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Synthesis and Muscarinic M_3 Pharmacological Activities of 1-Azabicyclo[2.2.2]octan-3-one Oxime Derivatives

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Abstract—A series of 1-azabicyclo[2.2.2]octan-3-one oximes and related 1-azabicyclo-[2.2.2]-octan-3-one hydroxylamines were synthesized and tested for muscarinic M_3 activity. All compounds showed at least some muscarinic binding properties, however, only one member of the series demonstrated mucarinic M_3 agonistic properties in vitro (contraction of guinea pig ileum) and in vivo (mydriasis, salivation). In addition, this compound partially reversed the cognitive deficit induced by central cholinergic depletion in two procedures testing memory in the rat, namely the delayed matching to position and swim maze tasks of spatial memory in the rat.

Introduction

Cholinergic deficits are thought to underlay some of the cognitive disturbances observed in patients suffering from Alzheimer's disease.[1] Degeneration of cholinergic neurons in the cortex of patients with Alzheimer's disease correlates with the reduction in mnemonic ability.[2]

Further studies have revealed a greater loss of presynaptic than postsynaptic receptors.[3,4] Replacement therapies for the loss of cholinergic input have concentrated on agonists that directly stimulate the surviving postsynaptic receptors and cholinesterase inhibitors that increase the efficacy of the remaining cholinergic neurons.[5–9]

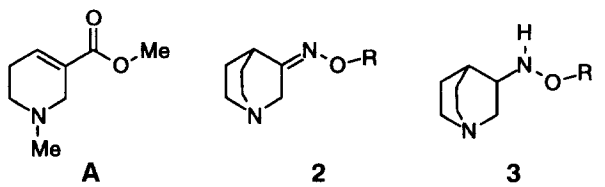
The search for cholinergic agonists has focused on ligands selective for M_1 receptors partly because they are present in large numbers in relevant brain structures[10] and partly because the other known muscarinic receptors are associated with strong peripheral effects. For example, stimulation of the M_2 receptors interferes with cardiovascular function and stimulation of the cholinergic M_3 receptors causes a number of smooth muscle effects. Recently we have observed that ligands for M_3 receptors appear to have much greater effects on CNS function[11] than their low density in the CNS might suggest.[10] Therefore, we have attempted to synthesize selective ligands for the M_3 receptor subtype, with the aim of identifying its relative importance in the CNS and the periphery using a number of in vitro and in vivo tests thought to measure M_3 functional activity and effects on memory. The present report details the effects of one series of compounds synthesized within this exploratory program.

Chemistry

A useful starting point for the design of the title series was provided by the consideration of the muscarinic activity of arecoline (**A**). The therapeutic benefit of this compound is rather limited because of its short duration of action due to fast hydrolysis of the ester function. In this paper we describe the bioisosteric replacement of arecoline (**A**) by 1-azabicyclo-[2.2.2]octan-3-one oxime derivatives which contain a hydrolysis resistant oxime function, namely **2**, (Fig. 1, Table 1), which overcomes this disadvantage. A similar chemical series has been described,[12,13] however, no M_3 selectivity was reported in the pharmacological profile of these compounds.

The general synthetic route for the preparation of the compounds is described in Scheme 1 (some analytical data are given in Table 1).

The 1-azabicyclo[2.2.2]octan-3-one oximes (**2**) were prepared in high yield by condensation of 1-azabicyclo[2.2.2]octan-3-one **1** with various hydroxyl amine derivatives. As far as the stereochemistry of the reaction is concerned, it is noteworthy that in this condensation only one of the two possible stereoisomers of the oxime is produced. This result can be rationalized by the assumption that the sterically



A **2** **3**

Figure 1.

Table 1. Analytical data

Compound	R	Molecular formula[a]	M W	mp [°C]
2a	H	$C_7H_{12}N_2O$, HCl	176.65	234
2b	CH_3	$C_8H_{14}N_2O$, HCl	190.67	216
2c	CH_2CH_3	$C_9H_{16}N_2O$, HCl	204.70	188
2d	$CH_2CH_2CH_3$	$C_{10}H_{18}N_2O$, HCl	218.73	128
2e	$CH(CH_3)_2$	$C_{10}H_{18}N_2O$, HCl	218.73	192
2f	$CH_2CH{=}CH_2$	$C_{10}H_{16}N_2O$, HCl	216.71	144
2g	$CH_2C{\equiv}CH$	$C_{10}H_{14}N_2O$, HCl	214.70	183
2h	$CH_2C_6H_5$	$C_{14}H_{18}N_2O$, HCl	266.77	215
2i	C_6H_5	$C_{13}H_{16}N_2O$, HCl	252.75	168
3a	H	$C_7H_{14}N_2O$, 2HCl	215.12	225
3b	CH_3	$C_8H_{16}N_2O$, 2HCl	229.15	205

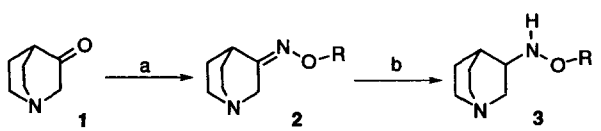
*Compounds gave satisfactory microanalyses for C, H, and N and satisfactory *m/z* data, respectively.

favored Z-isomers are formed selectively under the conditions used for this reaction. As an example, the stereochemical structure of one of the compounds (**2** R = Me) was proven unequivocally by single X-ray analysis[14] as being the Z-isomer. In order to investigate the scope and limitation for binding affinity to muscarinic receptors of compounds closely related to **2** the synthesis and testing of the derivatives **3** was planned.

Thus, reduction of two of the oximes **2** using TMA·BH_3 in a 3 N solution of HCl in methanol gave access to the corresponding hydroxylamine derivatives **3** in good yield.

Results

Following the primary screening program, all the compounds listed in Table 1 were submitted to a battery of tests to assess their affinity for muscarinic receptors (see Table 2). The binding studies suggested that one derivative (compound **2b**) may have agonistic potential. Previous reports have suggested that a large separation between agonist and antagonist binding values indicates potential agonistic properties.[15] From this series only one compound showed agonistic activity (compound **2b**, see Table 3). Further testing confirmed this compound as an agonist for effects mediated by the M_3 receptor such as contraction of the guinea pig ileum, induction of salivation or antagonism of mydriasis (Table 3). Additional testing suggested that the compound was a selective M_3 receptor agonist. This compound failed to inhibit activity in the isolated rat atrium, a model of M_2 functional activity,[11,15,16] and proved to be an antagonist with a pA_2 of 5.7. This compound also failed to stimulate the isolated rat vas



Scheme 1. (a) $NH_2OR \cdot HCl$, EtOH; (b) TMA·BH_3, 3 N HCl/EtOH.

Table 2. Ligand binding studies for muscarinic cholinergic receptors

Compound	Affinity (K_i in μM)		Ratio PZ:OXO-M
	OXO-M[a] $K_i \pm$SEM	Pz[b] $K_i \pm$SEM	
2a	4.5 ± 0.3	19 ± 4	4.2
2b	0.069 ± 0.005	4.3 ± 1.5	63
2c	0.017 ± 0.002	0.19 ± 0.04	11
2d	0.053 ± 0.009	0.13 ± 0.0	2.4
2e	0.023 ± 0.001	0.04 ± 0.008	1.7
2f	0.043 ± 0.003	0.17 ± 0.04	0.39
2g	0.0081 ± 0.0009	0.17 ± 0.03	21
2h	0.19 ± 0.01	0.86 ± 0.06	4.7
2i	0.16 ± 0.02	0.19 ± 0.06	1.2
3a	4.0	—	—
3b	2.3 ± 0.2	40	18

Data represent mean K_i values for at least three experiments.
[a][^{3}H]-Oxotremorine-M.
[b][^{3}H]-Pirenzepine in frontal cortex.

deferens (a model used to identify M_1- and M_4-like effects)[11,16,17] and exhibited antagonistic properties ($pA_2 = 5.9$).

Compound **2b** was able to partially reverse a mnemonic deficit induced by cholinergic depletion in two models of spatial memory (Fig. 2 and Fig. 3). This effect was dose-related but significant improvements in accuracy occurred only at doses which also altered noncognitive aspects of performance such as latency to respond and total trials completed in a session.

Discussion

A series of muscarinic ligands were synthesized and tested for muscarinic receptor activity in vitro and in vivo. Only one (compound **2b**) showed agonist properties at M_3 receptors. Subsequent in vitro testing using the vas deferens to assess M_1/M_4, and the isolated atrium to assess M_2 functional activity revealed that the compound was an agonist only at M_3 receptors.

All modifications of structure **2b** (R = Me) resulted in either a loss of binding affinity, agonist activity or both. For example, substituting larger groups at R (compounds **2c–2i**) results in very little change in OXO-M binding, but does radically alter pirenzepine binding and the agonist/antagonist binding ratio (Table 1, Table 2). A high ratio appears to predict, albeit weakly, agonist properties, while a low ratio predicts antagonist properties,[15] in this case **2b** has the highest ratio and is the only agonist present in the series (Table 3). The unsubstituted member of the series (compound **2a**, R = H) showed very poor binding and was a very weak antagonist. Reducing the double bond of the oxime function (e.g., **2b** to **3b**) significantly decreases both agonist and antagonist binding properties (Table 3).

Compound **2b** was tested for potential mnemonic effects in two models of spatial memory. A partial but significant reduction was observed in the performance

Table 3. M_3 Related muscarinic cholinergic activity in vitro and in vivo.[a]

Compound	R =	Contraction of Guinea Pig Ileum			
		Agonistic activity pD_2 α	Antagonistic activity PA_2	Induction of salivation (mg/kg)	Reversal of mydriasis (mg/kg)
2b	CH_3	5.3 1.4	<4.5	3	0.32
2c	CH_2CH_3	—	6.3	>10	>1
2d	$CH_2CH_2CH_2$	—	6.2	>10	>1
2e	$CH(CH_3)_2$	—	6.5	>10	>1
2f	$CH_2CH{=}CH_2$	—	6.2	>10	>1
2g	$CH_2C{\equiv}CH$	—	6.1	>10	>1
3b	CH_3	*	<4.5	⩾10	>1

[a]Muscarinic cholinergic activity mediated via M_3 receptor was measured by shifting a dose–response curve for acetylcholine to the right and calculating the pA_2-value as described previously.[16] Compounds that contracted the organ where tested for agonistic activity. The cholinergic character of the response was evaluated by measuring the sensitivity for atropine. Data refer to means of four or more experiments (see text under in vitro studies). Salivation and mydriasis experiments were conducted in anesthetized rats and mice as described under in vivo studies in the Experimental. *No agonism found.

deficit induced by cholinergic depletion in the delayed matching to position model of short-term spatial memory; this effect was repeated in a subsequent experiment. Similar positive effects on mnemonic performance were also observed in a separate model of memory based on the Morris swim maze. These data confirm a previous study indicating that selective M_3 agonists can improve compromized mnemonic function in rats.[11] In both procedures performance is not completely normalized, and some non-mnemonic aspects of performance deteriorate (Fig. 2 and Fig. 3). A recent study has indicated that potent M_3 agonists are unable to reverse scopolamine induced deficits in rats[18] and it may be that prominant side effects induced by potent M_3 agonists overshadow any minor positive effects on memory. This may explain why higher doses of compound **2b** have detrimental effects on performance; compound **2b** exerted effects on all the muscarinic M_3-dependent systems tested. However, higher doses of compound **2b** could be tested in the

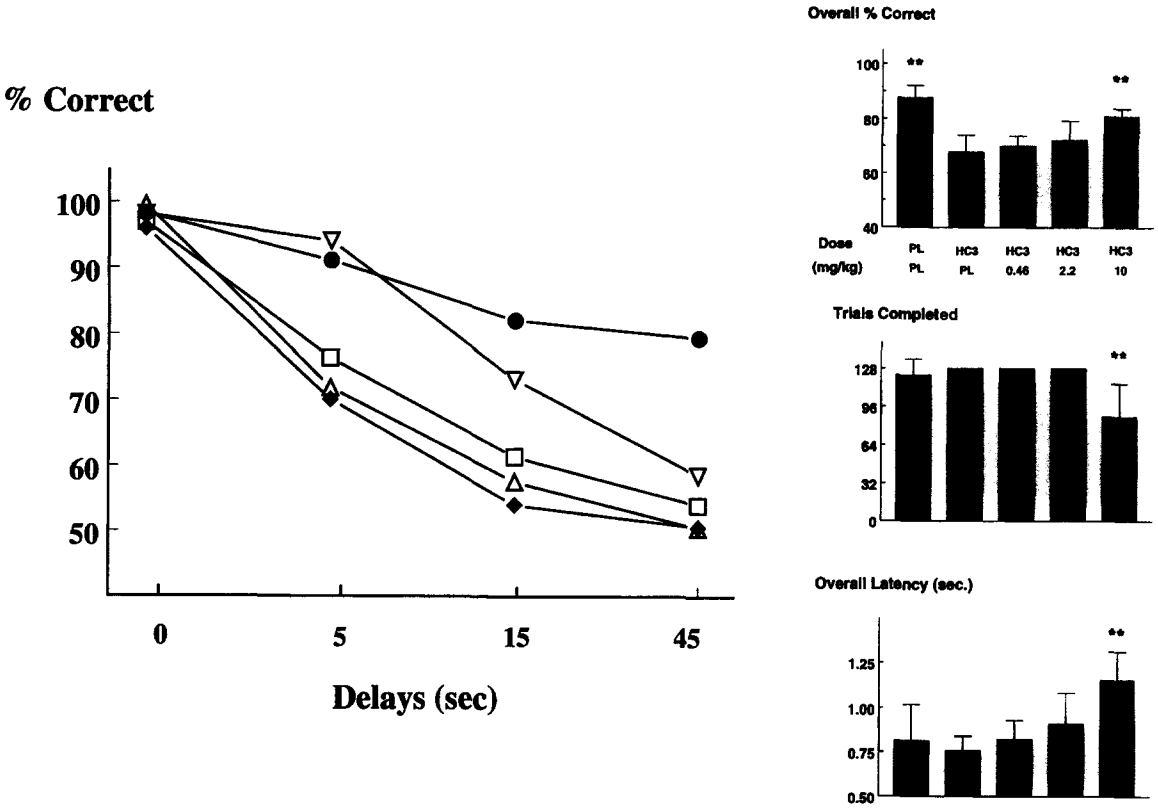


Figure 2. Ability of compound **2b** to reverse a mnemonic deficit in the delayed matching to position task due to central acetylcholine depletion induced by pretreatment with 1 mg hemicholinium-3 (HC3) icv. Main graph: mean % correct responding per delay; ● Placebo; ◆ HC3; △ HC3 + 0.46 mg/kg; □ HC3 + 2.2 mg/kg; ▽ HC3 + 10 mg/kg. Top right: % correct data collapsed over delays to show dose effect. Middle right: trials completed per session. Bottom right: speed of responding in each session. **Significantly different from HC3/placebo (PL) treated rats ($p<0.01$), data are mean and standard deviation of seven rats.

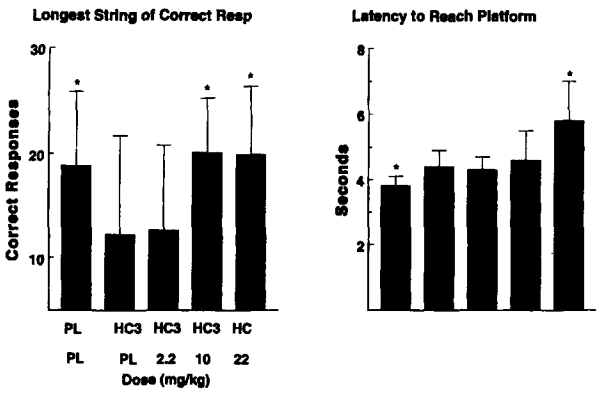


Figure 3. Ability of compound **2b** to reverse a mnemonic deficit in the swim maze due to central acetylcholine depletion induced by pretreatment with 5 mg hemicholinium-3 (HC3) icv. Left graph: longest sequence of correct response over a session of 30 trails. Right graph: mean latency to swim to a platform in the water maze. *Significantly different from HC3/placebo (PL) treated rats ($p > 0.05$), data are mean and standard deviation of nine rats.

swim maze than in the delayed matching procedure. This probably reflects the difference in the response requirements of the two procedures: the swim maze is essentially an aversively motivated escape task, whereas the delayed matching procedure is a positively reinforced procedure in which the consequences of not responding are minor (less food for one session). In general, interference from side effects is more readily observed in the delayed matching task than in the swim maze procedure allowing higher doses to be tested. These results suggest that M_3 agonism alone may be insufficient for successful treatment of cognitive disorders such as Alzheimer's disease due to concomitant activation of M_3 receptors in the periphery inducing unwanted side effects. Nevertheless, recent studies have indicated functional differences between M_3 receptors in different organs[19] and it may in the future be possible to target compounds more selectively at the CNS. It is possible that other cholinergic receptors may be involved in the in vivo response. M_1 antagonists are usually linked to amnestic effects, thus the antagonistic properties exhibited in the vas deferens is unlikely to have contributed to performance enhancement. These data indicate that weak M_1- and M_4-like antagonism is not automatically detrimental to cognitive function in some circumstances. It has been suggested that M_2 antagonism may have beneficial effects on cognition:[20] blockade of the presynaptically located M_2 receptors should lead to enhanced acetylcholine release. M_2 antagonism may therefore contribute to the efficacy of **2b** in both cognitive tests. However, other recent data has suggested that M_2 antagonists may not always exert positive effects on cognitive performance[21,22,27] and further research is required to clarify this issue.

In summary, this study shows that (a) it is possible to develop selective agonists for the M_3 receptor, (b) that the size of the substituent is critical for the observation of agonistic properties, and (c) that M_3 agonists may have useful effects on cognition in addition to those expected from muscarinic M_1 agonists. Although these data indicate that M_3 agonists may have some beneficial effects on memory, the effects are small and possibly subject to interference from peripheral effects. The preferred target for cholinergic agonists remains the M_1 receptor; however, additional M_3 activity may show synergistic effects on cognition. Currently, the separation of desirable from undesirable effects of M_3 stimulation remains difficult, therefore selective M_3 agonists are unlikely to be sufficently active for consideration in the treatment of cognitive disorders such as Alzheimer's disease.

Experimental

Chemistry

Melting points were taken on a Buechi capillary melting point apparatus and are uncorrected. The elemental analyses were within 0.45 of the theoretical values. Proton magnetic resonance spectra were measured on a Bruker WP200, AC200 or AM360 instrument. Chemical shifts are reported as δ-values (parts per million) relative to Me_4Si as an internal standard. Thin-layer chromatography (TLC) was carried out by using Merck precoated silica gel F-254 plates (thickness 0.25 mm). Spots were visualized with a UV handlamp and Cl_2/tetramethylbenzidine. Fast Atom Bombardment (FAB) mass spectra were recorded with a Finnigan MAT 90 mass spectrometer (Finnigan MAT, Bremen, FRG). Samples were dissolved in methanol and mixed with the matrix compounds on standard stainless steel targets. Exact masses of the protonated molecular ions were determined with the peak matching technique at a mass resolution of >8900 (10% valley definition) in the positive ion mode using two reference masses either from poly(ethylene glycol), average M W 400, or poly(propylene glycol), average M W 425. Average exact masses were calculated from at least 10 computer-controlled measurements using the bracketing method.

1-Azabicyclo-[2.2.2]octan-3-one oxime (2a). Hydroxylamine hydrochloride (1.3 g, 18.6 mmol) was added all at once to a stirred solution of quinuclidin-3-one hydrochloride (3.0 g, 18.6 mmol) in dry methanol at room temperature (rt) under nitrogen. The mixture was heated to reflux for 3 h. Then the solvent was removed in vacuo. Recrystallization from aqueous ethanol gave **2a** in 90% yield; mp 234 °C. ^{1}H NMR (200 MHz, D_2O) δ 4.30 (s, 2H), 3.65–3.30 (m, 4H), 2.90 (m, 1H), 2.40–1.90 (m, 4H); ^{13}C NMR (50 MHz, D_2O) δ 157.5 (s, C=N), 53.3 (t, $—CH_2$), 49.8 (t, 2 × $—CH_2$), 29.3 (d, —CH), 24.5 (t, 2 × $—CH_2$).

According to the procedure described above the following compounds were prepared, using O-alkyl or O-aryl hydroxylamines.

1-Azabicyclo[2.2.2]octan-3-one *O*-methyloxime (2b). Yield 81%; mp 216 °C. ^{1}H NMR (200 MHz, MeOD) δ

4.25 (s, 2H), 3.90 (s, 3H,), 3.60–3.40 (m, 4H), 2.85 (m, 1H), 2.35–1.95 (m, 4H).

1-Azabicyclo[2.2.2]octan-3-one *O*-ethyloxime (2c). Yield 87%; mp 188 °C. ^{1}H NMR (200 MHz, MeOD) δ 4.20 (s, 2H), 4.10 (q, 2H, O—CH_2), 3.60–3.40 (m, 4H), 2.85 (m, 1H), 2.35–1.95 (m, 4H), 1.25 (t, 3H, —CH_3); ^{13}C NMR (50 MHz, $CDCl_3$), δ 150.7 (s, C=N), 70.1 (t, —OCH_3), 50.9 (t, —CH_2), 46.7 (t, 2 × —CH_2), 26.8 (d, —CH), 22.5 (t, 2 × —CH_2), 14.2 (q, —CH_3).

1-Azabicyclo[2.2.2]octan-3-one *O*-propyloxime (2d). Yield, after purification by column chromatography (silica gel; CH_2Cl_2:MeOH, 9:1) 54%; mp 128 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.15–3.95 (m, 4H), 3.65–3.20 (m, 4H), 2.95 (m, 1H), 2.30–1.90 (m, 4H), 1.75–1.50 (m, 2H), 0.95 (t, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 151.0 (s, C=N), 76.3 (t, —OCH_2), 51.1(t, —CH_2), 46.7 (t, 2 × —CH_2), 27.1 (d, —CH), 22.9 (t, 2 × —CH_2), 22.2 (t, —CH_2), 10.3 (q, —CH_3).

1-Azabicyclo[2.2.2]octan-3-one *O*-(1-methylethyl)oxime (2e). Yield, after purification using column chromatography 51% (silica gel; CH_2Cl_2:MeOH, 9:1); mp 192 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.40–4.20 (m, 1H), 4.05 (s, 2H), 3.60–3.20 (m, 4H), 2.95 (m, 1H), 2.20–2.00 (m, 4H), 1.25 (d, 6H, 2 × CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 150.4 (s, C=N), 76.3 (t, —OCH_2), 51.2 (t, —CH_2), 46.6 (t, 2 × —CH_2), 27.1 (d, —CH), 22.9 (t, 2 × —CH_2), 21.5 (q, 2 × —CH_3).

1-Azabicyclo[2.2.2]octan-3-one *O*-(2-propenyl)oxime (2f). Yield 75%; mp 144 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.05–5.85 (m, 1H, =CH), 5.35–5.20 (m, 1H, —CH_2=), 4.55 (m, 2H, —O—CH_2), 4.10 (s, 2H), 3.60–3.25 (m, 4H), 2.95 (m, 1H), 2.30–2.00 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 151.7 (s, C=N), 133.3 (s, —C=), 118.3 (t, =CH_2) 75.4 (t, —OCH_2), 51.0 (t, —CH_2), 46.7 (t, 2 × —CH_2), 27.0 (d, —CH), 22.7 (t, 2 × —CH_2).

1-Azabicyclo[2.2.2]octan-3-one *O*-(2-propynyl)oxime (2g). Yield 60%; mp 182 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.65 (d, 2H, O—CH_2), 4.15 (s, 2H), 3.70–3.20 (m, 4H), 3.0 (m, 1H), 2.55 (t, 1H, ≡CH), 2.30–2.00 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 153.1 (s, C=N), 78.7 (s, —C≡), 75.1 (d, CH_2), 61.8 (t, —OCH_2), 50.8 (t, —CH_2), 46.6 (t, 2 × —CH_2), 26.9 (d, —CH), 22.4 (t, 2 × —CH_2).

1-Azabicyclo[2.2.2]octan-3-one *O*-benzyloxime (2h). Yield 83%; mp 215 °C. ^{1}H NMR (200 MHz, MeOD) δ 7.40–7.20 (m, 5H), 5.10 (s, 2H, —O—CH_2), 4.05 (s, 2H), 3.75 (m, 1H), 3.55–3.30 (m, 4H), 2.20–1.90 (m, 4H); ^{13}C NMR (50 MHz, D_2O) δ 158.7 (s, C=N), 139.8 (s, Ar C), 132.0–131.2 (d, 5 × C), 78.6 (t, —OCH_2), 54.1 (t, —CH_2), 49.6 (t, 2 × —CH_2), 25.2 (d, —CH), 23.1 (t, 2 × —CH_2).

1-Azabicyclo[2.2.2]octan-3-one *O*-phenyloxime (2i). Yield 87%; mp 168 °C. ^{1}H NMR (200 MHz, MeOD) δ 7.40–7.00 (m, 5H), 4.55 (s, 2H), 3.65–3.35 (m, 4H), 3.10 (m, 1H), 2.40–2.05 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 158.4 (s, C=N), 154.9 (s, Ar C), 129.3 (d, 2 × Ar C), 123.0 (d, Ar C), 114.4 (d, 2 × Ar C), 51.0 (t, —CH_2), 46.7 (t, 2 × —CH_2), 27.1 (d, —CH), 22.3 (t, 2 × —CH_2).

***N*-Hydroxy-azabicyclo[2.2.2]octan-3-amine (3a).** A solution of HCl in methanol (25 mL of a 4 N solution) was added dropwise to a stirred solution of **2a** (3.00 g, 18 mmol) and trimethylamine–borohydride complex (1.46 g, 20 mmol; Janssen Chimica) at rt under nitrogen. Then the mixture was stirred for 3 h at rt. The solvent was removed in vacuo. Recrystallization from methanol/ethylacetate gave white crystals in 77% yield; mp 225 °C. ^{1}H NMR (200 MHz, MeOD) δ 4.00–4.10 (m, 1H), 3.70–3.90 (m, 1H), 3.30–3.60 (m, 4H), 2.60 (m, 1H), 1.90–2.40 (m, 4H).

Compound **3b** was prepared according to the procedure described above.

***N*-Methoxy-1-azabicyclo[2.2.2]octan-3-amine(3b).** Yield 79%; mp 206 °C. ^{1}H NMR (200 MHz, MeOD) δ 4.10–4.20 (m, 1H), 4.00 (s, 3H) 3.70–3.90 (m, 1H,), 3.30–3.60 (m, 4H), 2.50–2.60 (m, 1H), 2.00–2.40 (m, 4H); ^{13}C NMR (50 MHz, MeOD) δ 63.2 (q, —CH_3), 55.7 (d, —CH), 48.5 (t, —CH_2), 47.6 (t, —CH_2), 47.3 (t, —CH_2), 22.9 (d, —CH), 18.7 (t, —CH_2).

In vitro studies

Binding of [methyl-^{3}H]-oxotremorine-M acetate (^{3}H-OXO-M) in homogenates of frontal cortex. The rapid filtration method of Freedman et al.[23] was used to measure the agonist character of muscarinic cholinergic drugs in rat cerebral cortex homogenates. For routine measurements, the concentration of [^{3}H]-OXO-M was 0.5 nM, tissue concentration was about 1 mg/mL original tissue, and incubation was for 40 min at 30 °C. Nonspecific binding was defined as the amount of binding of [^{3}H]-OXO-M in the presence of 2 mM atropine sulphate and represented about 10% of total binding.

Binding of [N-methyl-^{3}H]-pirenzepine [^{3}H-PZ] in homogenates of rat forebrain. The rapid filtration method of Freedman et al.[23] was used to characterize M_1 muscarinic cholinergic properties of drugs in rat forebrain membranes. For routine measurements the concentration of [^{3}H]-PZ was 1 nM, tissue concentration was about 10 mg/mL original tissue, and incubation was for 60 min at 25 °C. Nonspecific binding was defined as the amount of binding of [^{3}H]-PZ in the presence of 1 mM atropine sulphate and represented about 20% of total binding.

Evaluation of data. Displacement curves were obtained for the various compounds by measuring the specific binding in the presence of at least four different concentrations and IC_{50} values were obtained using a four parameter fitting procedure. K_i values were obtained from the IC_{50} values by using the

Chang–Prusoff equation $K_i = IC_{50}/1 + C/K_d$ in which C equals the radiolabelled ligand concentration and K_d equals the dissociation constant for the radiolabelled ligand. K_d values used for these calculations were as follows: [^{3}H]-OXO-M binding: $K_d = 0.7$ nM; [^{3}H]-PZ binding: $K_d = 8.3$ nM.

Interactions with M_3 receptors in isolated guinea pig ileum. Interactions with M_3 muscarinic cholinergic receptors were measured in the isolated guinea pig ileum. A fully automated method was used as described previously.[16] Contractions induced with acetylcholine as an agonist (pD_2-values between six and seven) were used to evaluate the potency of cholinergic antagonist. Antagonistic activity was measured as a shift to the right of the dose–response curve to acetylcholine in the presence of the compound.

In vivo studies

Antagonism of clonidine-induced mydriasis. Antagonism of clonidine-induced mydriasis was studied using the method described by Hagan et al.[24] Male Wistar rats (250–300 g, Harlan Zeist, the Netherlands) were anesthetized with 60 mg/kg of Nembutal, placed on a heated blanket, and the pupil diameter measured. Clonidine (0.3 mg/kg) or placebo is then administered sc and 20 min later the pupil diameter measured. Immediately following this measurement separate groups of rats (n = 6) are administered placebo or one of the drug doses in a 20 μL drop applied directly onto the eye. Thereafter the pupil is measured at 10, 30, and 60 min after application. Changes in pupil diameter are calculated with respect to levels induced by clonidine.

Induction of Salivation. Male CD-1 mice (30 g, Charles Rivers, Germany) were anesthetized using Avertine. After 10 min separate groups of mice (n = 6) were injected sc with the test drug in a saline/mulgofen vehicle (1–10 mg/kg) and placed on a filter paper. After 20 min the extent of any stain due to salivation was measured. The lowest dose to induce a stain of ≥ 1 cm^2 was recorded as the minimal effective dose.

Reversal of Hemicholinium-3-induced memory deficit. Drug effects on rat short-term spatial memory were assessed using a minor variation of the delayed matching to position procedure reported by Dunnett[25] and the two island swim task in the Morris swim maze as used by Hagan et al.[26] For both tasks male Long Evans rats (350 g, Harlan, Zeist, NL) were used; a group of seven rats for the delayed matching task, and a group of nine rats for the swim maze. Training in the delayed matching to position task has been described in detail elsewhere.[27] Rats were trained on the spatial matching task in standard operant chambers (Coulbourn Instruments, PA, U.S.A.) connected to a MEDLab interface (Med Associates, VT, U.S.A.) and controlled by an IBM PS2 PC. Training in the swim maze followed the procedure described in detail by Hagan et al.,[26] the same 2.1 m diameter water maze, cues and islands used by Hagan et al. were used in this study. After stabilization training, the rats were implanted with guide cannula into the lateral ventricle as described previously.[26,27] For the delayed matching experiment, rats were infused with 5 μL of merlys, or 5 μL of merlys containing 1 μg of the cholinergic depleting agent hemicholinium-3, 1 h before testing in the operant chambers. Rats in the swim maze received the same vehicle and infusion volume, but a concentration of 5 μg of hemicholinium-3. Hemicholinium-3 leads to a central depletion of acetylcholine and leaves both central and peripheral cholinoreceptors open for stimulation and may therefore allow a more relevant test of the cognitive effects of cholinergic agonist: Alzheimer's patients suffer from loss of transmitter, not blockade of receptors. Thirty minutes before the session, rats were administered the test drug sc in a saline vehicle. Each rat received five drug treatments in a latin square design. For the delayed matching the five treatments were: placebo icv and sc; hemicholinium-3 icv and placebo sc; hemicholinium-3 icv and 0.46 mg/kg of **2b**; hemicholinium-3 and 2.2 mg/kg of **2b**; hemicholinium-3 and 10 mg/kg of **2b**; for the swim maze: placebo icv and sc; hemicholinium-3 icv and placebo sc; hemicholinium-3 icv and 2.2 mg/kg of **2b**; hemicholinium-3 and 10 mg/kg of **2b**; hemicholinium-3 and 22 mg/kg of **2b**. Several parameters were recorded in both procedures. Data for correct responses, trials completed and reponse time latencies for both swim maze and delayed matching data were analyzed using ANOVA and, where significance was attained, by appropriate post hoc testing.

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