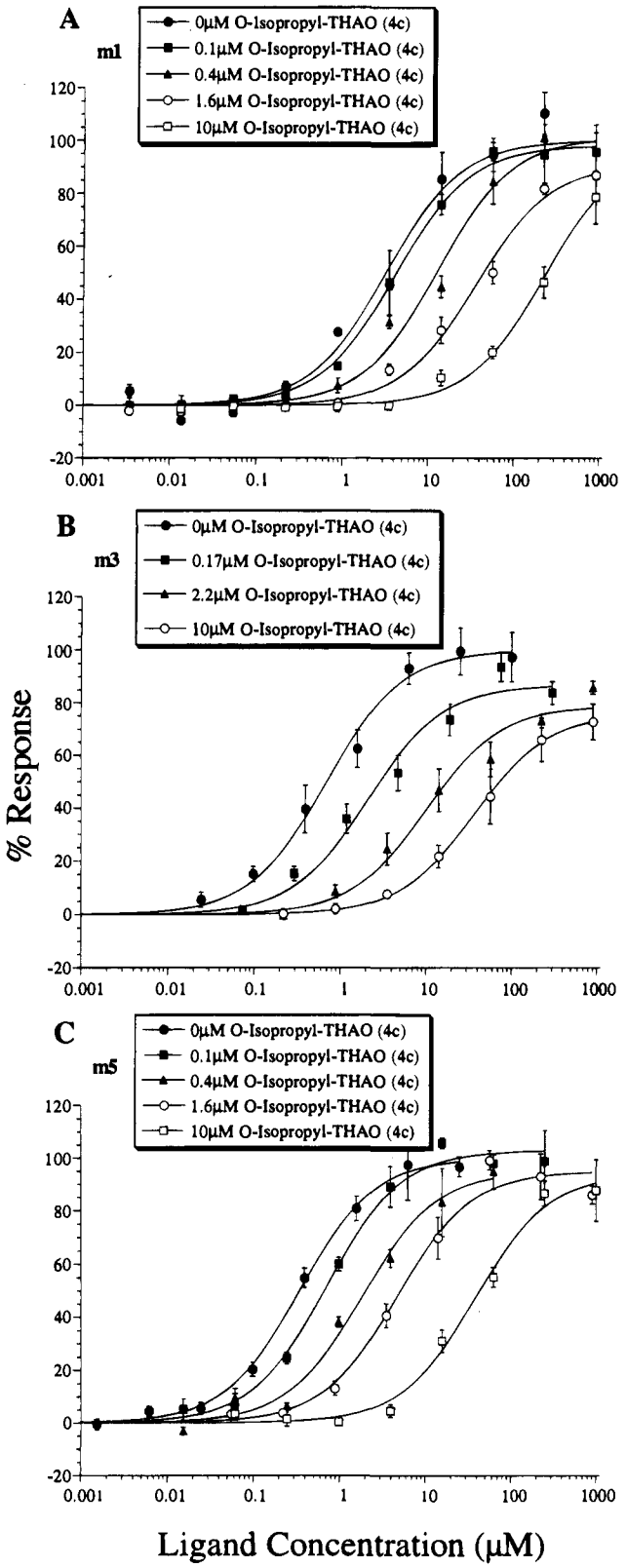


Figure 2. Pharmacological profile of *O*-isopropyl-THAO (**4c**) on the cloned human mAChR's as determined by receptor selection and amplification technology (R-SAT): (A) m1, (B) m3, and (C) m5. At higher doses of *O*-isopropyl-THAO (**4c**), there was a statistically significant ($p < 0.05$) suppression of the maximal response at m3. % response indicates responses relative to maximal responses by the full agonist, carbachol. Each datapoint is the mean value ± SEM of a representative experiment performed in triplicate.

subtype selectivity: m2 > m4 > m1 ≥ m3 > m5 (Table 2). The standard compounds, carbachol, arecoline, and oxotremorine, also were more potent at m2 and m4 than at m1, m3, and m5. Within the series of *O*-alkyl-THAO analogs, **4b** showed the highest degree of m1 selectivity (0.43) (Table 2) as predicted from the binding experiments, where **4b** showed the highest M_2/M_1 index value (0.97) (Table 1).

A striking aspect of the pharmacology of **4a**, **4b**, and **4d**, as compared with standard compounds, is their lack of selectivity at m5. Thus, all other compounds that we have tested so far have been more potent at m5 than at m1,[27] whereas **4a**, **4b**, and **4d** were 10-, 22-, and 7-fold, respectively, more potent at m1 than at m5. Furthermore, **4b** was shown to be a partial agonist at m1 but an antagonist at m5 (Figure 1 and Table 2).

O-Isopropyl-THAO (**4c**) was devoid of agonist effect at m1, m3, or m5 (data not shown). Schild analysis[30] of data from studies of effects of **4c** at m1 and m5 were consistent with **4c** acting as a competitive antagonist, i.e., no suppression of maximal carbachol response and a slope not significantly different from unity (Figure 2). A similar analysis of data from studies using m3 receptors gave a statistically significant suppression of carbachol responses at higher concentrations of **4c** (i.e., maximal response = 76 ± 2% at 2.2 μM **4c**, $n = 3$), thus indicating insurmountable antagonism of **4c** at m3 (Figure 2). As seen in Table 2, **4c** is equipotent at the m1, m3, and m5 receptors.

Discussion

The muscarinic agonist, arecoline, has been shown to improve significantly cognition when administered intravenously to AD patients[31] and to enhance learning in normal young humans and aged nonhuman primates.[32,33] The pharmacological effects of arecoline are short-lived, probably due to hydrolysis of the ester group,[34] and in attempts to develop muscarinic agonists more suitable for pharmacological studies, we have previously described the synthesis and pharmacology of annulated bicyclic analogs of arecoline containing the THPO[20-22] and the DHTO[19] ring systems and the hydrolysis-resistant 3-alkoxyisoxazole unit.[35,36] In order to further elucidate the structure–activity relationships for this particular class of arecoline ester bioisosteres, we have now synthesized and characterized pharmacologically a series of *O*-alkyl analogs of THAO (**4a–d**), which are ring homologs of the *O*-alkyl-THPO analogs (Scheme 1). To get a detailed insight into the molecular pharmacology of these latter compounds, they have been tested in receptor binding assays and in functional test systems using five cloned mAChRs (m1–m5) and using the newly developed R-SAT system.[27-29]

In agreement with the results of earlier conventional pharmacological studies on *O*-alkyl-THPO[21] and *O*-alkyl-DHTO[19] analogs, the relative intrinsic activities of **4a–d** were strictly dependent on the structure of the alkyl groups. Thus, compounds containing *O*-methyl, *O*-propargyl, *O*-ethyl, and *O*-isopropyl groups generally showed a decreasing order of intrinsic activity. Quite surprisingly, however, the potency of the compounds containing the THAO skeleton were typically 1 order of magnitude more potent that the respective analogs containing the THPO or DHTO ring systems. It is possible that the higher lipophilicity and increased level of molecular flexibility of the analogs of THAO are factors of major importance in this regard.[37] In general

agreement with previous studies on THPO and DHTO analogs, *O*-methyl-THAO (**4a**) as well as *O*-propargyl-THAO (**4d**) were partial agonists at m1–m5, the latter being the most potent compound at all five receptor subtypes.

O-Ethyl-THAO (**4b**) was shown to be a partial agonist at m1–m4 but an antagonist at m5 (Figure 1). Furthermore, **4b** is among the most m1-selective muscarinic compound that we have tested so far,[27] making **4b** an interesting lead for further design of partial muscarinic agonists of therapeutic interest in AD or SDAT. In accordance with the finding of *O*-isopropyl-THPO, which is an antagonist in conventional pharmacological test systems,[19,22] *O*-isopropyl-THAO (**4c**) proved to be an antagonist in binding studies and R-SAT. **4c** was equipotent at m1, m3, and m5 but showed signs of insurmountable antagonism only at the m3 receptor. We find this behavior interesting and are currently studying it in further detail.

The present molecular pharmacological studies have emphasized that even minor structural modifications of muscarinic receptor ligands can have dramatic effects on potency and pharmacological profile at mAChR subtypes. Further studies along these lines using potent and conformationally restricted ligands may lead to the development of detailed pharmacophore models for each of the m1–m5 receptors. Hopefully, such studies may shed further light on the mode of interaction between the mAChRs and muscarinic agonists, antagonist structurally related to agonists, and the more bulky classical antagonists. The ultimate goal of such systematic studies is to develop principles for rational design of muscarinic receptor ligands.

Experimental Section

Chemistry. Melting points were determined in capillary tubes and are uncorrected. Column chromatography (CC) was performed on silica gel 60 (70–230 mesh, ASTM, Merck). ^{1}H NMR spectra were recorded on a Varian EM-360 spectrometer. Chemical shifts are in parts per million with respect to TMS. The pH-stat consisted of a Radiometer PHM 82 pH-meter equipped with a TTT80 titrator. Elemental analyses were performed by Mr. G. Cornali, Microanalytical Laboratory, Leo Pharmaceutical Products, Denmark, and were within ±0.4% of the calculated values. Evaporations were performed under vacuum on a rotary evaporator at 15 mmHg. Compounds containing the 3-hydroxyisoxazole unit were visualized on TLC plates using UV light and a $FeCl_3$ spraying reagent. Compounds containing amino groups were visualized using a ninhydrin spraying reagent.

Ethyl (*RS*)-1-(Methoxycarbonyl)-3-oxoperhydroazepine-4-carboxylate (6) and Ethyl (*RS*)-1-(Methoxycarbonyl)-4-oxoperhydroazepine-3-carboxylate (7). A mixture of **6** and **7** was prepared as described earlier,[23] but the compounds were not separated.

Methyl 3-Hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine-5-carboxylate (10). A solution of hydroxylammonium chloride (1.14 g, 16.4 mmol) in 1 M NaOH (10 mL) was cooled to 0 °C, and the pH was adjusted to 10 by addition of 1 M NaOH. The solution was vigorously stirred while a mixture of **6** and **7** (4.0 g, 16.4 mmol) was added during 30 min, keeping the pH at 10 ± 0.2 by means of a pH-state loaded with 1 M NaOH. Stirring was continued for 30 min, and the mixture was poured into ice-cooled concentrated HCl (80 mL). The mixture was stirred overnight and evaporated. The residue was extracted with EtOAc (3 × 75 mL), and the combined extracts were evaporated. The oily residue was shown by TLC [eluent: toluene–EtOAc (1:1) containing 1% HOAc], spraying with $FeCl_3$ (**9** and **10** yellow dots; **8** and **11** brown dots), and using reference compounds[25] to be a mixture of **8**–**11**. The mixture was purified by CC [eluent: toluene containing EtOAc (0–25%) and HOAc (1%)]. The first compound eluted was **9** followed by **10**, **8**, and **11**, respectively. Only fractions containing **10** were collected and evaporated. Crude **10** was recrystallized from toluene–petroleum ether to give **10** (1.08 g, 31%): mp 144.5–145.5 °C. The IR and ^{1}H NMR spectra were identical with those of compound **10** prepared by the earlier published route.[25]

Methyl 3-Methoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine-5-carboxylate (13a). To a solution of **10** (490 mg, 2.31 mmol) in ether (20 mL) was added an excess of diazomethane. The mixture was stirred at room temperature for 1 h, and the excess of diazomethane was destroyed by addition of HOAc. The mixture containing **12a** and **13a** was evaporated and the residue purified by CC (eluent: toluene–EtOAc containing 1% of HOAc). Only fractions containing **13a** were collected and evaporated. The resulting oil was crystallized from toluene–petroleum ether to give 270 mg (52%) of **13a**: mp 68–70 °C; ^{1}H NMR ($CDCl_3$) δ 1.90 (2 H, m), 2.85 (2 H, t), 3.55 (2 H, m), 3.65 (3 H, s), 3.95 (3 H, s), 4.25 (2 H, s). Anal. ($C_{10}H_{14}N_2O_4$) C, H, N.

Methyl 3-Ethoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine-5-carboxylate (13b). To a solution of **10** (3.1 g, 14.6 mmol) in acetone (150 mL) was added K_2CO_3 (5.02 g, 36.5 mmol). The suspension was stirred at 50 °C for 1 h, and ethyl bromide (3.3 mL, 43.8 mmol) was added. The mixture was refluxed for 16 h and filtered. The filtrate was evaporated, and water (75 mL) was added. After stirring, the crystalline compound formed was collected and recrystallized from ether–petroleum ether to give 2.28 g of **13b** (65%): mp 52–53 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (3 H, t), 1.90 (2 H, m), 2.85 (2 H, t), 3.60 (2 H, m), 3.65 (3 H, s), 4.20 (2 H, s), 4.30 (2 H, q). Anal. ($C_{11}H_{16}N_2O_4$) C, H, N.

Methyl 3-Isopropoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo-[4,5-*c*]azepine-5-carboxylate (13c). The compound was prepared from **10** and isopropyl bromide using a procedure analogous with that described for the synthesis of **13b**. Compound **13c** was isolated as an oil in 57% yield: ^{1}H NMR ($CDCl_3$) δ 1.35 (6 H, d), 1.95 (2 H, m), 2.80 (2 H, m), 3.65 (2 H, m), 3.70 (3 H, s), 4.25 (2 H, s), 4.85 (1 H, m). Anal. ($C_{12}H_{18}N_2O_4$) C, H, N.

General Procedure for the Syntheses of 3-Alkoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepinium Chlorides 4a–c. To a solution of NaOH (20 mmol) in MeOH (5 mL) and water (1 mL) was added **13a**, **13b**, or **13c** (2 mmol), and the mixture was refluxed for 24 h. The reaction mixture was evaporated and the residue dissolved in water (10 mL). Extraction with CH_2Cl_2 (3 × 20 mL), drying, and evaporation of the organic phase gave an oil. The oil was dissolved in EtOH, and an excess of 4 M HCl was added. The mixture was evaporated and the residue recrystallized.

3-Methoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepinium chloride (4a): from MeOH–ether; yield 32%; mp 215–217 °C; ^{1}H NMR (D_2O) δ 2.05 (2 H, m), 2.95 (2 H, m), 3.55 (2 H, m), 3.95 (3 H, s), 4.05 (2 H, s). Anal. ($C_8H_{12}N_2O_2$·HCl) C, H, N, Cl.

3-Ethoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepinium chloride (4b): from acetonitrile; yield 84%: mp 202–203 °C; ^{1}H NMR (D_2O) δ 1.35 (3 H, t), 2.15 (2 H, m), 3.00 (2 H, m), 3.50 (2 H, m), 4.10 (2 H, s), 4.30 (2 H, q). Anal. ($C_9H_{14}N_2O_2$·HCl) C, H, N, Cl.

3-Isopropoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepinium chloride (4c): from acetonitrile; yield 37%: mp 218–220 °C; ^{1}H NMR (D_2O) δ 1.35 (6 H, d), 2.10 (2 H, m), 2.95 (2 H, m), 3.55 (2 H, m), 4.10 (2 H, s), 4.80 (1 H, m). Anal. ($C_{10}H_{16}N_2O_2$·HCl) C, H, N, Cl.

***tert*-Butyl 3-Hydroxy-5,6,7,8-tetrahydro-4*H*-isoxazolo-[4,5-*c*]azepine-5-carboxylate (15).** To a solution of 14[25] (1.38 g, 5.87 mmol) and K_2CO_3 (1.62 g, 11.7 mmol) in water (20 mL) was added a solution of di-*tert*-butyl dicarbonate (1.54 g, 7.06 mmol) in THF (20 mL), and the mixture was stirred at room temperature for 3 h. The reaction mixture was evaporated and the residue dissolved in water (20 mL). EtOAc (100 mL) was added, and the mixture was cooled in an ice bath. The mixture was acidified with 2 M HCl to pH 6 and subsequently to pH 3 with $KHSO_4$. The phases were sepa-

rated, and the aqueous phase was extracted with EtOAc (2 × 50 mL). The combined extracts were dried ($MgSO_4$) and evaporated. The residue was recrystallized from toluene–petroleum ether to give 887 mg of **15** (61%): mp 170–171 °C. Anal. ($C_{12}H_{18}N_2O_4$) C, H, N.

***tert*-Butyl 3-(2-Propynyloxy)-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine-5-carboxylate (17).** The compound was synthesized from **15** and 2-propynyl bromide using a procedure analogous with that described for the synthesis of **13b**. The reaction mixture containing **16** and **17** was separated using CC [eluent: toluene–EtOAc (3:1)]. Only fractions containing **17** were collected. Compound **17** was isolated as an oil in a yield of 58%: ^{1}H NMR ($CDCl_3$) δ 1.40 (9 H, s), 1.85 (2 H, m), 2.55 (1 H, t), 2.85 (2 H, t), 3.60 (2 H, m), 4.20 (2 H, br s), 4.85 (2 H, d). Anal. ($C_{15}H_{20}N_2O_4$) C, H, N.

3-(2-Propynyloxy)-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepinium Chloride (4d). To a solution of **17** (530 mg, 1.81 mmol) in EtOAc (10 mL) was added a 1 M solution of HCl in EtOAc (10 mL). The mixture was stirred at room temperature for 48 h. The precipitate was collected and recrystallized from acetonitrile–ether to give 267 mg (64%) of **4d**: mp 173–175 °C; ^{1}H NMR (D_2O) δ 2.15 (2 H, m), 3.05 (3 H, m), 3.60 (2 H, m), 4.15 (2 H, s), 4.95 (2 H, d). Anal. ($C_{10}H_{12}N_2O_2$·HCl) C, H, N, Cl.

Inhibition of Muscarinic Receptor Ligand Binding. [^{3}H]QNB binding to muscarinic receptor sites in rat cortical membranes was performed essentially as described by Watson *et al.*[38] Briefly, rat cortical membranes were homogenized in 100 vol (w/v) of ice-cold 10 mM sodium potassium phosphate buffer (pH 7.4) and diluted 1:10 with the same buffer. Aliquots (0.5 mg of tissue) were incubated with 0.12 nM [^{3}H]QNB (Amersham) alone or in the presence of test compounds in a total volume of 2.7 mL for 30 min at 37 °C. The reaction was stopped by adding ice-cold buffer and filtration through 0.1% poly(ethylenimine) presoaked Whatman GF/B filters by using a Brandel cell harvester. Each compound was tested in three different concentrations in triplicate in two independent experiments. Nonspecific binding was determined in the presence of 20 μM atropine.

The procedure for determinations of inhibition of [^{3}H]QNB binding to rat heart tissue was the same as that described above with the exception that the tissue was homogenized in an Ultraturax homogenizer followed by filtration through cheesecloth and that 4 mg of tissue was used per assay.

The inhibition of [^{3}H]PZ binding to rat cortical membranes was determined by a procedure analogous to that described above for [^{3}H]QNB binding to such membrane fractions: 3 mg of tissue were incubated with 1.0 nM [^{3}H]PZ (DuPont NEN) at 25 °C for 60 min in a total volume of 1.5 mL. Nonspecific binding was estimated in the presence of 10 μM atropine.

The procedure for determination of inhibition of [^{3}H]Oxo-M binding to rat cortical membranes was analogous to that described above for [^{3}H]QNB binding to such membrane fractions: 5 mg of tissue were incubated with 0.2 nM [^{3}H]Oxo-M (DuPont NEN) at 30 °C for 40 min in a total volume of 1.5 mL of buffer. Nonspecific binding was determined in the presence of 10 μM atropine.

Receptor Selection and Amplification Technology. R-SAT was performed as described earlier.[27–29] NIH 3T3 cells (ATCC no. CRL 1658) were grown in a 37 °C humidified CO_2 atmosphere in Dulbecco's Modified Eagle's Media supplemented with 4500 mg/L glucose, 862 mg/L L-alanyl-L-glutamine, 50 units/mL penicillin G, 50 units/mL streptomycin (Gibco, Paisley, Scotland), and 10% calf serum (HyClone, UT).

One day prior to transfection, cells were plated in a density of 2 × 10^6 cells per 15 cm of tissue culture dish.

Cells were transfected by calcium phosphate–DNA precipitation as described by Wigler *et al.*[39] using 10 μg of receptor DNA,[40,41] 10 μg of p-SV-β-galactosidase DNA (Promega, WI), and 40 μg of salmon sperm DNA (Sigma, MO). Cells transfected with m2 or m4 receptor DNA were further cotransfected with 10 μg of the chimeric G-protein, Gq-i5.[42]

Media was exchanged the day after transfection. Two days after transfection, cells were split into two 96-well plates, and ligands were added into a total volume of 200 μL/well.

After four (m1, m3, and m5) or five (m2 and m4) days of ligand incubation, the rate of β-galactosidase induction was measured as described by Lim and Chae.[43] Each well was incubated in 200 μL of PBS containing 3.5 mM *o*-nitrophenyl β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma, MO). After 16–24 h the plates were read at 420 nm on a plate reader (Molecular Devices).

Data Analysis. In binding experiments the inhibitory constants (K_i's) were calculated from the Cheng–Prusoff equation.[44]

$$K_i = \frac{IC_{50}}{(1 + ([^3H]\text{ligand}/K_d))}$$

where the IC_{50} values were determined by conventional methods and K_d values were derived from Scatchard analyses following procedures analogous with those determined earlier.[38]

In analogy with a published method for estimation of muscarinic agonist efficacy,[26] the ratio between the K_i values of the compounds determined in [^{3}H]QNB (brain) and [^{3}H]Oxo-M (brain) binding experiments was used as a muscarinic agonist index of the compounds. According to Freedman *et al.*,[26] values of this index above 4000 predict full agonism, values below 10 predict antagonism, and values in between predict partial agonism of muscarinic compounds:

$$\text{agonist index} = \frac{K_i(\text{QNB, brain})}{K_i(\text{Oxo-M, brain})} = \frac{IC_{50}(\text{QNB, brain})}{IC_{50}(\text{Oxo-M, brain})} \times 0.162$$

The ratio between K_i values of a compound determined in QNB (heart) and PZ (brain) binding experiments was used as an index of M_1 selectivity (M_2/M_1 index), higher values of this index indicating higher degrees of M_1 selectivity.[22]

$$M_2/M_1 \text{ index} = \frac{K_i(\text{QNB, heart})}{K_i(\text{PZ, brain})} = \frac{IC_{50}(\text{QNB, heart})}{IC_{50}(\text{PZ, brain})} \times 0.125$$

Agonist data from R-SAT experiments were fitted to the four parameter equation:[45,46]

$$R = \frac{D + (A - D)}{1 + (x/c)^b}$$

For agonists: A = minimum response, D = maximum response, and c = EC_{50}. For antagonists: A = maximum response, D = minimum response, and c = IC_{50} (R = response, x = ligand concentration, and b = slope at EC_{50} or IC_{50}). The b value was set to unity.

K_i values were calculated from IC_{50} values by the method of Cheng and Prusoff[44] as described by McKinney *et al.*[47] and Craig.[48]

Antagonists that were unable to fully inhibit a carbachol response were subjected to Schild analysis.[30] Schild analysis is invalid if the maximal agonist response is suppressed by the addition of antagonist, which is a sign of noncompetitive antagonism. Noncompetitive antagonists were analyzed as described by Kenakin.[49]

Curves were generated by nonweighted least-squares fits using the program KaleidaGraph 2.1 (Abelbeck Software) for the Macintosh computer. Statistical significance was evaluated by *t*-test using the program StatView 1.0 (Abacus Concepts) for the Macintosh computer. $p \leq 0.05$ was considered statistical significant.

Acknowledgment. This work was supported by grants from the Danish Medical and Technical Research Councils and the Lundbeck Foundation. The secretarial assistance of Mrs. A. Nordly and Ms. D. Persson and the technical assistance of Mrs. A. Kristensen and Mrs. T. Lindgreen are gratefully acknowledged. Finally, we wish to acknowledge Dr. Bruce R. Conklin for providing the Gq-i5 construct.

References

(1) Bartus, R. T.; Dean, R. L., III; Beer, B.; Lippa, A. S. The cholinergic hypothesis of geriatric memory dysfunction. *Science* **1982**, *217*, 408–417.

(2) Perry, E. Acetylcholine and Alzheimer's disease. *Br. J. Psychiat.* **1988**, *152*, 737–740.

(3) Smith, C. J.; Perry, E. K.; Perry, R. H.; Candy, J. M.; Johnson, M.; Bonham, J. R.; Dick, D. J.; Fairbairn, A.; Blessed, G.; Birdsall, N. J. M. Muscarinic cholinergic subtypes in hippocampus in human cognitive disorders. *J. Neurochem.* **1988**, *50*, 847–856.

(4) Wurtman, R. J., Corkin, S., Growdon, J. H., Ritter-Walker, E., Eds. *Advances in Neurology, Vol. 51. Alzheimer's Disease*; Raven Press: New York, 1990.

(5) Coyle, J. T.; Price, D. L.; DeLong, M. R. Alzheimer's disease: A disorder of cholinergic innervation. *Science* **1983**, *219*, 1184–1190.

(6) Bartus, R. T.; Flicker, C.; Dean, R. L., III; Fisher, S.; Pontecorvo, M.; Figueiredo, J. Behavioral and biochemical effects of nucleus basalis magnocellularis lesions: implications and possible relevance to understanding or treating Alzheimer's disease. *Prog. Brain Res.* **1986**, *70*, 345–361.

(7) Perry, E. K.; Tomlinson, B. E.; Blessed, G.; Bergmann, K.; Gibson, P. H.; Perry, R. H. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *Br. Med. J.* **1978**, *ii*, 1457–1459.

(8) Mash, D.; Flynn, D. D.; Potter, L. T. Loss of M2 muscarinic receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. *Science* **1985**, *228*, 1115–1117.

(9) Aubert, I.; Araujo, D. M.; Cecyre, D.; Robitaille, Y.; Gauthier, S.; Quirion, R. Comparative alterations of nicotinic and muscarinic binding sites in Alzheimer's and Parkinson's diseases. *J. Neurochem.* **1992**, *58*, 529–541.

(10) Levey, A. I.; Kitt, C. A.; Simonds, W. F.; Price, D. L.; Brann, M. R. Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J. Neurosci.* **1991**, *11*, 3218–3226.

(11) Wall, S. J.; Yasuda, R. P.; Hory, F.; Flagg, S.; Martin, B. M.; Ginns, E. I.; Wolfe, B. B. Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. *Mol. Pharmacol.* **1991**, *39*, 643–649.

(12) Wall, S. J.; Yasuda, R. P.; Li, M.; Ciesla, W.; Wolfe, B. B. The ontogeny of m1–m5 muscarinic receptor subtypes in rat forebrain. *Dev. Brain Res.* **1992**, *66*, 181–185.

(13) Pomara, M.; Bagne, C. A.; Stanley, M.; Yarbrough, G. G. Prospective strategies for cholinergic interventions in Alzheimer's disease. *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* **1986**, *10*, 553–569.

(14) Mitchelson, F. Muscarinic receptor differentiation. *Pharmacol. Ther.* **1988**, *37*, 357–423.

(15) Whitehouse, P. J. Cholinergic therapy in dementia. *Acta Neurol. Scand.* **1993**, Suppl. 149, 42–45.

(16) Hu, J.; Wang, S. Z.; el-Fakahany, E. E. Effects of agonist efficacy on desensitization of phosphoinositide hydrolysis mediated by m1 and m3 muscarinic receptors expressed in chinese hamster ovary cells. *J. Pharmacol. Exp. Ther.* **1991**, *257*, 938–945.

(17) Pontzer, N. J.; Crews, F. T. Densensitization of muscarinic stimulated hippocampal cell firing is related to phosphoinositide hydrolysis and inhibited by lithium. *J. Pharmacol. Exp. Ther.* **1990**, *253*, 921–929.

(18) Thompson, A. K.; Fisher, S. K. Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J. Pharmacol. Exp. Ther.* **1990**, *252*, 744–752.

(19) Sauerberg, P.; Falch, E.; Meier, E.; Lembøl, H. L.; Krogsgaard-Larsen, P. Heterocyclic muscarinic agonists. Synthesis and biological activity of some bicyclic sulfonium arecoline bioisosteres. *J. Med. Chem.* **1988**, *31*, 1312–1316.

(20) Sauerberg, P.; Larsen, J. J.; Falch, E.; Krogsgaard-Larsen, P. A novel class of conformationally restricted heterocyclic muscarinic agonists. *J. Med. Chem.* **1986**, *29*, 1004–1009.

(21) Sauerberg, P.; Fjalland, B.; Larsen, J. J.; Bach-Lauritsen, T.; Falch, E.; Krogsgaard-Larsen, P. Pharmacological profile of a novel class of muscarinic acetylcholine receptor agonists. *Eur. J. Pharmacol.* **1986**, *130*, 125–131.

(22) Krogsgaard-Larsen, P.; Falch, E.; Sauerberg, P.; Freedman, S. B.; Lembøl, H. L.; Meier, E. Bioisosteres of arecoline as novel CNS-active muscarinic agonists. In *Subtypes of Muscarinic Receptors III*; Levine, R. R., Birdsall, N. J. M., North, R. A., Holman, M., Watanabe, A., Iversen, L. L., Eds.; Elsevier: Amsterdam, 1988; pp 69–74.

(23) Krogsgaard-Larsen, P.; Hjeds, H. Synthesis of 1-acyl-3-piperidones and ring expansion of methyl 3-oxopiperidine-1-carboxylate with ethyl diazoacetate. *Acta Chem. Scand.* **1976**, *B30*, 884–888.

(24) Jacobsen, N.; Kolind-Andersen, H.; Christensen, J. Synthesis of 3-isoxazolols revisited. Diketene and β-ketoesters as starting materials. *Can. J. Chem.* **1984**, *62*, 1940–1944.

(25) Krogsgaard-Larsen, P. Muscimol analogues. II. Synthesis of some bicyclic 3-isoxazolol zwitterions. *Acta Chem. Scand.* **1977**, *B31*, 584–588.

(26) Freedman, S. B.; Harley, E. A.; Iversen, L. L. Relative affinities of drugs acting at cholinoceptors in displacing agonist and antagonist radioligands: the NMS/Oxo-M ratio as an index of efficacy at cortical muscarinic receptors. *Br. J. Pharmacol.* 1988, *93*, 437–445.

(27) Bräuner-Osborne, H.; Brann, M. R. Pharmacology of muscarinic acetylcholine receptor subtypes (m1–m5): High throughput assays in mammalian cells. Submitted.

(28) Burstein, E. S.; Spalding, T. A.; Hill-Eubanks, D.; Brann, M. R. Structure-function of muscarinic receptor coupling to G proteins. *J. Biol. Sci.* **1995**, *270*, 3141–3146.

(29) Burstein, E. S.; Spalding, T. A.; Bräuner-Osborne, H.; Brann, M. R. Constitutive activation of muscarinic receptors by the G-protein Gq. *FEBS Lett.* In press.

(30) Arunlakshana, O.; Schild, H. O. Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.* **1959**, *14*, 48–58.

(31) Christie, J. E.; Shering, A.; Ferguson, J.; Glen, A. I. M. Physostigmine and arecoline: effects of intravenous infusions in Alzheimer presenile dementia. *Br. J. Psychiat.* **1981**, *138*, 46–50.

(32) Sitaram, N.; Weingartner, H.; Gillin, J. C. Human serial learning: enhancement with arecoline and choline impairment with scopolamine. *Science* **1978**, *201*, 274–276.

(33) Bartus, R. T.; Dean, R. L.; Beer, B. Memory deficits in aged cebus monkeys and facilitation with central cholinomimetics. *Neurobiol. Aging* **1980**, *1*, 145–152.

(34) Nieschulz, O.; Schmersahl, P. On the pharmacology of the active principle of betel. Part 2: Transformation of arecoline to arecaidine. *Arzneim.-Forsch.* **1968**, *18*, 222–225.

(35) Krogsgaard-Larsen, P.; Johansen, J. S.; Falch, E. Deuterium labelling of the GABA agonists THIP, piperidine-4-sulphonic acid and the GABA uptake inhibitor THPO. *J. Labelled Compd.* **1982**, *19*, 689–702.

(36) Bowden, K.; Crank, G.; Ross, W. J. The synthesis of pantherine and related compounds. *J. Chem. Soc. C* **1968**, 172–185.

(37) Lagersted, A.; Falch, E.; Ebert, B.; Krogsgaard-Larsen, P. Conformational aspects of the muscarinic receptor interactions of bicyclic isoxazole ester bioisosteres of arecoline. *Drug Design Disc.* **1993**, *9*, 237–250.

(38) Watson, M.; Yamamura, H. I.; Roeske, W. R. A unique regulatory profile and regional distribution of [3H]-pirenzepine binding in the rat provide evidence for distinct M1 and M2 muscarinic receptor subtypes. *Life Sci.* **1983**, *32*, 3001–3011.

(39) Wigler, M.; Silverstein, S.; Lee, L.-S.; Pellicer, A.; Cheng, Y.-C.; Axel, R. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **1977**, *11*, 223–232.

(40) Bonner, T. I.; Buckley, N. J.; Young, A. C.; Brann, M. R. Identification of a family of muscarinic acetylcholine receptor genes. *Science* **1987**, *237*, 527–532.

(41) Bonner, T. I.; Young, A. C.; Brann, M. R.; Buckley, N. J. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1988**, *1*, 403–410.

(42) Conklin, B. R.; Farfel, Z.; Lustig, K. D.; Julius, D.; Bourne, H. R. Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. *Nature* **1993**, *363*, 274–276.

(43) Lim, K.; Chae, C.-B. A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase. *BioTechniques* **1989**, *7*, 576–579.

(44) Cheng, Y.-C.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(45) Healy, M. J. R. Statistical analysis of radioimmunoassay data. *Biochem. J.* **1972**, *130*, 207–210.

(46) Rodbard, D.; Hutt, D. M. Statistical analysis of radioimmunoassays and immunoradiometric (labelled antibody) assays. In *Radioimmunoassay and Related Procedures in Medicine*; International Atomic Energy Agency: Vienna, 1974; Vol. 1, pp 165–192.

(47) McKinney, M.; Anderson, D. J.; Vella-Rountree, L.; Connolly, T.; Miller, J. H. Pharmacological profiles for rat cortical M1 and M2 muscarinic receptors using selective antagonists: comparison with N1E-115 muscarinic receptors. *J. Pharmacol. Exp. Ther.* **1991**, *257*, 1121–1129.

(48) Craig, D. A. The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharmacol. Sci.* **1993**, *14*, 89–91.

(49) Kenakin, T. Allotopic, noncompetitive, and irreversible antagonism. In *Pharmacologic Analysis of Drug-Receptor Interaction*, 2nd ed.; Raven Press: New York, 1993; pp 323–343.

JM950040R