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Muscarinic agonists. Syntheses and structure–activity relationships of bicyclic isoxazole ester bioisosteres of norarecoline

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Summary — (*RS*)-3-Methoxy-8-methyl-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine (*O*,8-di-Me-THAO, **2c**) and (*RS*)-8-methyl-3-propargyloxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine (8-Me-*O*-propargyl-THAO, **2d**) have been synthesized and evaluated as muscarinic receptor ligands in receptor binding assays and in *in vitro* functional assays. The corresponding compounds without methyl groups at C-8, *ie O*-Me-THAO (**2a**) and *O*-propargyl-THAO (**2b**), have previously been shown to exhibit muscarinic agonistic profiles with very little discrimination between M_1- and M_2-receptor sites. Based on functional assays, **2c** and **2d** were found to be less efficacious than **2a** and **2b**, respectively, and **2d** proved to be an M_1-selective partial agonist. The affinity and M_1 efficacy of **2c** and **2d** were comparable to those of the corresponding six-membered ring analogues, (*RS*)-3-methoxy-7-methyl-4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridine (*O*,7-di-Me-THPO, **1c**) and (*RS*)-7-methyl-3-propargyloxy-4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridine (7-Me-*O*-propargyl-THPO, **1d**). However, neither compound **1c** nor compound **1d** displayed M_1 selectivity. In summary, within this class of bicyclic muscarinic agonists, replacement of 3-methoxy by 3-propargyloxy groups generally increases muscarinic affinity without affecting the efficacy at M_1 receptors significantly. Introduction of a methyl group into the saturated ring at the position α to the C-5 of the isoxazole ring (α-position) leads to compounds exerting lower efficacy and, in the case of compound **2d**, an increased selectivity with respect to M_1 agonism.

muscarinic acetylcholine receptor / muscarinic agonist ligand / partial muscarinic agonist / norarecoline bioisostere / bicyclic isoxazole derivative / *O*-alkyl-THPO / *O*-alkyl-THAO / 7-Me-*O*-alkyl-THPO / 8-Me-*O*-alkyl-THAO

Introduction

Dementia of the Alzheimer type (DAT) is a neurodegenerative disorder characterized by progressive loss of memory, cognitive abilities and personality, and finally leading to the death of the patient [1, 2]. Post mortem studies of brains from DAT patients have revealed a marked reduction of the cholinergic neurons that project from the basal forebrain to the cerebral cortex and the hippocampus [1–4]. According to the 'cholinergic hypothesis' these cholinergic deficiencies are related directly to the cognitive impairments [2]. Replacement therapies with cholinergic agonists may, therefore, improve the defective cholinergic function and thereby ameliorate some of the symptoms seen in DAT patients [5, 6].

Five different human muscarinic receptor subtypes, m1–m5, have been cloned and characterized in functional test systems [7, 8]. By the use of selective antagonists the pharmacologically defined muscarinic receptor subtypes, M_1–M_3, have been characterized [8, 9]. It seems that the antagonist affinities determined for the cloned m2 and m3 receptors generally correlate well with those of the pharmacologically defined M_2 and M_3 receptors, respectively [10], whereas the antagonist binding properties for both m1 and m4 receptors appear to be similar to those of the M_1 receptor [10].

It has been proposed that the postsynaptic M_1 receptor in the brain is involved in memory formation and higher cognitive functions [1, 2]. Pharmacological activation of the M_1 receptor is therefore believed to improve memory function, whilst activation of the M_2 receptor may be associated with undesirable peripheral side effects [11].

The main problems encountered in clinical trials with muscarinic agonists are either unsatisfactory pharmacokinetic properties or distressing side effects of the available compounds, which frequently lack receptor subtype selectivity [11].

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The conformationally restricted heterocyclic analogue of norarecoline, 3-methoxy-4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridine (*O*-Me-THPO, **1a**) and the corresponding seven-membered ring analogue, 3-methoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]-azepine (*O*-Me-THAO, **2a**) (fig 1) have previously been described as potent muscarinic agonists [12–16]. Replacement of the 3-*O*-methyl group by various *O*-alkyl groups has shown that for the THPO as well as for the THAO series the 3-*O*-propargyl derivatives exert the highest muscarinic receptor affinity and M_1 selectivity [12–16], in agreement with previously described observations by Mutschler and Hultzsch [17].

As part of our current project on the search for M_1-selective agonists, we have now synthesized the derivatives of *O*-Me-THAO and *O*-propargyl-THAO with methyl groups in the 8-positions (fig 1). Since the originally described method for the synthesis of the bicyclic ring system [18] proved to be unsuitable for the preparation of 8-methyl-substituted analogues, a new synthetic route for this particular bicyclic ring system has been developed. The receptor subtype selectivities (at M_1- and M_2-receptor sites) and the agonist potency of the compounds were estimated on the basis of functional *in vitro* tests. The biological activities of the synthesized compounds were compared with the activities of the corresponding unmethylated THAO derivatives and to the previously described [16] 7-methylated THPO derivatives.

Chemistry

(*RS*)-3-(3-Methoxyisoxazol-5-yl)butanol **3** [19] was converted into the 4-methoxybenzyl-protected derivative **4** by conventional methods (scheme 1). Carboxylation of the 4-position of the isoxazole ring is a key step in the synthesis. According to the literature [20–23] this carboxylation reaction is highly dependent on the nature of the substituents in the 3- and 5-positions of the isoxazole ring, and the yields are often poor. However, in this case, the synthesis was optimized to give **5** in 90–95% yield using *n*-butyllithium and solid CO_2. Conversion of the isoxazole-4-carboxylic acid, **5**, into the corresponding amide, **6**, was accomplished by conventional methods. Attempts to reduce compound **6** to the corresponding 4-aminomethyl isoxazole by use of complex metal hydrides resulted in either decomposition or virtually complete recovery of the starting material. Instead, the amide **6** was dehydrated by treatment with trichloroacetyl chloride and triethylamine (TEA) to afford the isoxazole-4-carbonitrile derivative **7**. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was used for oxidative removal of the 4-methoxybenzyl group to give compound **8**.

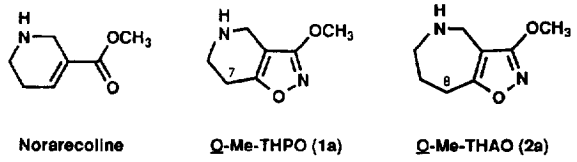


Fig 1. Structures of norarecoline, *O*-Me-THPO **1a** and *O*-Me-THAO **2a**.

Reduction of the isoxazole-4-carbonitrile **8** to the corresponding 4-aminomethyl isoxazole **9** was accomplished by treatment with alane. The amino-alcohol **9** was converted into the corresponding chloride by use of thionyl chloride, and the final cyclization, in order to give (*RS*)-3-methoxy-8-methyl-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine **2c**, was accomplished by treatment with aqueous sodium hydroxide.

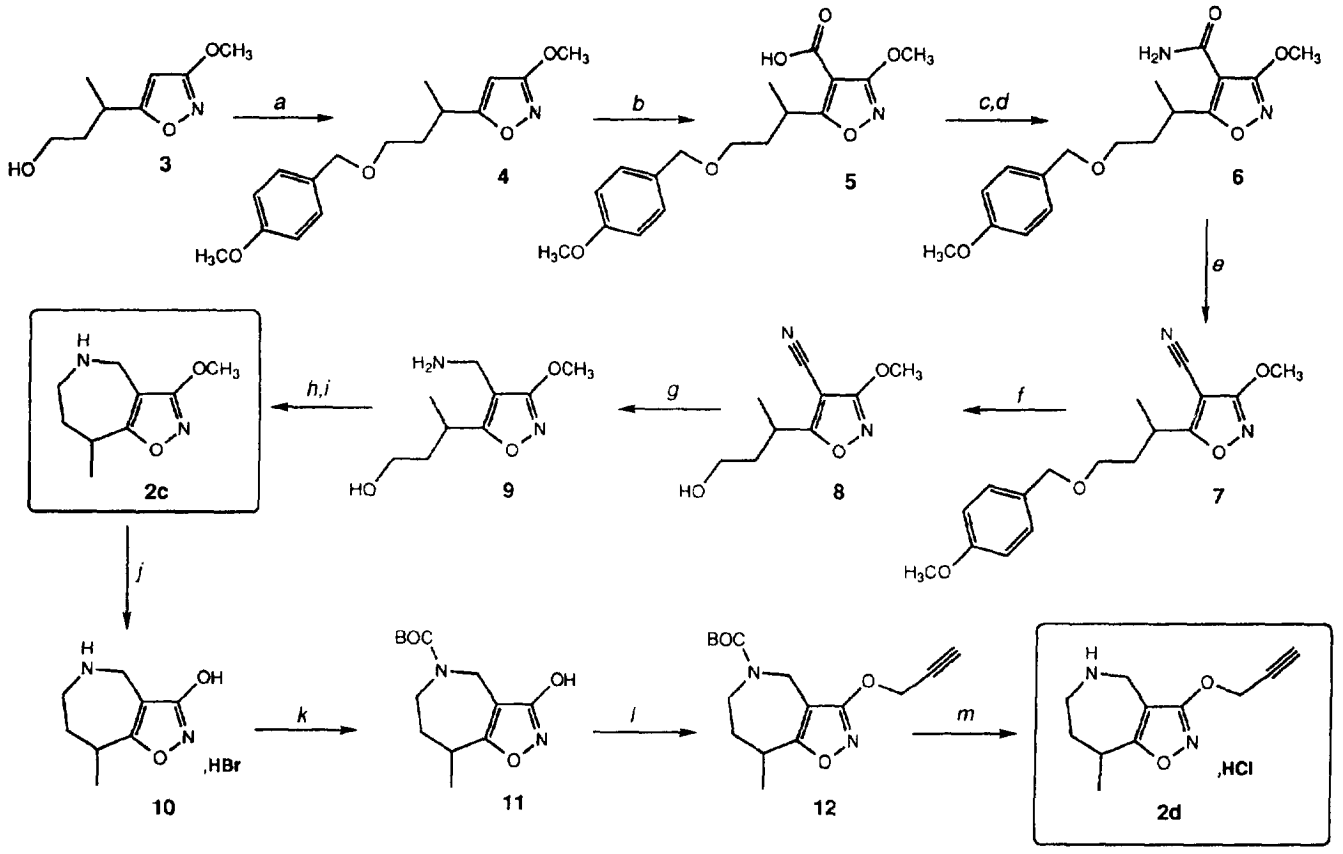
The 3-*O*-propargyl-THAO derivative **2d** was synthesized by use of compound **2c** as an intermediate (scheme 1). Removal of the 3-*O*-methyl group was accomplished by treatment with hydrobromic acid in glacial acetic acid to afford **10** as the hydrobromide salt. The readily removable *t*-butoxycarbonyl group (BOC) was used as a protection group for the amino group, and alkylation of the 3-hydroxyisoxazole **11** with propargyl bromide gave the desired propargyloxy compound **12**. Deprotection of the amino group was carried out by use of HCl in diethyl ether to give compound **2d** as the hydrochloride salt.

The structures of all new compounds were confirmed by ^{1}H-NMR and in some cases were supported by ^{13}C-NMR spectroscopy.

Results and discussion

The pharmacological test models are described in detail in the *Experimental protocols*. The K_i values for inhibition of binding of tritiated muscarinic ligands by the compounds studied in this work are shown in table I together with the corresponding agonist index and M_2/M_1 index values. Functional *in vitro* activities of the compounds are shown in table II.

Tritiated quinuclidinyl benzilate (QNB) and pirenzepine (PZ) were used as unselective and M_1-selective muscarinic antagonist ligands, respectively. Tritiated oxotremorine-M (Oxo-M) was used as a muscarinic agonist ligand. The ratio between the K_i values for a compound, determined in [^{3}H]QNB (brain) and [^{3}H]Oxo-M (brain) binding experiments, was used as a muscarinic agonist index (agonist index) of the compound for estimation of the efficacy at muscarinic receptors in analogy with the method described by Freedman *et al* [24–26]. In our test set-up, agonist



Scheme 1. Synthesis of (*RS*)-3-methoxy-8-methyl-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine **2c** and (*RS*)-8-methyl-3-propargyloxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepine **2d**. *a*: *p*-Methoxybenzyl chloride/NaH/DMF; *b*: *n*-BuLi/CO_2/THF; *c*: $SOCl_2$; *d*: NH_3(aq); *e*: $Cl_3CCOCl/TEA/CH_2Cl_2$; *f*: $DDQ/CH_2Cl_2/H_2O$; *g*: $LiAlH_4/AlCl_3/Et_2O$; *h*: $SOCl_2/CH_2Cl_2$; *i*: NaOH/MeOH; *j*: HBr/HOAc; *k*: di-*t*-butyl pyrocarbonate/K_2CO_3; *l*: $BrCH_2C{\equiv}CH/K_2CO_3$; *m*: HCl/Et_2O.

index values above 100 can be grossly considered to reflect full agonism, whereas values between 10 and 100 indicate partial agonism. Values below 10 are considered to indicate antagonistic profiles of muscarinic agents.

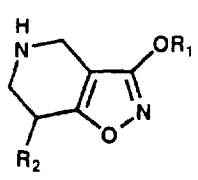
The ratio between K_i values of a compound determined in [^{3}H]QNB (heart) and [^{3}H]PZ (brain) binding experiments was used as an index for M_1-selectivity (M_2/M_1 index). M_2/M_1 index values are considered proportional to the M_1-selectivity [12].

Such ratios are, of course, purely empirical and in this work further testing in functional assays were performed to substantiate the functional efficacy and selectivity of the compounds, as indicated by the agonist and M_2/M_1 indices. The functional assays applied were depolarisation of the rat superior cervical ganglion (measures M_1 receptor activity [27]), depression of atrial contractility in guinea-pig left atrium (measures M_2 receptor activity [28]) and contraction of guinea-pig ileum (measures a mixture of M_1, M_2 and M_3 receptor activity [28]).

As seen in table I, *O*-Me-THPO (**1a**) is less potent than norarecoline in all binding tests. *O*-Me-THAO (**2a**), on the other hand, is more potent than norarecoline, in agreement with previous findings [14, 16, 29]. Based on the agonist index values, both of these bicyclic compounds seem to be less efficacious agonists than norarecoline [29]. In the functional tests, both compounds are partial agonists at M_1 receptors, but full agonists at M_2 receptors (table II).

Replacements of the 3-*O*-methyl groups in **1a** and **2a** with 3-*O*-propargyl groups result in compounds **1b** and **2b**, respectively, which exert significantly higher affinities in all binding tests than their methylated counterparts. Likewise, the potencies in the functional assays are enhanced for these compounds (table II). The agonist efficacies of **1b** and **2b** are largely unaffected by this structural modification as indicated by

Table I. *In vitro* affinities for muscarinic receptors[a].



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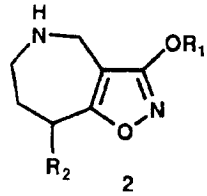

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Compound	R_1	R_2	*QNB (brain)*	*QNB (heart)*	*PZ (brain)*	*Oxo-M (brain)*	*Agonist index*[b]	M_2/M_1 *index*[c]
Norarecoline			2600	35	2200	4.8	540	0.02
1a	CH_3	H	4700	300	5500	44	110	0.05
1b	Prg[d]	H	950	200	570	5.0	190	0.35
1c	CH_3	CH_3	2500	990	280	120	21	3.5
1d	Prg	CH_3	480	230	39	9.2	52	5.9
2a	CH_3	H	230	5.1	160	2.0	120	0.03
2b	Prg	H	22	1.0	10	0.2	110	0.10
2c	CH_3	CH_3	730	370	190	78	9.4	2.0
2d	Prg	CH_3	37	43	7.1	7.0	5.3	6.1

[a]K_i values in nM. [b]Agonist index = K_i(QNB (brain))/K_i(Oxo-M (brain)). [c]M_2/M_1 index = K_i(QNB (heart))/K_i(PZ (brain)). [d]Prg = propargyl. The inhibitory constants (K_i) of the compounds derived from $K_i = IC_{50}/(1 + S/K_D)$, where K_D is the dissociation constant of the binding of the radioactive ligand. K_D for QNB (brain) = 13.7 ± 0.9 pM, QNB (heart) = 9.1 ± 0.7 pM, PZ (brain) = 1.8 ± 0.1 nM, and Oxo-M (brain) = 0.48 ± 0.03 nM.

Table II. Functional *in vitro* effects at rat superior cervical ganglion, guinea-pig left atrium and guinea-pig ileum[a].

Compound	*Ganglion (M_1)*		*Atrium (M_2)*		*Ileum ($M_1/M_2/M_3$)*	
	EC_{50} (SD-factor)	*RM ± SD*	*EC_{50} (SD-factor)*	*RM ± SD*	*EC_{50} (SD-factor)*	*RM ± SD*
Norarecoline	≥ 2700[b] (1.7)	≥ 1.05 ± 0.07	370 (1.4)	1.02 ± 0.05	620 (1.3)	1.06 ± 0.10
1a	≥ 3300 (1.7)	≥ 0.69 ± 0.06	1300 (1.6)	0.98 ± 0.07	2900 (1.4)	1.04 ± 0.02
1b	≥ 780 (1.3)	≥ 0.61 ± 0.25	300 (1.5)	0.98 ± 0.02	1200 (1.3)	1.03 ± 0.04
1c	≥ 5400 (2.3)	≥ 0.37 ± 0.11	7900 (1.3)	0.67 ± 0.16	ant[c] –	– –
1d	≥ 2000 (1.5)	≥ 0.37 ± 0.13	620 (1.5)	0.45 ± 0.03	ant –	– –
2a	≥ 260 (1.1)	≥ 0.63 ± 0.12	170 (1.6)	1.04 ± 0.05	240 (1.2)	0.91 ± 0.15
2b	≥ 75 (1.3)	≥ 0.73 ± 0.05	13 (1.3)	1.00 ± 0.01	50 (1.2)	1.07 ± 0.03
2c	≥ 6900 (1.4)	≥ 0.31 ± 0.06	2300 (1.4)	0.49 ± 0.05	> 25 000 (1.7)	> 0.34 ± 0.07
2d	≥ 1300 (1.5)	≥ 0.28 ± 0.18	> 51 000 –	ND[d]	ant –	– –

[a]EC_{50} values in nM (log mean with standard deviation factor) and RM values, *ie* relative maximum effects (mean with standard deviation). Values are means of three separate experiments. For explanation of RM, see *Experimental protocols*. [b]For explanation of the ≥ sign, see *Experimental protocols*. [c]Compounds exerting antagonistic properties in the ileum test are marked 'ant'. The antagonist strengths of these compounds were not determined. [d]Not determined.

the agonist index (table I) and substantiated in the functional tests (table II). As indicated, both by the M_2/M_1 index values and by the functional tests, compounds **1b** and **2b** display M_1/M_2 agonism with some preference for M_2 receptors. In the α-methylated series, a similar increase in affinity is observed when going from 3-*O*-methyl derivatives to 3-*O*-propargyl derivatives, as demonstrated by comparing compounds **1c** to **1d** and **2c** to **2d** (tables I and II). However, of all the compounds studied in this work, the α-methylated 3-*O*-propargyl derivative **2d** is the only compound that exerts functional agonism at M_1 receptors combined with functional antagonism at M_2/M_3 receptors (table II).

Introductions of methyl groups into the 7- and 8-positions of **1a** and **2a**, respectively, (to give **1c** and **2c**) reduce the affinities in [^{3}H]Oxo-M (brain) bindings. [^{3}H]QNB (brain) affinities are slightly increased for **1c** and reduced for **2c**. Since the agonist index is decreased both compounds are expected to become less efficacious agonists relative to the unmethylated compounds **1a** and **2a**. These results are confirmed in the functional tests (table II). Compound **1c** actually exerts antagonistic properties in the ileum test.

Introductions of 7- and 8-methyl groups into the *O*-propargyl derivatives **1b** and **2b**, respectively, to give **1d** and **2d**, result in less efficacious agonists, as determined by the agonist index (table I). This change of profile is similar to the change observed in the *O*-methyl series, as mentioned above. Based on the low agonist index value, compound **2d** might be expected to exert antagonistic properties. This is actually the case in the ileum test, but **2d** is a low-efficacy agonist in the ganglion test (table II). Compound **2d** does not display M_2 agonism, as shown in the atrium test, whereas **1d** is a partial agonist at both M_1 and M_2 receptors.

The ring expansion of the cyclic amine in the THPO derivatives leading to homologous THAO derivatives generally increases the affinities in all binding tests. For compounds without an α-methyl substituent, **2a** and **2b**, the efficacies and functional selectivities appear to be unaffected by the ring expansion, whereas a change in functional selectivity is seen for the α-methylated compounds **2c** and **2d**.

In conclusion, compound **2d** is a functionally selective, partial M_1 agonist. Further pharmacological characterization must be performed in order to clarify whether compound **2d** exerts the *in vivo* pharmacological profile, relevant in treatment of DAT. It should also be noted that the α-methylated compound **2d** is a racemic mixture and, as seen for other muscarinic agonists [30, 31], resolution might be expected to produce enantiomers with different pharmacological profiles.

Experimental protocols

Chemistry

Melting points, determined in capillary tubes, are uncorrected. High performance liquid chromatography (HPLC) and column chromatography (CC) were performed on silica gel C60-H (230-400 mesh, Rhône-Poulenc). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Brucker 250 MHz spectrometer (250.13 MHz for ^{1}H-NMR and 62.90 MHz for ^{13}C-NMR) using TMS as an internal standard. Chemical shift data are given as δ (ppm) values. The MS spectrum (EI; 70 eV) of compound **2c** was recorded on a Varian MAT 311-A, and was kindly carried out by J Møller, Department of Chemistry, Odense University, Denmark. Elemental analyses, as indicated by elemental symbols, were within ±0.4% of the theoretical values and they were performed by the Analytical Research Department, H Lundbeck A/S. All evaporations were performed *in vacuo* using a rotatory evaporator.

(RS)-3-Methoxy-5-[4-(4-methoxybenzyloxy)-2-butyl]isoxazole 4

Compound **3** (132 g; 0.77 mol) [19] was added to a suspension of NaH (80% in oil, 69 g; 2.3 mol) in dry DMF (800 ml), and the mixture was stirred at room temperature for 1 h. 4-Methoxybenzyl chloride (125 ml; 0.92 mol) was added dropwise and the reaction mixture was cooled in an ice bath to keep the temperature at 25°C. After stirring for 24 h, methanol (75 ml) was added and the mixture was poured into ice cold water (2.5 l). The pH of the aqueous solution was adjusted to 7 using acetic acid and extracted with diethyl ether (3 x 500 ml). The combined organic layers were washed with a saturated aqueous $CaCl_2$ solution (500 ml), dried ($MgSO_4$) and concentrated. The residue was subjected to CC (heptane/THF, 4:1) and **4** was isolated as an oil (149 g; 66%). ^{1}H-NMR ($CDCl_3$) δ 7.25–6.85 (dd, 4H, phenyl-*H*); 5.55 (s, 1H, isoxazole-*H*); 4.40 (s, 2H, OCH_2-phenyl); 3.94 (s, 3H, isoxazole-OCH_3); 3.80 (s, 3H, phenyl-OCH_3); 3.53–3.37 (m, 2H, $CH(CH_3)CH_2CH_2$); 3.13–2.98 (m, 1H, $CH(CH_3)CH_2CH_2$); 2.04–1.74 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.27–1.24 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR ($CDCl_3$) δ 177.49; 171.43; 158.39; 129.73; 128.29 (2C); 112.83 (2C); 90.07; 71.62; 66.23; 55.62; 54.04; 34.24; 28.96; 17.60.

(RS)-3-Methoxy-5-[4-(4-methoxybenzyloxy)-2-butyl]isoxazol-4-carboxylic acid 5

A solution of **4** (109 g; 0.37 mol) in dry THF (750 ml) was cooled to –78°C. *n*-Butyllithium (15% in hexane; 275 ml; 0.44 mol) was added dropwise during 45 min. After stirring for additional 1.5 h, the dark-red solution was poured into a suspension of solid CO_2 (washed 3 times with dry THF) in dry THF, and the mixture was allowed to reach room temperature. After evaporation of the mixture, the residue was dissolved in water (750 ml) and extracted with diethyl ether (2 x 500 ml). The aqueous solution was acidified with concentrated HCl to pH 3 and extracted with diethyl ether (3 x 750 ml). The diethyl ether extracts were washed with a saturated aqueous NaCl solution, dried ($MgSO_4$) and concentrated to afford **5** (120 g; 96%) as an oil. ^{1}H-NMR ($CDCl_3$) δ 11.90–11.60 (s large, 1H, COO*H*); 7.20–6.83 (dd, 4H, phenyl-*H*); 4.35 (s, 2H, OCH_2-phenyl); 4.04 (s, 3H, isoxazole-OCH_3); 3.90–3.80 (m, 1H, $CH(CH_3)CH_2CH_2$); 3.77 (s, 3H, phenyl-OCH_3); 3.52–3.34 (m, 2H, $CH(CH_3)CH_2CH_2$); 2.20–1.86 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.32–1.28 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR (DMSO-d_6) δ 183.89; 169.36; 165.14; 158.60; 129.42; 128.64 (2C); 113.12 (2C); 98.40; 71.89; 66.72; 56.89; 54.45; 33.34; 29.84; 17.59.

(RS)-3-Methoxy-5-[4-(4-methoxybenzyloxy)-2-butyl]isoxazol-4-carboxamide 6
A mixture of **5** (120 g; 0.36 mol), $SOCl_2$ (120 ml) and CH_2Cl_2 (500 ml) was refluxed for 1.5 h. After evaporation, the remaining oil was dissolved in THF (500 ml) and added dropwise to an aqueous solution of ammonia (25%, 500 ml) at –5°C. The reaction mixture was stirred for 3 h and allowed to reach room temperature. After evaporation of the THF, the residue was extracted with diethyl ether (2 x 500 ml). The extracts were washed with a saturated aqueous NaCl solution, dried ($MgSO_4$) and concentrated to give **6** (103 g; 86%) as an oil. ^{1}H-NMR ($CDCl_3$) δ 7.23–6.83 (dd, 4H, phenyl-*H*); 6.70–6.50 (s large, 1H, $CONH_2$); 6.40–6.20 (s large, 1H, $CONH_2$); 4.35 (s, 2H, OCH_2-phenyl); 4.05 (s, 3H, isoxazole-OCH_3); 4.00–3.90 (m, 1H, $CH(CH_3)CH_2CH_2$); 3.78 (s, 3H, phenyl-OCH_3); 3.50–3.38 (m, 2H, $CH(CH_3)CH_2CH_2$); 2.20–1.85 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.31–1.27 (d, 3H, $CH(CH_3)CH_2CH_2$).

(RS)-3-Methoxy-5-[4-(4-methoxybenzyloxy)-2-butyl]isoxazol-4-carbonitrile 7
To an ice-cold solution of **6** (103 g; 0.31 mol) and TEA (99 ml; 0.72 mol) in CH_2Cl_2 was added trichloroacetyl chloride (41 ml; 0.36 mol) in CH_2Cl_2 (40 ml), and the mixture was stirred at room temperature for 1 h. Water (500 ml) was added and the two phases were separated. The organic layer was washed with water (2 x 400 ml), dried ($MgSO_4$) and concentrated. Purification by CC of the residue (ethyl acetate/heptane, 1:1) gave **7** (45 g; 46%) as an oil. ^{1}H-NMR ($CDCl_3$) δ 7.24–6.84 (dd, 4H, phenyl-*H*); 4.40–4.38 (d, 2H, OCH_2-phenyl); 4.00 (s, 3H, isoxazole-OCH_3); 3.80 (s, 3H, phenyl-OCH_3); 3.53–3.28 (m, 3H, $CH(CH_3)CH_2CH_2$); 2.14–1.88 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.39–1.35 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR ($CDCl_3$) δ 184.62; 169.54; 158.70; 129.63; 128.80 (2C); 113.18 (2C); 109.28; 81.60; 72.17; 66.23; 57.27; 54.62; 34.00; 30.52; 17.45.

(RS)-3-Methoxy-5-(4-hydroxy-2-butyl)isoxazol-4-carbonitrile 8
To a solution of **7** (25 g; 79.0 mmol) in CH_2Cl_2 (750 ml) were added water (1 ml) and DDQ (20 g; 88.1 mmol) dissolved in CH_2Cl_2 (800 ml). The reaction mixture was stirred at room temperature for 1.5 h, and water (350 ml) was added. After stirring for additional 30 min, the two phases were separated, and the organic layer was washed with water (100 ml), dried ($MgSO_4$) and concentrated. The residue was purified by CC (ethyl acetate/heptane, 1:1) and **8** was isolated as an oil (12 g; 77%). ^{1}H-NMR ($CDCl_3$) δ 4.30 (s, 3H, OCH_3); 3.74–3.56 (m, 2H, $CH(CH_3)CH_2CH_2$); 3.43–3.27 (m, 1H, $CH(CH_3)CH_2CH_2$); 2.80–2.50 (s large, 1H, CH_2OH); 2.13–1.84 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.44–1.40 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR ($CDCl_3$) δ 185.18; 169.96; 109.58; 81.84; 59.23; 57.75; 36.65; 30.34; 17.65.

(RS)-4-Aminomethyl-3-methoxy-5-(4-hydroxy-2-butyl)isoxazole 9
Compound **8** (12 g; 61.1 mmol) was added to a mixture of AlH_3 in dry diethyl ether prepared *in situ* from $LiAlH_4$ (6 g; 157.9 mmol) in dry diethyl ether (400 ml) and $AlCl_3$ (6 g; 45.0 mmol) in dry diethyl ether (100 ml). The reaction mixture was refluxed for 1.5 h, cooled to room temperature and hydrolyzed in succession with water (6 ml) and aqueous NaOH (1.8 M; 25 ml). After stirring for 30 min, the precipitate was removed by filtration and washed with CH_2Cl_2. The combined organic phases were concentrated to give **9** as an oil (7.5 g; 61%). ^{1}H-NMR ($CDCl_3$) δ 3.98 (s, 3H, OCH_3); 3.72–3.44 (dd, 2H, CH_2NH_2); 3.58–3.17 (m, 3H, $CH(CH_3)CH_2CH_2$); 3.20–2.75 (s large, 1H, CH_2OH); 1.98–1.67 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.34–1.30 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR ($CDCl_3$) δ 173.01; 170.32; 104.77; 57.60; 56.35; 38.07; 31.94; 27.32; 18.17.

(RS)-3-Methoxy-8-methyl-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-c]azepine 2c
To a solution of **9** (3.8 g; 19.1 mmol) in chloroform (50 ml) was added a saturated solution of HCl in diethyl ether (50 ml). After stirring for 30 min, the mixture was concentrated and the remaining oil dissolved in CH_2Cl_2 (150 ml). $SOCl_2$ (50 ml) was added and the reaction mixture refluxed for 6 h. The reaction mixture was concentrated, and the residue dissolved in methanol (275 ml). This solution was added dropwise over 90 min to 0.2 N NaOH (660 ml) at 90°C. The resulting mixture was stirred for additional 90 min at 90°C and extracted with CH_2Cl_2 (3 x 500 ml). The combined organic layers were dried ($MgSO_4$) and concentrated to afford **2c** (1.7 g; 49%) as an oil. ^{1}H-NMR ($CDCl_3$) δ 3.87 (s, 3H, OCH_3); 3.62–3.45 (dd, 2H, isoxazole-CH_2-NH); 3.25–3.14 (m, 1H, $CH(CH_3)CH_2CH_2$); 3.03–2.85 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.90–1.45 (m, 3H, $CH(CH_3)CH_2CH_2NH$); 1.28–1.24 (d, 3H, $CH(CH_3)CH_2CH_2$). MS (30°C) *m/z* (rel int): 182 ($M^{+\bullet}$, 53); 181 (100); 153 (28); 138 (45); 110 (35); 83 (24); 81 (39); 53 (72); 41 (44); 30 (68).

Addition of fumaric acid to **2c**, dissolved in acetone, yielded the hemifumarat salt, **2c**·$0.5C_4H_4O_4$ (1.9 g; 85%), mp 183–185°C. CHN: calc 54.98; 6.73; 11.66, found 54.85; 6.80; 11.57. ^{1}H-NMR (D_2O) δ 6.33 (s, 1H, 0.5 HOOC*CH*=*CH*-COOH); 4.02–3.85 (dd, 2H, isoxazol-CH_2NH); 3.81 (s, 3H, OCH_3); 3.60–3.27 (m, 2H, $CH(CH_3)CH_2CH_2NH$); 3.20–3.04 (m, 1H, $CH(CH_3)CH_2CH_2$); 2.09–1.73 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.21–1.17 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR (methanol-d_3) δ 174.45; 171.50; 169.17 (2C); 134.78 (2C); 99.22; 55.86; 46.33; 37.86; 32.18; 30.86; 14.96.

(RS)-8-Methyl-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-c]azepin-3-olhydrobromide 10
A mixture of **2c** (1.52 g; 8.3 mmol) and 33% HBr in acetic acid (50 ml) was stirred at room temperature for 24 h. The reaction mixture was concentrated. The resulting crude crystals were recrystallized from acetone to give **10** (1.9 g; 92%), mp 230–232°C (dec). CHN: calc 38.57; 5.27; 11.25, found 38.52; 5.35; 11.32. ^{1}H-NMR (D_2O) δ 4.04–3.87 (dd, 2H, isoxazole-CH_2NH); 3.62–3.29 (m, 2H, $CH(CH_3)CH_2CH_2$); 3.20–3.04 (m, 1H, $CH(CH_3)CH_2CH_2$); 2.11–1.75 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.20–1.16 (d, 3H, $CH(CH_3)CH_2CH_2$).

t-Butyl (RS)-3-hydroxy-8-methyl-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-c]azepine-5-carboxylate 11
To a solution of **10** (1.9 g; 7.6 mmol) and K_2CO_3 (3.3 g; 23.9 mmol) in water (100 ml) was added THF (150 ml). The reaction mixture was cooled to 5°C and di-*t*-butyl pyrocarbonate (1.8 g; 8.4 mmol) was added. The mixture was stirred at 5°C for 4 h and at room temperature for 1 h. The THF was evaporated and water (100 ml) was added. The reaction mixture was extracted with ethyl acetate (3 x 250 ml). The extracts were dried (Na_2CO_3) and evaporated to afford **11** (1.8 g; 88%) as an oil. ^{1}H-NMR ($CDCl_3$) δ 4.40–4.10 (m, 2H, isoxazole-CH_2NH); 3.90–3.40 (m, 2H, $CH(CH_3)CH_2CH_2$); 3.15–3.00 (m, 1H, $CH(CH_3)CH_2CH_2$); 2.05–1.60 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.45 (s, 9H, $COC(CH_3)_3$); 1.34–1.30 (d, 3H, $CH(CH_3)CH_2CH_2$).

t-Butyl (RS)-8-methyl-3-propargyloxy-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-c]azepine-5-carboxylate 12
A mixture of **11** (600 mg; 2.2 mmol) and K_2CO_3 (490 mg; 3.5 mmol) in acetone (80 ml) was heated to reflux and propar-

gyl bromide (0.43 ml; 4.8 mmol) was added dropwise. The reaction mixture was refluxed for an additional 2 h, filtered and concentrated. After CC of the remaining oil (ethyl acetate/heptane/methanol, 5:5:1) **12** (350 mg; 52%) was isolated as an oil. ^{1}H-NMR ($CDCl_3$) δ 4.87–4.85 (d, 2H, $OCH_2C{\equiv}CH$); 4.40–4.05 (m, 2H, isoxazole-CH_2NH); 3.90–3.65 (m, 1H, $CH(CH_3)CH_2CH_2$); 3.55–2.95 (m, 2H, $CH(CH_3)CH_2CH_2$); 2.57–2.52 (m, 1H, $OCH_2C{\equiv}CH$); 2.05–1.60 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.40 (s, 9H, $COC(CH_3)_3$); 1.33–1.29 (d, 3H, $CH(CH_3)CH_2CH_2$).

(RS)-8-Methyl-3-propargyloxy-5,6,7,8-tetrahydro-4H-isoxazolo[4,5-c]azepine hydrochloride **2d**

To a solution of **12** (200 mg; 0.7 mmol) in diethyl ether (5 ml) a saturated solution of HCl in diethyl ether (10 ml) was added at 10°C, and the reaction mixture was stirred for 24 h at room temperature. The mixture was concentrated and the resulting crude crystals recrystallized from acetone/ethanol to give **2d** (110 mg; 69%), mp 157–159°C. CHN: calc 54.15; 6.35; 11.71, found 54.43; 6.24; 11.54. ^{1}H-NMR (methanol-d_3) δ 4.98–4.96 (d, 2H, $OCH_2C{\equiv}CH$); 4.13–4.08 (d, 2H, isoxazole-CH_2NH); 3.78–3.39 (m, 2H, $CH(CH_3)CH_2CH_2$); 3.37–3.20 (m, 1H, $CH(CH_3)CH_2CH_2$); 3.14–3.07 (m, 1H, $OCH_2C{\equiv}CH$); 2.29–1.88 (m, 2H, $CH(CH_3)CH_2CH_2$); 1.46–1.42 (d, 3H, $CH(CH_3)CH_2CH_2$). ^{13}C-NMR (methanol-d_3) δ 175.03; 167.54; 97.63; 76.24; 75.92; 56.74; 46.31; 37.54; 32.06; 29.92; 14.74.

Pharmacology

Receptor binding

[^{3}H]QNB binding to muscarinic receptor sites in membrane fractions prepared from rat brains was examined essentially, as described by Watson *et al* [32]. Briefly, rat brains were homogenized in 100 vol (w/v) 10 mM sodium potassium phosphate buffer (pH 7.4) and diluted 1:10 with the same buffer. Aliquots (0.5 mg tissue) were incubated in the presence of the test compound in a total volume of 4 ml buffer for 10 min at 37°C before addition of 1.0 ml 0.60 nM [^{3}H]QNB (Amersham; 1.63 TBq/mmol) and further incubation for 30 min at 37°C. Final concentration of [^{3}H]QNB was 0.12 nM. The reaction was stopped by adding 5 ml of ice-cold buffer and rapid filtration through Whatman GF/B-filters presoaked in 0.1% polyethylenamine. The filters were immediately washed twice with the same volume of ice-cold buffer and bound radioactivity estimated by liquid scintillation counting. Specific binding was defined as total binding minus binding in the presence of 20 μM atropine. The dissociation constant (K_D) for the binding of [^{3}H]QNB to rat brain membranes was determined to 13.7 ± 0.9 pM based on computer-assisted Scatchard analysis of binding data.

The procedure for determination of inhibition of [^{3}H]QNB binding to rat heart tissue was analogous to that described above for [^{3}H]QNB binding to rat brain membranes [32] with the exceptions that the tissue was homogenized in an ultrathorax homogenizer and 2 mg of tissue was used per assay. A Scatchard analysis of data from [^{3}H]QNB binding to rat heart tissue gave a K_D value of 9.1 ± 0.7 pM.

The procedure for determination of inhibition of [^{3}H]PZ binding to rat brain membranes was the same as described above for [^{3}H]QNB binding to such membrane fractions [32]: 3 mg of tissue was incubated with the test compound in a total volume of 1.25 ml of buffer for 10 min at 25°C before addition of 0.25 ml 6.0 nM [^{3}H]PZ (New England Nuclear; 3.22 TBq/mmol) and further incubation for 60 min at 25°C. The final concentration of [^{3}H]PZ was 1.0 nM. The reaction was stopped by filtration under reduced pressure followed by three washes with 4 ml ice-cold buffer. Specific binding was estimated by subtracting non-specific binding in the presence of 10 μM atropine. The K_D value of 1.8 ± 1.0 nM for the binding of [^{3}H]PZ to rat brain membranes was derived from a Scatchard analysis.

The procedure for determination of inhibition of [^{3}H]Oxo-M to rat brain membranes was performed as modified from the binding assay for [^{3}H]QNB binding to such membrane fractions [32]: 5 mg of tissue was incubated with the test compound in a total volume of 1.25 ml of buffer for 10 min at 30°C before addition of 0.25 ml 1.20 nM [^{3}H]Oxo-M (New England Nuclear, 3.22 TBq/mmol) and further incubation for 40 min at 30°C. The final concentration of [^{3}H]Oxo-M was 0.2 nM. The reaction was stopped by adding 5 ml of ice-cold buffer, rapid filtration and one wash with the same volume of buffer. Specific binding was estimated by subtracting non-specific binding in the presence of 10 μM atropine. A K_D value of 0.48 ± 0.03 nM for the binding of [^{3}H]Oxo-M to rat brain membranes was determined by Scatchard analysis.

Each compound was tested in five different concentrations and IC_{50} values estimated from hand-drawn log concentration–response curves. All determinations were made in triplicate, and each displacement experiment was repeated at least twice.

In a series of IC_{50} value determinations, the variance of the log ratio (VAR_R) between double determinations in each binding assay was determined according to the following formula:

$$VAR_R = \frac{1}{2n} \Sigma (\log R_i)^2$$

where R_i is the ith ratio and n is the number of observations. The VAR_R is equivalent to the square of the standard deviation of the log ratio (SD_R^2). The following SD_R-values were obtained: QNB (brain) 1.5 (n = 100), QNB (heart) 1.5 (n = 100), Oxo-M 1.5 (n = 100) and PZ 1.6 (n = 100).

Functional assays

The ability of the compounds to depolarize isolated rat superior cervical ganglion was used to estimate their M_1 agonistic effect and efficacy. Details have been described previously [33]. The potencies of the agonists are expressed by their EC_{50} values, *ie* the concentrations, which have effects equal to 50% of the individual maximum effects. The apparent efficacies of the agonists are expressed by their RM value, *ie* the maximum effect of agonists, relative to the maximum effect of muscarine, determined on separate ganglia. All EC_{50} values and RM values estimated for the ganglion are expressed as equal to or greater than the values calculated, because the cumulative concentration–response curve does not reach a 100% steady-state level at the highest drug concentration tested.

The ability of the compounds to depress the electrically stimulated contraction of isolated guinea-pig left atrium was used to estimate their M_2 agonistic effect and efficacy. Details have been described previously [33]. The potencies of the agonists are expressed by their EC_{50} values. The apparent efficacies are estimated by the RM value, *ie* the maximum depression of agonists, relative to the maximum depression of carbachol, measured on the same atrium.

The ability of the compounds to contract isolated guinea-pig ileum was used to estimate their mixed $M_1/M_2/M_3$ agonistic effect and efficacy. Details have been described previously [33]. The potencies of the agonists are expressed by their EC_{50} values. The apparent efficacies are estimated by the RM value, *ie* the maximum effect of the agonists, relative to the maximum effect of carbachol, measured on the same ileum strip.

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