Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 795–809

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Muscarinic Receptor Pharmacology of a Series of 4,5,6,7-Tetrahydroisothiazolo[4,5-*c*]pyridine Bioisosteres of Arecoline

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Received 17 August 1998; accepted 19 October 1998

Abstract—A series of *O*- and ring-alkylated derivatives of 4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridin-3-ol was synthesized via treatment of appropriately substituted 4-benzylamino-1,2,5,6-tetrahydropyridine-3-carboxamides with hydrogen sulfide and subsequent ring closure by oxidation with bromine. The muscarinic receptor affinity as well as estimated relative efficacy and subtype selectivity of this series of bicyclic arecoline bioisosteres were determined using rat brain membranes and a number of tritiated muscarinic receptor ligands. The effects at the five cloned human muscarinic receptor subtypes of a selected series of chiral analogues, with established absolute stereochemistry, were studied using receptor selection and amplification technology (R-SAT™). The potency, relative efficacy, and receptor subtype selectivity of these compounds were related to the structure of the *O*-substituents and the position and stereochemical orientation of the piperidine ring methyl substituents.

Introduction

Accumulating evidence supports 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] 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. In light of these observations, there is a therapeutic interest in 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 than full agonists 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 the synthesis and pharmacology of annulated bicyclic muscarinic agonists and partial agonists bioisosterically derived from arecoline (**1**) as exemplified by

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0968-0896/99/$ - see front matter
PII: S0968-0896(99)00033-4

3-methoxy-5-methyl-4,5,6,7-tetrahydroisoxazolo[4,5-*c*]-pyridinium (**2**)[19–21] and 3-methoxy-5-methyl-6,7-dihydro-4*H*-thiopyrano[3,4-*d*]isoxazol-5-ium (**3**)[22] halides (Fig. 1).

Structure–activity studies on a series of 3-alkoxy-5,6,7,8-tetrahydro-4*H*-isoxazolo[4,5-*c*]azepines, including **4–6** (Fig. 1), emphasized the determinative influence of the structure of the 3-alkoxy group on the pharmacology of these compounds.[23] In order to further elucidate the importance of the structure of the 'bottom part' of this class of conformationally restricted mAChR ligands, we have now synthesized and pharmacologically characterized a series of 3-alkoxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridines (**7–11**) (Fig. 1).

Synthesis

Methyl 3-hydroxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]-pyridine-5-carboxylate **12**, which is the precursor for compounds **7a–c** (Scheme 1) was prepared as described previously.[24] The syntheses of the 6- and 7-methylated analogues of **12**, compounds **23** (Scheme 2) and **30** (Scheme 3), respectively, were based on the corresponding β-oxo amides **21** and **28**, respectively, as key intermediates. In Scheme 2, the syntheses of the target molecules, **8c,d** and **9c**, via **21** and **23** are outlined. Methyl crotonate (**14**) was refluxed with benzylamine in methanol to give the amino ester **15** in nearly quantitative yield. The benzyl group was removed by treatment with ammonium formate in methanol, in the presence of palladium on activated carbon, in 96% yield. Treatment of the resulting aminoester **16** with acrylonitrile gave **17** in 95% yield, which was regioselectively cyclized with potassium *tert*-butoxide in toluene. The product, β-oxo nitrile **18**, was isolated as the ethyl carbamate in 35% yield. Treatment of **18** with sulphuric acid did not afford the desired amide **21**, and a different approach had to be used. Compound **18** was converted into the 1,3-dioxane **19** in nearly quantitative yield. After oxidative hydrolysis of **19** with basic hydrogen peroxide and subsequent acid hydrolysis of the 1,3-dioxane ring, **21** was obtained in 39% yield. The keto amide **21** was converted into the corresponding benzyl enamine **22** by treatment with benzylamine in refluxing xylene in a yield of 76%. The 3-isothiazolol unit was then established by treating enamine **22** with excess hydrogen sulfide in dimethylformamide (DMF) and subsequent treatment of the crude product with bromine in ethyl acetate to give **23** in 31% yield, following a previously described procedure.[24] In the first step of the conversion of **23** into the final products, **8c,d** and **9c**, a *tert*-butyloxycarbonyl (Boc) group was substituted for the ethoxycarbonyl group of **23** to give **24** (Scheme 2). Thus, after removal of the latter group, using hydrogen bromide in glacial acetic acid, the resulting amine salt was treated with potassium carbonate and $(Boc)_2O$ in a water:tetrahydrofuran (THF) mixture. Under these conditions, the 3-hydroxy group also became acylated, but the 3-carbonate group formed was selectively cleaved in methanol containing catalytic amounts of potassium carbonate.[25] The overall yield of these de- and reprotection reactions was 33%. The *O*-alkylations of **24**

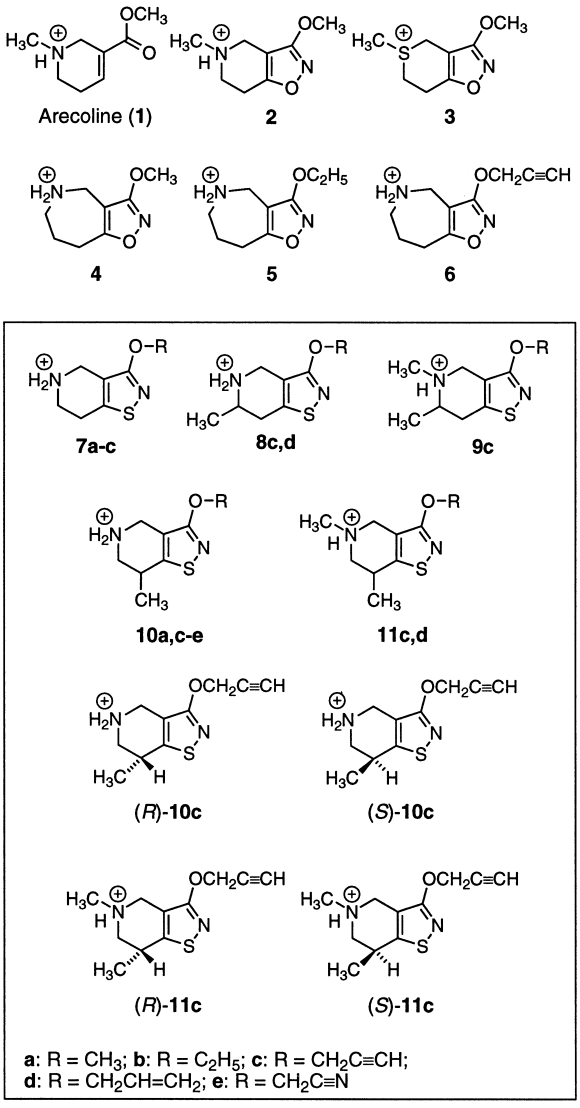
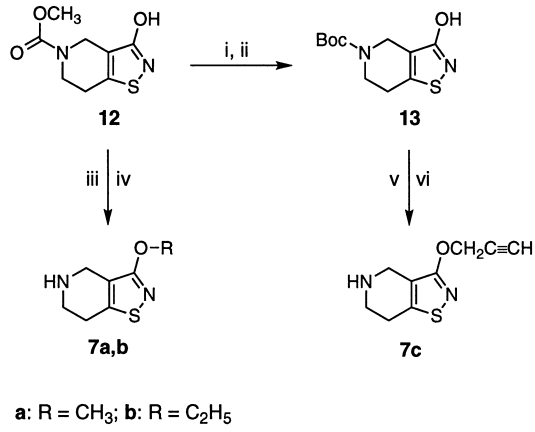
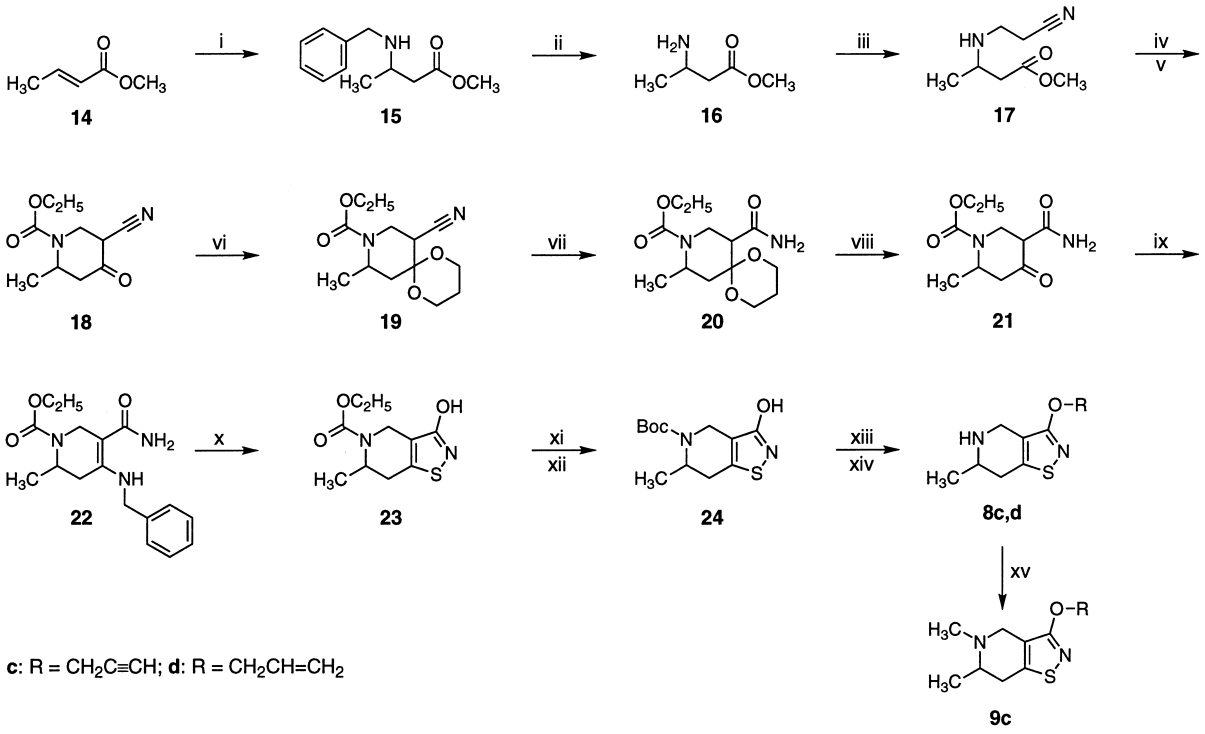


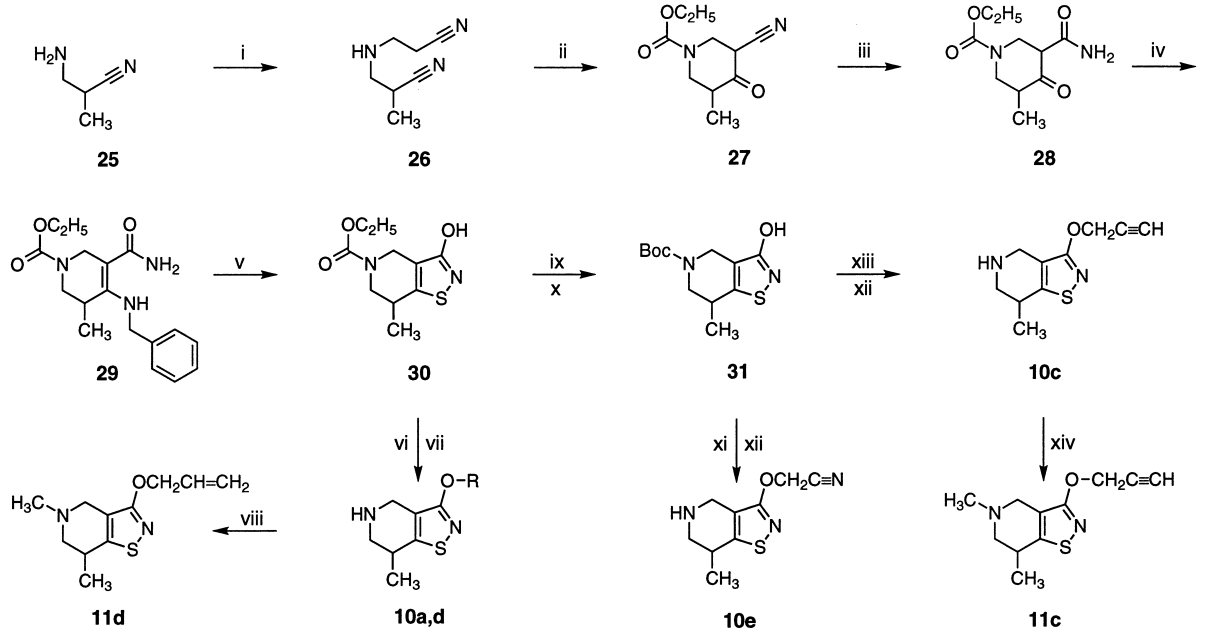
Figure 1. Structures of a number of known (**1–6**) and new (**7–11**) agonist, partial agonist, or antagonist ligands at central mAChRs.



Scheme 1. *Reagents and conditions*: (i) HBr/AcOH (33%); (ii) $(Boc)_2O$, K_2CO_3, K_2CO_3 (1%)/CH_3OH; (iii) CH_2N_2 (**a**), $(C_2H_5)_2SO_4$, $(C_4H_9)_4N{\cdot}HSO_4$, NaOH (**b**); (iv) KOH/CH_3OH; (v) $CH{\equiv}CCH_2Br$, K_2CO_3, $(C_4H_9)_4N{\cdot}HSO_4$; (vi) HCl, ether, NaOH.



Scheme 2. *Reagents and conditions*: (i) $C_6H_5CH_2NH_2$, C_2H_5OH; (ii) NH_4·HCOO, Pd-C (5%); (iii) $CH_2=CHCN$, CH_3OH; (iv) $KOC(CH_3)_3$, toluene; (v) $ClCOOC_2H_5$, K_2CO_3; (vi) $HOCH_2CH_2CH_2OH$, TsOH, toluene, reflux; (vii) H_2O_2, NaOH, C_2H_5OH; (viii) HCl, H_2O, THF; (ix) $C_6H_5CH_2NH_2$, toluene, reflux; (x) H_2S, DMF, Br_2; (xi) HBr/AcOH (33%); (xii) $(Boc)_2O$, K_2CO_3; (xiii) $CH\equiv CCH_2Br$, K_2CO_3, $(C_4H_9)_4N\cdot HSO_4$ (**c**), $CH_2=CHCH_2Br$, K_2CO_3 (**d**); (xiv) HCl, ether; (xv) HCOOH, CH_2O, reflux.



Scheme 3. *Reagents and conditions*: (i) $CH_2=CHCN$, C_2H_5OH; (ii) $KOC(CH_3)_3$, toluene, $ClCOOC_2H_5$; (iii) H_2SO_4 (85%), 60–70 °C; (iv) $C_6H_5CH_2NH_2$, xylene, reflux; (v) H_2S, DMF, Br_2; (vi) $(CH_3)_2SO_4$, $(C_4H_9)_4N\cdot HSO_4$, NaOH (**a**), $CH_2=CHCH_2Br$, $(C_4H_9)_4N\cdot HSO_4$, K_2CO_3 (**d**); (vii) KOH/CH_3OH; (viii) HCOOH, CH_2O, reflux; (ix) HBr/AcOH (33%); (x) $(Boc)_2O$, K_2CO_3; (xi) $CNCH_2Cl$, $(C_4H_9)_4N\cdot HSO_4$, K_2CO_3; (xii) HCl, ether, NaOH; (xiii) $CH\equiv CCH_2Br$, $(C_4H_9)_4N\cdot HSO_4$, K_2CO_3; (xiv) HCOOH, CH_2O, reflux.

Table 1. Alkylation reactions and resolutions after formation of the 4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridin-3-ol ring system

Starting compound	Alkylating agent	Yield (%) of *N*-deprotected product	Yield (%) of optical resolution	Yield (%) of *N*-methylated product
12	CH_2N_2	**7a**,HCl (45%)		
12	$(C_2H_5)_2SO_4$	**7b**, maleate (29%)		
13	$CH{\equiv}CCH_2Br$	**7c**, hemifumarate (55%)		
24	$CH{\equiv}CCH_2Br$	**8c**, HCl (45%)		**9c**, oxalate (32%)
			(−)-**8c**, fumarate (18%)	(−)-**9c**, oxalate (33%)
			(+)-**8c**, hemifumarate (24%)	(+)-**9c**, oxalate (31%)
24	$CH_2{=}CHCH_2Br$	**8d**, fumarate (32%)		
30	$(CH_3)_2SO_4$	**10a**, fumarate (18%)		
30	$CH_2{=}CHCH_2Br$	**10d**, fumarate (36%)		**11d**, oxalate (40%)
			(−)-**10d**, hemi-L-tartrate (11%)	
			(+)-**10d**, hemi-D-tartrate (8%)	
31	$CH{\equiv}C{-}CH_2Br$	**10c**, fumarate (41%)		**11c**, oxalate (57%)
			(*R*)-(−)-**10c**, fumarate (22%)	(*R*)-(−)-**11c**, oxalate (56%)
			(*S*)-(+)-**10c**, fumarate (26%)	(*S*)-(+)-**11c**, oxalate (57%)
31	$N{\equiv}CCH_2Cl$	**10e**, fumarate (33%)		

were performed using the appropriate alkyl halides in DMF in the presence of potassium carbonate and tetrabutylammonium hydrogen sulphate. In both cases, parallel alkylations at N-2 were observed, but no attempts were made to purify and characterize these by-products. The Boc-protected and *O*-alkylated products were deprotected by hydrogen chloride in ether to give the corresponding secondary amines **8c,d**. Compound **8c** was N-5-methylated by a standard Eschweiler–Clarke reaction involving heating in formic acid in the presence of formaldehyde. The yields of these reactions, starting with compound **24**, and the salts of these C-6-methylated final products isolated are listed in Table 1.

The syntheses of the C-7-methylated target compounds, **10a,c–e** and **11c,d**, are outlined in Scheme 3. The reaction sequence for the preparation of the key intermediates, β-oxo amide **28** and 3-isothiazolols **30** and **31**, are with a few exceptions analogous with those shown in Scheme 2. Thus, **28** was synthesized from 2-methyl-3-aminopropionitrile **25**.[26] Addition of acrylonitrile gave dinitrile **26**, which under Thorpe–Ziegler cyclization conditions, using *tert*-butoxide in toluene, and subsequent acid hydrolysis and protection of the secondary amine gave the desired β-oxo nitrile **27** in 87% yield. Hydrolysis of the nitrile group of **27** gave β-oxo amide **28** in 64% yield. The results of the transformations of 3-isothiazolols **30** and **31** into the final products containing secondary amino groups (**10a,c–e**) and those containing *N*-methyl tertiary amino groups (**11c,d**) are listed in Table 1.

On the basis of the results of the pharmacological studies, it was decided to subject compounds **8c**, **10c**, and **10d** to optical resolution procedures. Subsequently, the enantiomers of **8c** and **10c** were transformed into the respective enantiomers of the N-5-methylated analogues, **9c** and **11c**, using Eschweiler–Clarke reaction conditions. The optical resolutions of **8c** and **10c** were accomplished by precipitation of diastereomeric salts with (2*S*,3*S*)-(+)-*O*,*O*′-dibenzoyltartaric acid [D-(+)-dibenzoyltartaric acid] and (2*R*,3*R*)-(−)-*O*,*O*′-diben-

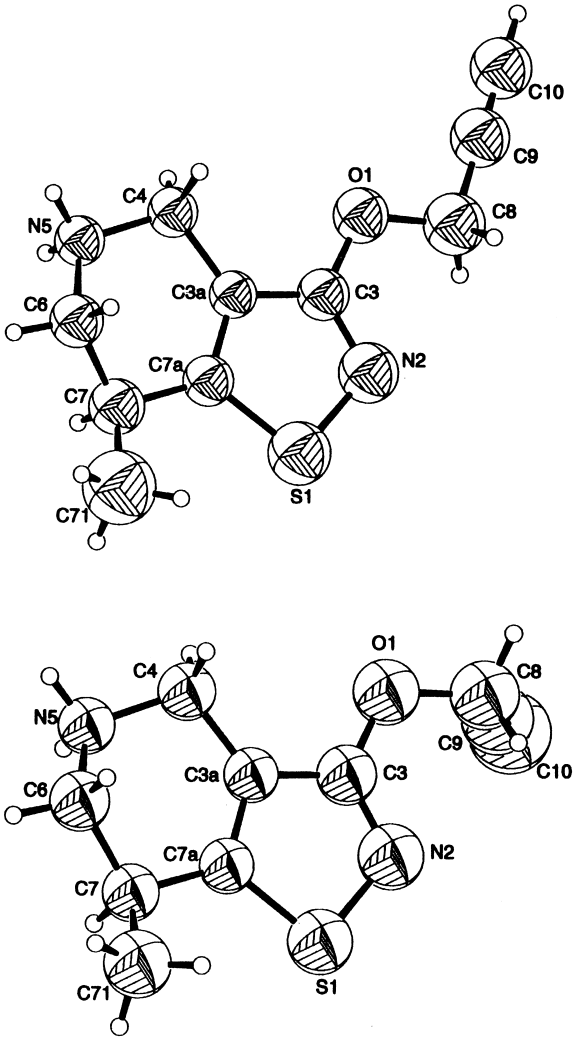


Figure 2. Perspective drawings[27] of the two conformationally different cations of compound (*S*)-(+)-**10c** crystallized as a salt with D-(+)-dibenzoyltartaric acid. Spheres representing the isotropic or equivalent isotropic displacement parameters of the non-hydrogen atoms are shown at the 50% probability level Hydrogen atoms in calculated positions are represented by spheres of arbitrary size.

zoyltartaric acid [L-(−)-dibenzoyltartaric acid]. Compound **10d** was resolved by precipitation of diastereomeric salts with D-(−)- and L-(+)-tartaric acids. The results of these resolution procedures are shown in Table 1. The optical purity was determined by HPLC, using a chiral stationary phase, or by ^{1}H NMR spectroscopy, using the chiral shift reagent (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol which had sufficient enantioselective influence on the chemical shifts of the methyl groups at C-6 of the enantiomers of **8c** or the methyl groups at C-7 of the enantiomers of **10c** and **10d**. The absolute configuration of (*R*)-(−)- and (*S*)-(+)-**10c** and, thus of (*R*)-(−)- and (*S*)-(+)-**11c** was established by an X-ray crystallographic analysis.

X-ray crystallographic analysis

The X-ray analysis established the absolute configuration of (+)-**10c**, crystallized as a salt with D-(+)-dibenzoyltartaric acid, to be *S*. This salt is a 2:1 complex of (*S*)-(+)-**10c** to the D-(+)-dibenzoyltartrate dianion together with an ethanol molecule of solvation from the recrystallization. Thus, the absolute configuration of (*S*)-(+)-**10c** was based on the salt with an anion of known absolute stereochemistry. The geometry of cationic (*S*)-(+)-**10c** with atomic labeling is shown in Figure 2.

Receptor binding

The compounds **5–11** were studied in receptor binding assays using rat brain and rat brain stem 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 radioligand binding studies, M_2/M_1 index[21] and agonist index[21,28] values were calculated (Table 2). The former index is indicative of selectivity for the M_1 receptor site, higher M_2/M_1 index values predicting greater M_1 selectivity.[21] The agonist index values, on the other hand, have been shown to be predictive of relative efficacy of compounds at mAChRs,[21,28] and in our test setup, values above 100 and between 100 and 10 are considered indicative of full and partial mAChR agonism, respectively, whereas values below 10 are predictive of antagonism.[29] It must be emphasized that these indexes are empirical[21,28] and should only be used to predict effects at mAChRs in relative terms within series of structurally related compounds. On the basis of calculated M_2/M_1 index and agonist index values (Table 2) a few compounds of particularly interesting apparent pharmacological profile were selected for studies using the five cloned human mAChRs (m1–m5) using the functional assay, receptor selection and amplification technology (R-SAT™)[23,30–32] (see subsequent section).

Table 2. Receptor binding data of muscarinic ligands using rat brain and heart tissues

	Receptor binding					
	[^{3}H]Oxo-M[a] (brain)	[^{3}H]PZ[b] (brain)	[^{3}H]QNB[c] (brain)	[^{3}H]QNB[d] (brain stem)	Agonist[e] index	M2/M1[f] index
Compound	K_i values (nM)					
Oxotremorine	0.27	35	130	3.6	480	0.1
Arecoline (**1**)	1.3	650	1100	42	850	0.06
5	0.35	7.1	19	2.7	54	0.4
6	0.24	10	22	1.0	92	0.1
7a	12	1000	2300	370	192	0.4
7b	20	240	290	33	15	0.1
7c	4.4	34	230	50	52	1.5
8c	14	14	120	92	8.6	6.6
(−)-**8c**	11	19	100	85	9.1	4.5
(+)-**8c**	41	37	330	250	8.0	6.8
8d	65	57	310	210	4.8	3.7
9c	6.1	7.7	34	24	5.6	3.1
(−)-**9c**	1.6	1.2	28	20	18	17
(+)-**9c**	7.7	6.0	81	54	15	9.0
10a	17	84	290	110	17	1.3
10c	1.3	4.4	33	13	25	3.0
(*R*)-(−)-**10c**	0.49	5.0	23	11	47	2.2
(*S*)-(+)-**10c**	1.1	3.1	20	20	18	6.5
10d	3.7	7.1	26	18	7.0	2.5
(−)-**10d**	7.0	23	42	36	6.0	1.6
(+)-**10d**	9.2	10	49	32	5.3	3.2
10e	28	31	430	160	15	5.2
11c	3.7	4.3	54	61	15	14
(*R*)-(−)-**11c**	2.7	2.8	35	32	13	11
(*S*)-(+)-**11c**	13	9.0	190	180	15	20
11d	23	17	160	160	7.0	9.4

[a][^{3}H]Oxo-M binding on rat brain homogenate.
[b][^{3}H]PZ binding on rat brain homogenate.
[c][^{3}H]QNB binding on rat brain homogenate.
[d][^{3}H]QNB binding on rat brain stem homogenate. For details see ref 43.
[e]Agonist index = K_i (QNB_{brain})/K_i (Oxo-M).
[f]M2/M1 index = K_i ($QNB_{brain\ stem}$)/K_i (PZ).

Within the group of 3-alkoxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridines without substituents in the tetrahydropyridine ring, compounds **7a–c** (Fig. 1), only **7a** shows the characteristics of a full mAChR agonist (agonist index = 192) (Table 2), whereas **7c** shows the highest degree of M_1 selectivity (M_2/M_1 index = 1.5). This receptor selectivity, which is of particular pharmacological interest, could be increased by incorporation of methyl groups at various sites of the molecule of **7c**. Thus, the C-6 methyl substituted analogue of **7c**, compound **8c**, appears to show at least 4 times higher M_1 selectivity (M_2/M_1 index = 6.6) than **7c**. Whereas methylation of **7c** at C-7, to give **10c**, results in a slightly increased M_1 selectivity, *N*-methylation of **10c** markedly increases M_1 selectivity, **11c** showing an M_2/M_1 index value of 14, which is about an order of magnitude higher than that of the parent compound, **7c**.

In agreement with earlier observations[21,22] substitution of a propargyloxy group for the methoxy group of **7a**, to give **7c**, increases mAChR affinity in the [^{3}H]Oxo-M binding assay, assumed to reflect the affinity for the agonist conformation of the mAChRs.[33] Similarly, compound **10c**, containing an *O*-propargyl group, is about an order of magnitude more potent than the corresponding *O*-methyl analogue, **10a**, in this binding assay (Table 2). Whereas the *O*-alkyl analogue, **10d**, is slightly weaker, the *O*-cyanomethyl analogue, **10e**, is markedly weaker than **10c**. The compounds **8c**, **10c**, and **10d** were selected for optical resolution, and the enantiomers of these compounds were obtained via diastereomeric salt formation using enantiomers of tartaric acid or dibenzoyltartaric acid as resolving agents. The receptor affinity data and calculated agonist index and M_2/M_1 index values for these five pairs of enantiomers are compared with the respective data for the parent racemates in Table 2. In all cases, a remarkably low degree of stereoselectivity was observed.

Effects on cloned human mAChRs

The effects of the enantiomeric pairs of **10c** and **11c** at cloned human m1–m5 were studied using the functional assay, R-SAT™.[23,30–32] In agreement with the receptor binding data (Table 2), all four compounds were potent mAChR ligands, showing EC_{50} (agonists) or K_i (antagonists) values in the nanomolar range (Table 3, Fig. 3). Both enantiomers of **10c** showed partial agonism at m1, m2, and m4, (*R*)-(−)-**10c** having the highest relative efficacy at all three receptors. Both compounds showed higher relative efficacy at m2 and m4 than at m1, a tendency which has been seen for a number of standard compounds (Table 3), and which, at least partially, is an inherent result of the R-SAT™ technology.[30] Compared to the standard compound **6**,[23] both enantiomers of **10c** showed lower relative efficacy at all five human mAChRs, but at m3 and m5 neither compound displayed any detectable activity. A Schild analysis[34] revealed that (*R*)-(−)-**10c** as well as (*S*)-(+)-**10c** showed antagonist effects at m3 and m5 (Table 3). The Schild analysis of data from studies on the m5 receptor was consistent with both enantiomers of **10c** acting as competitive antagonists (no suppression of maximal response to the standard full agonist carbachol, and a slope not significantly different from unity). A similar analysis of the effects of the **10c** enantiomers at m3 did, on the other hand, show signs of insurmountable antagonism (statistically significant suppression of maximal responses at higher antagonist concentrations) (Fig. 4).

At all five receptors, except m3, (*S*)-(+)-**10c** was slightly more potent than (*R*)-(−)-**10c**. Both compounds were

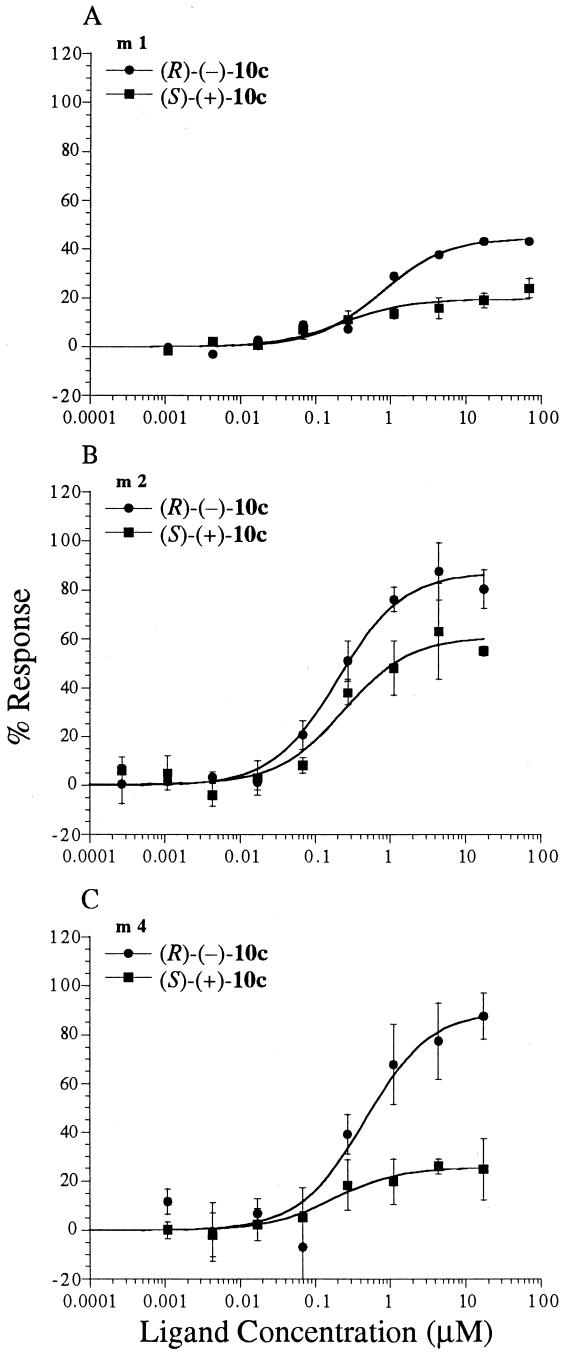


Figure 3. Pharmacological profile of (*R*)-(−)-**10c** and (*S*)-(+)-**10c** using the cloned human mAChRs as determined by R-SAT: (A) m1, (B) m2, and (C) m4. % Response indicates responses relative to maximal responses by the full agonist carbachol. Each datapoint is the mean value ± SEM of a representative experiment performed in triplicate (m1) or quadruplicate (m2 and m4).

slightly more potent at m2 and m4 than at m1, m3, and m5, a trend also seen for a number of standard compounds such as carbachol, arecoline (**1**), oxotremorine, **5**, and **6** (Table 3). This can be partially explained by a higher receptor reserve/G-protein coupling of m2 and m4 in R-SAT.[30,35]

The *N*-methylated analogues of the enantiomers of **10c**, (*R*)-(−)-**11c** and (*S*)-(+)-**11c**, showed no agonist effects at m1, m3, or m5. A Schild analysis of data from studies of effects of these enantiomers at m1 and m5 was consistent with competitive antagonism by both compounds with no suppression of maximal responses and with slopes not significantly different from unity. Similar analyses on the effects of (*R*)-(−)-**11c** and (*S*)-(+)-**11c** at m3 did, however, show significant inhibition of maximal responses at higher antagonist concentrations, thus indicating insurmountable antagonism. The (*R*)-enantiomer was the more potent compound at all three receptors.

Discussion

The naturally occurring alkaloid, arecoline (**1**) is an agonist at mAChRs and has been shown to improve cognition when administered to AD patients[36] and to enhance learning in young humans and aged non-human primates.[37,38] The pharmacological effects of **1** are, however, shortlived, probably due to hydrolysis of the ester group, and the adverse effects of **1** after administration in man may reflect lack of M_1 receptor selectivity and non-optimal relative agonist efficacy at subtypes of central mAChRs. As attempts to develop mAChR agonists or partial agonists more suitable for therapeutic use, we have previously synthesized and pharmacologically described different series of conformationally restricted bicyclic analogues of **1** containing the ring systems of **2**,[19,20] **3**,[22] and **4–6**.[23]

Structure–activity studies on these previously described series of mAChR ligands led to the conclusion that the

Table 3. Pharmacological parameters of muscarinic agonists and antagonists at the five cloned human muscarinic receptor subtypes determined by receptor selection and amplification technology (R-SAT)

	Receptor response (% of maximal carbachol response)					
	m1	m2	m3	m4	m5	EC_{50}(m2)/EC_{50}(m1)
Compound	pEC_{50} or pK_B					
Carbachol[a]	5.3 ± 0.0 (100%)	7.1 ± 0.0 (100%)	5.9 ± 0.1 (100%)	7.1 ± 0.1 (100%)	6.5 ± 0.0 (100%)	0.016
Arecoline (**1**)[a]	5.5 ± 0.1 (86 ± 3%)	7.6 ± 0.0 (105 ± 0%)	6.5 ± 0.1 (66 ± 9%)	7.0 ± 0.2 (70 ± 3%)	6.2 ± 0.0 (77 ± 2%)	0.078
Oxotremorine[a]	6.4 ± 0.1 (75 ± 10%)	7.9 ± 0.3 (105 ± 6%)	6.7 ± 0.1 (66 ± 5%)	7.5 ± 0.2 (102 ± 3%)	7.3 ± 0.0 (74 ± 2%)	0.049
5[a]	7.1 ± 0.1 (42 ± 2%)	7.4 ± 0.1 (85 ± 6%)	6.4 ± 0.2 (34 ± 6%)	7.2 ± 0.1 (89 ± 5%)	*5.8 ± 0.2*[b]	0.43
6[a]	6.9 ± 0.1 (59 ± 9%)	8.3 ± 0.2 (86 ± 2%)	6.9 ± 0.1 (70 ± 8%)	7.5 ± 0.1 (105 ± 4%)	6.4 ± 0.2 (36 ± 5%)	0.048
(*R*)-(−)-**10c**	6.1 ± 0.0 (39 ± 3%)	6.7 ± 0.1 (80 ± 11%)	*6.6 ± 0.1*[c]	6.7 ± 0.1 (94 ± 6%)	*6.1 ± 0.3*[d]	0.24
(*S*)-(+)-**10c**	6.6 ± 0.0 (14 ± 2%)	6.9 ± 0.2 (67 ± 5%)	*6.4 ± 0.1*[c]	6.9 ± 0.1 (34 ± 6%)	*6.6 ± 0.2*[d]	0.57
(*R*)-(−)-**11c**	*6.7 ± 0.0*[d]	nd	*6.3 ± 0.1*[c]	nd	*6.3 ± 0.2*[d]	
(*S*)-(+)-**11c**	*6.6 ± 0.2*[d]	nd	*6.1 ± 0.2*[c]	nd	*6.0 ± 0.1*[d]	

[a]From ref 23.
[b]pK_B value calculated from IC_{50} by the Cheng–Prusoff equation.[44]
[c]Schild analysis shows signs of insurmountable antagonism (depressions of maximal response at higher antagonist doses); pK_B values calculated as described by Kenakin.[53]
[d]pK_B values calculated from Schild analysis.[34]
nd, not determined. Data represent the mean (± SEM) of two to five experiments. Antagonism values are shown in italics.

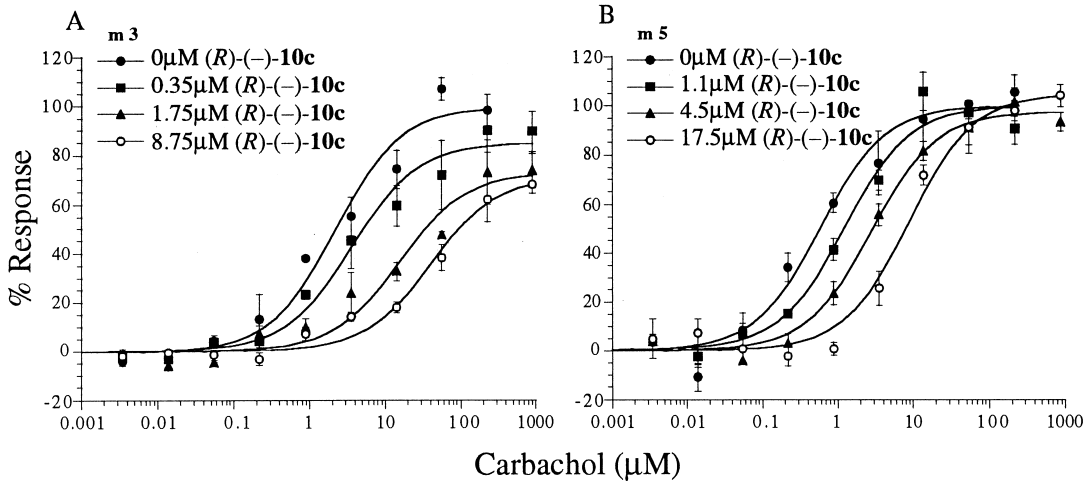


Figure 4. Pharmacological profile of (*R*)-(−)-**10c** using the cloned human mAChRs as determined by R-SAT: (A) m3 and (B) m5. % Response indicates responses relative to maximal responses by the full agonist carbachol. At higher concentrations of (*R*)-(−)-**10c** there was a statistically significant supression of maximal responses ($p < 0.05$). Each datapoint is the mean value ± SEM of a representative experiment performed in triplicate.

structure of the 'top part' of the molecules, including the structure of the alkoxy groups and the substitution at the cationic centers, largely determine the pharmacology of the compounds.[21] Although compounds **4–6** did not show degrees of M_1 selectivity or relative agonist efficacies markedly different from those of their lower ring homologues containing tetrahydropyridine rings, their 10–20 times higher potency promoted the idea that the pharmacological parameters of such mAChR ligands might be optimized by structural manipulation of the 'bottom part' of the molecules, assumed to interact with a lipophilic part of the receptors.[39] In the new compounds, **7–11** (Fig. 1), we have therefore varied not only the structure of the 'top part' of the molecules, but we have also introduced, in a stereospecific manner, methyl groups into different positions of the 'bottom part'. The isoxazole ring of previously described compounds, exemplified by compounds **2–6**, has been replaced by an isothiazol ring in order to increase the lipophilicity of the 'bottom part' of the molecules (Fig. 1).

As in previous structure–activity studies on mAChR ligands, M_1 selectivity and relative agonist efficacy were estimated on the basis of M_2/M_1 and agonist index values, respectively, derived from mAChR binding data using different radioligands[21,28,29] (Table 2). Two pairs of enantiomers, (*R*)-(−)- and (*S*)-(+)-**10c** and (*R*)-(−)- and (*S*)-(+)-**11c** were further characterized pharmacologically and compared with a series of standard mAChR ligands using the five cloned human receptors m1–m5 (Table 3). The relative order of M_1 selectivity has been estimated using the M_2/M_1 index (Table 2) and determined using the EC_{50} (m2)/EC_{50} (m1) ratio (Table 3). Higher values of these two series of data indicate higher M_1 selectivity, and the relative order of these two sets of data actually are similar, though not identical. As examples, the relative order of M_1 selectivity of **5** and **6** and of (*R*)-(−)-**10c** and (*S*)-(+)-**10c** are identical.

All of the new compounds, **7–11**, are potent inhibitors of the binding of mAChR radioligands, and in the [^{3}H]Oxo-M agonist binding assays, K_i values are typically found in the low nanomolar range (Table 2). With the exception of **7a**, all of the compounds show agonist index values below 100, indicating partial agonism (values between 100 and 10) or antagonism (values below 10),[29] a majority of compounds showing the characteristics of low-efficacy partial agonists (Table 2). In the functional assays, the enantiomers of **10c** show partial agonist or antagonist effects, whereas the enantiomers of **11c** show antagonism of the receptors tested (Table 3). This suggests that the compounds preferentially interact with the antagonist conformation(s) of the mAChRs,[33] and may explain why most of the new compounds show comparable effects in the mAChR agonist binding assay using [^{3}H]Oxo-M as the radioligand (Table 2). In other series of structurally related mAChR ligands, as exemplified by **2**,[21] **3**,[22] and **4–6**,[23] which typically show higher degrees of relative agonist efficacies, structural changes of the compounds typically have more marked influence on agonist binding affinities.

Five of the new compounds, **8c**, **9c**, **10c**, **10d** and **11c**, were resolved, and the absolute stereochemistry of the enantiomers of **10c** and **11c** was established by an X-ray analysis. Generally, these pairs show a remarkably low degree stereoselectivity in the binding (Table 2) and functional (Table 3) assays. This may reflect that the chiral centers of these compounds are located at sites (C-6 or C-7), which, as previously postulated,[39] interact with very lipophilic and perhaps not particularly chiral parts of the mAChR sites.

Some of the compounds described in this paper show pharmacological profiles, which make them interesting candidates for clinical studies in, for example, AD patients. These aspects will not be described and discussed in the present paper.

Experimental

Chemistry

Column chromatography (CC) was performed on silica gel 60, 230–400 mesh, ASTM. Evaporations were performed under vacuum at approximately 20 mmHg. Melting points were determined in capillary tubes and are not corrected. ^{1}H NMR Spectra were recorded at 80 MHz on a Bruker WP 80 DS spectrometer or at 250 MHz on a Bruker AC 250 spectrometer with TMS as an internal standard; where nothing is stated, the spectrum was recorded at 80 MHz. Chemical shifts are expressed in ppm values and are relative to internal TMS. The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t = triplet, q = quartet, qui = quintet, m = multiplet. NMR signal corresponding to acidic protons are omitted. Contents of H_2O in crystalline compounds was determined by Karl Fischer titration. Microanalyses, Karl Fischer titrations and optical rotations were performed by Lundbeck Analytical Department, and results obtained for microanalyses were within ±0.4% of the theoretical values unless otherwise stated.

3-Methoxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (7a) hydrochloride. To a solution of methyl 3-hydroxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-5-carboxylate (**12**)[24] (1.60 g, 7.5 mmol) in Et_2O (50 mL) and EtOH (2 mL) was added excess diazomethane. The mixture was stirred at room temperature for 1 h, and excess diazomethane was destroyed by addition of AcOH. The mixture was evaporated and the residue was eluted from silica gel (CC) with toluene/EtOAc 1/1. The isolated *O*-methyl isomer (0.89 g, 3.9 mmol) was dissolved in MeOH (9 mL) containing KOH (2.0 g), and the mixture was refluxed for 20 h. The reaction mixture was evaporated. The residue was dissolved in H_2O (30 mL) and extracted with $CHCl_3$ (3×50 mL). Standard workup of the combined organic phases yielded an oil which crystallized as the hydrochloric salt from Et_2O/EtOAc. Yield: 0.70 g (45%); mp 234–35 °C. ^{1}H NMR (DMSO-d_6) 2.85–3.20 (m, 2H), 3.18–3.48 (m, 2H), 3.99 (s, 2H), 3.95 (s, 3H). Anal. ($C_7H_{10}N_2OS$·HCl) H; C: calcd, 40.66; found, 42.79. N: calcd, 13.55; found 12.51.

3-Ethoxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (7b) maleate. A solution of **12**[24] (1.50 g, 7.0 mmol), tetrabutylammonium hydrogen sulphate (0.1 g, 0.31 mmol) and NaOH (0.6 g, 15 mmol) in CH_2Cl_2 (6 mL) and H_2O (6 mL) was stirred for 10 min. Et_2SO_4 (1 mL, 7.63 mmol) was added and the mixture was refluxed for 16 h. Concentrated NH_4OH (10 mL) was added and reflux was continued for 1 h. The mixture was diluted with CH_2Cl_2 (100 mL) and the phases were separated. Conventional workup of the organic phase gave an oil (2.5 g) which was eluted from silica gel (CC) with EtOAc/heptane (1/2) to give 1.1 g (4.5 mmol, 65%) of the protected *O*-ethyl derivative which was deprotected analogously to the preparation of **7a**. Yield of **7b**: 0.40 g (50%); mp 163–164 °C (EtOAc); ^{1}H NMR (DMSO-d_6) 1.30 (t, 3H), 2.90–3.25 (m, 2H), 3.25–3.55 (m, 2H), 4.05 (s, 2H), 4.40 (q, 2H), 6.05 (s, 2H). Anal. ($C_8H_{12}N_2OS \cdot C_4H_4O_4$) C, H, N.

***tert*-Butyl 3-hydroxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]-pyridine-5-carboxylate (13).** A solution of **12**[24] (6.40 g, 30 mmol) in 33% HBr in AcOH (100 mL) was stirred for 72 h. The mixture was evaporated, and EtOH (10 mL) was added and evaporated. The residue was dissolved in H_2O (60 mL) and was washed with CH_2Cl_2 (2×25 mL). The aqueous phase was cooled on an ice bath and K_2CO_3 (7 g, 51 mmol) was added below 20 °C. The aqueous phase was washed with CH_2Cl_2 (100 mL), and was cooled to 5 °C. A solution of $(Boc)_2O$ (10 g, 52 mmol) in THF (100 mL) was added below 15 °C. After addition the mixture is stirred for 16 h. The phases were separated, and the aqueous phase was extracted with Et_2O (4×30 mL). The combined organic phases were washed with saturated NaCl solution (3×30 mL). Drying over $MgSO_4$ and activated carbon and evaporation yielded 9.5 g of a semicrystalline material (mostly diBoc-derivative), which was dissolved in MeOH (200 mL). Anhydrous K_2CO_3 (3.5 g, 25 mmol) was added, and the mixture was stirred for 14 h. MeOH was removed, and the residue was dissolved in H_2O (50 mL) and washed with Et_2O (3×30 mL). The aqueous phase was acidified to pH 6 with concentrated HCl and extracted with EtOAc (4×30 mL). The EtOAc phases were washed with saturated NaCl solution (2×30 mL), dried over $MgSO_4$ and evaporated to yield 7.0 g of crude product.

3-Propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (7c) hemifumarate. To a solution of crude *tert*-butyl 3-hydroxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-5-carboxylate **13** (1.50 g, ca. 5.6 mmol) in DMF (30 mL) was added tetrabutylammonium hydrogen sulphate (0.1 g, 0.31 mmol), K_2CO_3 (2.0 g, 14.5 mmol) and 3-bromopropyne (1.5 mL, 16.8 mmol) and the mixture was stirred for 4 h at 70 °C. The solvent was removed, and the residue was dissolved in Et_2O (100 mL). The organic phase was washed with saturated $CaCl_2$ solution (3×50 mL) and was then worked up in a conventional manner. The residue was dissolved in Et_2O (20 mL) and a concentrated solution of HCl in Et_2O (20 mL) was added. After stirring for 3 h at room temperature the solvent was removed and the oily residue was dissolved in H_2O (50 mL). The aqueous phase was made basic with 2 M NaOH solution and the free base of **7c** was isolated in a conventional manner. The hemifumarate was crystallized from Me_2CO. Yield: 0.60 g (55%); mp 186–188 °C; ^{1}H NMR (DMSO-d_6) 2.75–3.25 (m, 4H), 3.55 (t, 1H), 3.75 (s, 2H), 5.0 (d, 2H), 6.50 (s, 1H). Anal. ($C_9H_{10}N_2OS \cdot 0.5C_4H_4O_4$) C, H, N.

Methyl (*RS*)-3-(*N*-benzylamino)butyrate (15). A solution of **14** (1188 g, 11.9 mol) and benzylamine (980 g, 9.2 mol) in EtOH (1 L) was heated to reflux for 16 h. Removal of the solvent gave the title product as an oil, which was used for the synthesis of **16** without further purification. Yield: 1890 g (99%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.20 (d, 3H),1.80 (s, 1H), 2.45 (dd, 2H), 3.20 (sextet, 1H), 3.70 (s, 3H), 3.80 (s, 2H).

Methyl (*RS*)-3-aminobutyrate (16). To a solution of crude **15** (485 g, ca. 2.34 mol) in MeOH (1.7 L) was added ammonium formate (269 g, 3.5 mol) and 5% palladium on charcoal (59 g). The mixture was refluxed for 3 h. Filtration and removal of the solvent gave the title product as an oil, which was used for the synthesis of **17** without further purification. Yield: 232 g (94%). ^{1}H NMR ($CDCl_3$, 250 MHz) 1.20 (d, 3H), 2.80 (s, 2H), 2.40–2.55 (m, 2H), 3.25–3.70 (m, 1H), 3.75 (s, 3H).

Methyl *rac*-3-amino-*N*-(2-cyanoethyl)butyrate (17). A solution of crude **16** (248 g, ca. 2.11 mol) and acrylonitrile (140 mL, 2.13 mol) in MeOH (490 mL) was heated to reflux for 60 h. Removal of the solvent gave the title compound as an oil, which was used for the synthesis of **18** without further purification. Yield: 318 g (89%). ^{1}H NMR ($CDCl_3$, 250 MHz) 1.20 (d, 3H), 2.00 (s, 1H), 2.25–2.75 (m, 4H), 2.80–3.45 (m, 3H), 3.75 (s, 3H).

Ethyl *rac*-3-cyano-6-methyl-4-oxopiperidine-1-carboxylate (18). A mixture of potassium *tert*-butoxide (213 g, 1.90 mol) in toluene (2.2 L) was heated to 70 °C with stirring. Crude **17** (318 g, ca. 1.87 mol) was added while keeping the temperature at 70–80 °C. After the addition the mixture was heated to reflux for 30 min. After cooling to 10 °C on an ice bath the potassium enolate was filtered off and was dissolved in ice H_2O (2 L) (the filtercake must be kept wet with toluene to avoid decomposition). K_2CO_3 (310 g, 2.25 mol) was added, and ethyl chloroformate (196 mL, 2.05 mol) was added dropwise at 10 °C. After the addition the mixture was stirred for 16 h at room temperature. EtOAc (1 L) was added and the organic phase was worked up in the usual manner, giving the 4-enolcarbonate of **18** as an oil (167 g, 0.59 mol). The aqueous phase was acidified with 4 M HCl and was worked up with EtOAc in the usual manner giving the title compound (109 g, 0.52 mol). The **18**-4-enol carbonate was dissolved in MeOH (1 L) containing K_2CO_3 (50 g) and the mixture was stirred for 16 h at room temperature. The solvent was removed, and the residue was dissolved in H_2O (1 L). The aqueous phase was acidified with 4 M HCl and was worked up with EtOAc in the usual manner giving the title product (99 g, 0.47 mol), which was used for the synthesis of **19** without further purification. Total yield: 208 g (53%). A sample was crystallized from EtOAc: Mp 63–65 °C. ^{1}H NMR ($CDCl_3$, 250 MHz) 1.60 (d, 3H), 1.70 (t, 3H), 2.80

(dd, 1H), 3.10 (dd, 1H), 3.70–3.90 (m, 1H), 4.00 (dd, 1H), 4.60 (q, 2H), 5.0–5.45 (m, 2H). Anal. ($C_{10}H_{14}N_2O_3$) H, N; C: calcd, 57.12; found, 57.62.

Ethyl *rac*-7-cyano-11-methyl-1,5-dioxa-9-azaspiro[5.6]-undecane-9-carboxylate (19). A solution of crude **18** (270.0 g, ca. 1.28 mol), 1,3-propanediol and *p*-toluenesulphonic acid monohydrate (5 g, 0.026 mol) in toluene (1.15 L) was refluxed with a Dean–Stark water separator for 16 h. After cooling to room temperature, H_2O was added, and the phases were separated. The aqueous phase was extracted with EtOAc, and the combined organic phases were washed with dilute sodium hydroxide solution and worked up in the usual manner giving the title product (340.7 g, 1.26 mol, 98%) as an oil, which was used for the synthesis of **20** without further purification. ^{1}H NMR ($CDCl_3$) 1.30 (m, 6H), 1.75 (qui, 2H), 2.00 (dd, 1H), 2.75 (m, 2H), 3.25–4.75 (m, 9H).

Ethyl *rac*-7-carboxamido-11-methyl-1,5-dioxa-9-azaspiro-[5.6]undecane-9-carboxylate (20). To a solution of crude **19** (174.4 g, ca. 0.671 mol) in EtOH (720 mL) was added a solution of NaOH (30 g, 0.75 mol) in H_2O (50 mL). The mixture was heated to 60 °C with stirring, and 30% H_2O_2 (560 mL, 9.9 mol) was added keeping the temperature at 60–65 °C. After the addition the mixture was stirred for 2 h at 60 °C and then left overnight at room temperature. The mixture was concentrated, and the residue was dissolved in CH_2Cl_2. The layers were separated and the organic phase was worked up in the usual manner giving the title product (120.4 g, 0.433 mol, 65%) as an oil, which was used for the synthesis of **21** without further purification. ^{1}H NMR ($CDCl_3$) 1.20 (d, 3H), 1.30 (t, 3H), 1.40–2.25 (m, 2H), 2.45 (dd, 1H), 2.90 (dd, 1H), 3.10–4.65 (m, 10H), 6.70 (broad d, 2H (NH_2)).

Ethyl *rac*-3-carboxamido-6-methyl-4-oxopiperidine-1-carboxylate (21). To a solution of crude **20** (718.4 g, ca. 2.51 mol) in THF (1 L) was added 6 M HCl (2.62 L) with cooling and stirring. The mixture was stirred for 2 h at room temperature, and then the THF was removed, and pH was adjusted to 7.0 with dilute NaOH solution. The mixture was then worked up in the usual manner with CH_2Cl_2 yielding the title product as an oil (220.7 g, 0.97 mol, 39%), which was used for the synthesis of **22** without further purification. ^{1}H NMR ($CDCl_3$, 250 MHz) 1.20 (d, 3H), 1.30 (t, 3H), 2.05 (d, 1H), 2.70 (dd, 1H), 3.65 (dd, 1H), 4.20 (q, 2H), 4.45 (d, 1H), 4.55–4.70 (m, 2H), 5.45 (broad, 2H (NH_2)).

Ethyl (*RS*)-4-benzylamino-3-carboxamido-6-methyl-1,2,5,6-tetrahydropyridine-1-carboxylate (22). A mixture of crude **21** (220 g, ca. 976 mmol), benzylamine (130 mL, 1.04 mol), and xylene (mixture of isomers, 1.5 L) was refluxed with a Dean–Stark water separator for 90 min. The reaction mixture was evaporated under vacuum to constant weight to give crude **22**, which was used for the synthesis of **23** without further purification. Yield: 249.1 g (0.787 mol, 81%) as an oil. ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.10 (d, 3H), 1.25 (t, 3H), 2.20 (dd, 1H), 2.45 (dd, 1H), 3.75 (d, 1H), 4.15 (q, 2H), 4.30 4.55 (m, 4H), 4.95 (s, 2H (NH_2)), 7.20–7.40 (m, 5H), 10.2 (t, 1H).

Ethyl (*RS*)-3-hydroxy-6-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-5-carboxylate (23). H_2S (93 g, 2.74 mol) was dissolved with cooling in DMF (1.5 L) and crude **22** (124.5 g, ca. 0.39 mol) was added. The reaction mixture was stirred for 16 h. Then DMF and excess H_2S was removed by evaporation. The residue was dissolved in EtOAc (1 L) and washed with saturated $CaCl_2$ solution (4×150 mL), dried over $MgSO_4$ and evaporated to a foamy product, which was dissolved in EtOAc (0.5 L). To this solution was added Br_2 (45 mL, 0.84 mol) in CH_2Cl_2 with cooling at 20 °C. After addition the reaction mixture was stirred for 16 h at room temperature, and evaporated. The residue was dissolved in CH_2Cl_2 (0.6 L) and washed with ice cold saturated NH_4Cl solution (3×0.3 L). The organic phase was dried over $MgSO_4$ and evaporated to give crude **23** as an oil. Yield: 29.4 g (0.12 mol, 31%). A sample was crystallized from EtOAc: mp 148–150 °C. ^{1}H NMR ($CDCl_3$) δ 1.15 (d, 3H), 1.30 (t, 3H), 2.65 (d, 1H), 3.05 (dd, 1H), 3.95 (d, 1H), 4.20 (q, 2H) 4.80 (d, 1H), 4.90–5.05 (m, 1H). Anal. ($C_{10}H_{14}N_2O_3S{\cdot}H_2O$) C, H, N.

***tert*-Butyl (*RS*)-3-hydroxy-6-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-5-carboxylate (24).** A solution of crude **23** (29.3 g, ca. 121 mmol) in 33% HBr in AcOH (400 mL) was stirred for 72 h. The mixture was evaporated, and EtOH (25 mL) was added and evaporated. The residue was dissolved in H_2O (200 mL) and was washed with CH_2Cl_2 (2×50 mL). The aqueous phase was cooled on an ice bath and K_2CO_3 (28 g, 203 mmol) was added below 20 °C. The aqueous phase was washed with CH_2Cl_2 (100 mL), and was cooled to 5 °C. A solution of $(Boc)_2O$ (43.3 g, 208 mmol) in THF (100 mL) was added below 15 °C. After addition the mixture was stirred for 16 h. The phases were separated, and the aqueous phase was extracted with Et_2O (4×100 mL). The combined organic phases were washed with saturated NaCl solution (3×100 mL). Drying over $MgSO_4$ and activated carbon and evaporation yielded 38.2 g of a semicrystalline material (mostly diBoc-derivative), which was dissolved in MeOH (400 mL). Anhydrous K_2CO_3 (14.0 g, 102 mmol) was added, and the mixture was stirred for 14 h. MeOH was removed, and the residue was dissolved in H_2O (200 mL) and washed with Et_2O (3×100 mL). The aqueous phase was acidified to pH 6 with concentrated HCl and extracted with EtOAc (4×100 mL). The EtOAc phases were washed with saturated NaCl solution (2×100 mL), dried over $MgSO_4$ and evaporated to give crude **24**. Yield: 10.2 g (0.040 mol, 33%) as an oil. ^{1}H NMR ($CDCl_3$) 1.20 (d, 3H), 1.50 (s, 9H), 2.40 (d, 1H), 3.00 (dd, 1H), 3.80 (dd, 1H), 4.70 (dd, 1H), 4.75–5.15 (m, 1H).

(*RS*)-6-Methyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (8c) hydrochloride. The title product was prepared from crude **24** and 3-bromopropyne as described for the preparation of **7c**. Yield: 41%. Mp 169–171 °C; ^{1}H NMR (DMSO-d_6) 1.45 (d, 3H), 2.65–3.25 (m, 2H), 3.30–3.60 (m, 1H), 4.05 (s, 3H), 5.05 (d, 2H). Anal. ($C_{10}H_{12}N_2OS{\cdot}HCl$) C, H, N.

(+)-6-Methyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(+)-8c] hemifumarate and (−)-6-methyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(−)-8*c*] fumarate. To a stirred solution of **8c** base (10.3 g, 49.2 mmol) in EtOH (20 mL) was added a solution of D-(+)-dibenzoyltartaric acid (4.4 g, 12.3 mmol) in EtOH (20 mL). The mixture was cooled to 10 °C, and the crystals (10.2 g) were filtered off. Three recrystallizations from 80% EtOH gave 5.76 g of a hemi-D-(+)-dibenzoyltartrate salt with mp 180–181 °C. The base was set free with NaOH yielding 2.82 g, which was precipitated from EtOH with fumaric acid (1.7 g). Yield of (+)-**8c** hemifumarate: 3.86 g (11.8 mmol, 24%). Mp 180–181 °C. $[\alpha]_D^{25}$ +52.9° (*c* 0.1, H_2O). Anal. ($C_{10}H_{12}N_2OS{\cdot}0.5C_4H_4O_4{\cdot}0.91H_2O$) C, H, N. ^1H NMR spectroscopy [(+)-**8c** base (15 mg) and (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol (60 mg)] gave baseline splitting of the doublet corresponding to the 6-methyl group. On the basis of peak intensities, enantiomeric excess was estimated to be 95.0%. To the crude free base of (−)-**8c**, isolated from the filtrate from the first crystallization of (+)-**8c**, hemi-D-(+)-dibenzoyltartrate was added L-(−)-dibenzoyltartaric acid monohydrate (5.0 g), and (−)-**8c** fumarate was obtained as described above. Yield: 3.86 g (11.8 mmol, 24%). Mp 155–7 °C. $[\alpha]_D^{25}$ −48.6° (*c* 0.1, H_2O). Anal. ($C_{10}H_{12}N_2OS{\cdot}C_4H_4O_4$) C, H, N. Enantiomeric excess of (−)-**8c** was estimated by ^1H NMR spectroscopy, as described above for (+)-**8c**, to be 92.0%.

(*RS*)-3-Allyloxy-6-methyl-4,5,6,7-tetrahydroisothiazolo-[4,5-*c*]pyridine (8d) fumarate. The title product was prepared from crude **24** and allyl bromide as described for the preparation of **7c**. Yield: 32%. Mp 169–171 °C; ^1H NMR (DMSO-d_6) 1.25 (d, 3H), 2.75 (d, 1H), 2.95–3.25 (m, 3H), 3.70 (s, 2H), 4.70 (dt, 2H), 5.15–5.50 (m, 2H), 6.05 (ddd, 1H), 6.55 (s, 2H). Anal. ($C_{10}H_{14}N_2OS{\cdot}C_4H_4O_4$) C, H, N.

(*RS*)-5,6-Dimethyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (9c) dioxalate. A solution of **8c** (2.0 g, 9.5 mmol) in formic acid (70 mL) and formaldehyde (37% solution, 25 mL) was refluxed for 4 h. The reaction mixture was concentrated, and the residue was dissolved in 2 M NaOH solution (25 mL) and EtOAc (50 mL). The aqueous phase was extracted with two 50 mL portions of EtOAc, and the combined organic phases were worked up in the usual manner giving 1.02 g of an oil, which was crystallized as oxalate. Yield: 0.95 g, (3.4 mmol, 32%). Mp 154–156 °C (from Me_2CO); ^1H NMR (DMSO-d_6) 1.30 (d, 3H), 2.80 (s, 3H), 2.95–3.30 (m, 2H), 3.40–3.90 (m, 1H), 3.60 (t, 1H), 4.20 (s, 2H), 5.05, (d, 2H). Anal. ($C_{11}H_{14}N_2OS{\cdot}2C_2H_2O_4$) C, H, N.

(−)-5,6-Dimethyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(−)-9c] oxalate. From (−)-**8c** as described for the preparation of **9c** in 33% yield. Mp 62–64 °C (from Me_2CO, amorphous). $[\alpha]_D^{25}$ −19.6° (*c* 0.5, H_2O). Anal. ($C_{11}H_{14}N_2OS{\cdot}C_2H_2O_4$) C, H; N: calcd, 8.97; found, 8.49.

(+)-5,6-Dimethyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(+)-9c] oxalate. From (+)-**8c** as described for the preparation of **9c** in 31% yield. Mp 61–63 °C (from Me_2CO, amorphous). $[\alpha]_D^{25}$ +19.1° (*c* 0.5, H_2O). Anal. ($C_{11}H_{14}N_2OS{\cdot}C_2H_2O_4{\cdot}0.275\ H_2O$) C, H; N: calcd, 8.83; found, 8.40.

(*RS*)-*N*-(2-Cyanoethyl)-2-methyl-3-aminopropionitrile (26). A solution of (*RS*)-2-methyl-3-aminopropionitrile (**25**)[26] (1032 g, 12.27 mol) and acrylonitrile (718 g, 13.53 mol) in EtOH (2.15 L) was refluxed for 16 h. The solution was evaporated to give crude **26** as a light oil (1604 g, ca. 11.7 mol, 95.3%). ^1H NMR ($CDCl_3$) 1.35 (d, 3H), 1.50 (s, 1H, NH), 2.55 (t, 2H), 2.70–2.95 (m, 3H), 3.00 (t, 2H).

Ethyl *rac*-3-cyano-5-methyl-4-oxopiperidine-1-carboxylate (27). A stirred mixture of potassium *tert*-butoxide (589 g, 5.26 mol) in toluene (4.6 L) was heated to 80 °C. Without heating crude **26** (656 g, 4.78 mol) was added at a rate that kept the mixture at gentle reflux. After the addition, the mixture was refluxed for 0.5 h, cooled to 10 °C and filtered with suction (the filtercake must be kept wet with toluene to avoid decomposition). The filtercake was washed with toluene (1 L) and poured on ice (1.2 kg). The mixture was acidified with concentrated HCl (1.52 L) and refluxed for 0.5 h. The reaction mixture was cooled on an ice bath and was made basic with 28% NaOH solution (2.6 L) keeping the temperature below 30 °C. Then the mixture was cooled to 10 °C and ethyl chloroformate (571 g, 5.26 mol) was added at 10 °C. After the addition the mixture was stirred for 0.5 h on the ice bath. Then the organic phase was discharged, and the aqueous phase was washed once with EtOAc (600 mL), and was then acidified with concentrated HCl (600 mL). The product was extracted with CH_2Cl_2 (3×600 mL), and the combined organic phases were washed once with saturated NaCl solution, dried over $MgSO_4$ and evaporated to yield crude **27** as an oil, which solidified on standing (874 g, ca. 4.16 mol, 87%). Mp 59–61 °C; ^1H NMR (250 MHz, $CDCl_3$, recorded at 57 °C, 1/1 mixture of keto-enol forms) 1.10 (two d, 6H), 1.30 (two t, 6H), 2.55 (heptet, 1H), 2.80 (dd, 1H), 2.90–3.20 (m, 1H), 3.30 (dd, 1H), 3.50–3.75 (m, 2H), 4.15–4.35 (m, 3H), 4.45 (ddd, 1H), 4.80 (ddd, 1H). Anal. ($C_{10}H_{14}N_2O_3$) H, N; C: calcd, 57.12; found, 57.64.

Ethyl *rac*-3-carboxamido-5-methyl-4-oxopiperidine-1-carboxylate (28). To 2 L of 85% H_2SO_4, kept at 65 °C, molten **27** (600 g, 2.85 mol) was added with cooling at 60–70 °C. After stirring for 0.5 h at 60–70 °C the mixture is poured on ice (5 kg), CH_2Cl_2 (2 L), and NaCl (0.5 kg) with efficient stirring. The phases were separated, and to the aqueous phase was added 28% NaOH solution (1 L), and it was extracted with CH_2Cl_2 (3×1 L). The combined organic phases were washed with saturated NaCl solution (2×0.5 L) and evaporated to 620 g of crude **28**, which was dissolved in hot EtOAc (600 mL). Compound **28** crystallized on cooling yielding 418 g (1.83 mol, 64%). Mp 133–138 °C; ^1H NMR ($CDCl_3$, 250 MHz) 1.20 (d, 3H), 1.30 (t, 3H), 2.45–2.60 (m, 1H), 3.40 (dd, 1H), 3.55–3.70 (m, 1H), 4.05 (d, 1H), 4.15 (d, 1H), 4.20 (q, 2H), 5.4–5.7 (broad, 2H (NH_2)), 14.1 (s, 1H (enol H)). Anal. ($C_{10}H_{16}N_2O_4$) C, H, N.

Ethyl (*RS*)-4-Benzylamino-3-carboxamido-5-methyl-1,2,5,6-tetrahydropyridine-1-carboxylate (29). A mixture of **28** (600 g, 2.63 mol), benzylamine (350 mL, 2.8 mol), and xylene (mixture of isomers, 3.9 L) was refluxed with a Dean–Stark water separator for 45 min. Then more benzylamine was added, and reflux was continued for another 45 min. On cooling to 20 °C the title product crystallized. The crystals were filtered off and washed with xylene yielding 737.9 g of **29** (2.32 mol, 88%). Mp 157–166 °C; ^{1}H NMR ($CDCl_3$, 250 MHz) 1.20 (d, 3H), 1.25 (t, 3H), 2.65 (m, 1H), 2.95 (t, 1H), 3.70–4.05 (m, 2H), 4.15 (q, 2H), 4.30–4.50 (m, 3H), 5.05 (broad, 2H (NH_2)), 7.20–7.40 (m, 5H), 10.15 (t, 1H). Anal. ($C_{17}H_{23}N_3O_3$) C, H, N.

Ethyl (*RS*)-3-hydroxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-5-carboxylate (30). H_2S (155 g, 4.56 mol) was dissolved with cooling in DMF (2.5 L), and **29** (205 g, 0.65 mol) was added. The reaction mixture was stirred for 16 h. Then DMF and excess H_2S was removed by evaporation. The residue was dissolved in EtOAc (1.5 L) and washed with saturated $CaCl_2$ solution (4×250 mL), dried over $MgSO_4$ and evaporated to a foamy product, which was dissolved in EtOAc (0.8 L). To this solution was added Br_2 (75 mL, 1.4 mol) in CH_2Cl_2 with cooling at 20 °C. After addition, the reaction mixture was stirred for 16 h at room temperature, and evaporated. The residue was dissolved in CH_2Cl_2 (1 L) and washed with ice cold saturated NH_4Cl solution (3×0.5 L). The organic phase was dried over $MgSO_4$ and evaporated to an oil (116 g) from which **30** was crystallized from EtOAc. Yield: 65 g (0.27 mol, 41%). Mp 144–146 °C; ^{1}H NMR ($CDCl_3$, 250 MHz) 1.30 (d, 3H), 1.35 (t, 3H), 3.05–3.20 (m, 2H), 4.10 4.25 (m, 4H), 4.60 (d, 1H). Anal. ($C_{10}H_{14}N_2O_3S$) C, H, N.

(*RS*)-3-Methoxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (10a) fumarate. The title product was prepared from **30** and Me_2SO_4 as described for the preparation of **7b**. Yield: 18%. Mp 167–168 °C (from Me_2CO); ^{1}H NMR (DMSO-d_6, 250 MHz) 1.25 (d, 3H), 2.65 (dd, 1H), 3.10–3.25 (m, 1H), 3.30 (dd, 1H), 3.75 (d, 1H), 3.80 (d, 1H), 3.95 (s, 3H), 6.65 (s, 2H). Anal. ($C_8H_{12}N_2OS{\cdot}C_4H_4O_4$) C, H, N.

(*RS*)-3-Allyloxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (10d) fumarate. The title product was prepared from **30** and allyl bromide. The alkylation was performed as described for **7c**, and the deprotection as described for **7a**. Yield: 36%. Mp 162–164 °C (from Me_2CO); ^{1}H NMR (DMSO-d_6, 250 MHz) 1.25 (d, 3H), 2.70 (dd, 1H), 3.2 (sextet, 1H), 3.35 (dd, 1H), 3.75 (d, 1H), 3.85 (d, 1H), 4.85 (dt, 2H), 5.25 (dd, 1H), 5.40 (dd, 1H), 6.00–6.20 (m, 1H), 6.55 (s, 2H). Anal. ($C_{10}H_{14}N_2OS{\cdot}C_4H_4O_4$) C, H, N.

(−)-3-Allyloxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(−)-10d] hemi-L-(+)-tartrate and (+)-3-allyloxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(+)-10d] hemi-D-(−)-tartrate. To a solution of **10d** base (18.75 g, 0.089 mol) in EtOH (50 mL) was added a solution of L-(+)-tartaric acid (3.34 g, 0.022 mol) in EtOH (20 mL). The mixture was left overnight for crystallization. The crystals were filtered off and dried yielding 14.5 g with Mp 158–160 °C, which was transformed to base (10.42 g) and again precipitated with 0.25 equiv L-(+)-tartaric acid. After a total of 5 precipitations from EtOH the yield was 2.79 g (9.8 mmol, 11%) of hemi-L-(+)-tartrate salt. Mp 192–3 °C. $[\alpha]_D^{25}$ −39.5° (*c* 1, MeOH on the free base). Anal. ($C_{10}H_{14}N_2OS{\cdot}0.5C_4H_6O_6$) C, H, N. ^{1}H NMR spectroscopy [(−)-**10d** base (15 mg) and (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol (60 mg)] gave baseline splitting of the doublet corresponding to the 7-methyl group. On the basis of the peak intensities enantiomeric excess was estimated to 95.6%. The filtrates from the crystallizations of (−)-**10d** hemi-L-(+)-tartrate were combined and the base was isolated (15.2 g). After 5 precipitations as described above with D-(−)-tartaric acid the yield was 1.95 g (6.8 mmol, 8%) of hemi-D-(−)-tartrate salt. Mp 191–2 °C. $[\alpha]_D^{25}$: +39.7° (*c* = 1, MeOH on the free base). Anal. ($C_{10}H_{14}N_2OS{\cdot}0.5C_4H_6O_6$) C, H, N. Enantiomeric excess was estimated as described above to 90.6%.

(*RS*)-3-Allyloxy-5,7-dimethyl-4,5,6,7-tetrahydroisothiazolo[4,5-c]pyridine (11d) oxalate. A solution of **10d** (2.0 g, 9.5 mmol) in formic acid (70 mL) and formaldehyde (37% solution, 25 mL) was refluxed for 4 h. The reaction mixture was concentrated, and the residue was dissolved in 2 M NaOH solution (25 mL) and EtOAc (50 mL). The aqueous phase was extracted with two 50 mL portions of EtOAc, and the combined organic phases were worked up in the usual manner giving 1.02 g of an oil, which was crystallized as oxalate. Yield: 1.19 g, (3.8 mmol, 40%) of oxalate salt. Mp 162–3 °C (from Me_2CO); ^{1}H NMR (DMSO-d_6) 1.25, 2.75 (s, 3H), 2.80 (dd, 1H), 3.20–3.55 (m, 2H), 3.70 (d, 1H), 4.00 (d, 1H), 4.85 (dt, 2H), 5.20–5.55 (m, 2H), 6.05 (ddd, 1H). Anal. ($C_{11}H_{16}N_2OS{\cdot}C_2H_2O_4$) C, H, N.

***tert*-Butyl (*RS*)-3-hydroxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine-5-carboxylate (31).** A solution of **30** (366 g, 1.51 mol) in 33% HBr in AcOH (4.5 L) was stirred for 72 h. The mixture was evaporated, and EtOH (250 mL) was added and evaporated. The residue was dissolved in H_2O (2.3 L) and was washed with CH_2Cl_2 (2×300 mL). The aqueous phase was cooled on an ice bath and K_2CO_3 (350 g, 2.53 mol) was added below 20 °C. The aqueous phase was washed with CH_2Cl_2 (0.5 L), and was cooled to 5 °C. A solution of $(Boc)_2O$ (541 g, 2.6 mol) in THF (1 L) was added below 15 °C. After addition, the mixture was stirred for 16 h. The phases were separated, and the aqueous phase was extracted with Et_2O (4×0.5 L). The combined organic phases were washed with saturated NaCl solution (3×0.5 L). Drying over $MgSO_4$ and activated carbon and evaporation yielded 477 g of a semicrystalline material (mostly diBoc-derivative), which was dissolved in MeOH (4 L). Anhydrous K_2CO_3 (175 g, 1.27 mol) was added, and the mixture was stirred for 14 h. MeOH was removed, and the residue was dissolved in H_2O (2 L) and washed with Et_2O (3×0.5 L). The aqueous phase was acidified to pH 6 with concentrated HCl and extracted with EtOAc (4×0.5 L). The EtOAc phases were washed with saturated NaCl solution (2×0.5 L),

dried over $MgSO_4$ and evaporated to yield 350 g of crude product from which **31** could be crystallized from Et_2O. Yield: 235 g (0.869 mol, 58%), mp 143–145 °C; ^1H NMR ($CDCl_3$) 1.25 (d, 3H), 1.50 (s, 9H), 2.95–3.15 (m, 2H), 4.00–4.25 (m, 2H), 4.50 (d, 1H). Anal. ($C_{12}H_{18}N_2O_3S$) C, H, N.

(*RS*)-3-Cyanomethyloxy-7-methyl-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (10e) fumarate. The title compound was prepared from **31** and chloroacetonitrile as described for the preparation of **7c**. Yield: 33%. Mp 196–197 °C (from EtOAc); ^1H NMR (DMSO-d_6, 250 MHz) 1.25 (d, 3H), 2.60 (dd,1H), 3.05–3.25 (m, 1H), 3.25 (dd, 1H), 3.70 (d, 1H), 3.80 (d, 1H), 5.30 (s, 2H), 6.55 (s, 2H). Anal. ($C_9H_{11}N_3OS{\cdot}C_4H_4O_4$) C, H, N.

(*RS*)-7-Methyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (10c) fumarate. The title product was prepared from **31** and 3-bromopropyne as described for the preparation of **7c**. Yield: 41%. Mp 184–186 °C; ^1H NMR (DMSO-d_6) 1.25 (d, 3H), 2.75 (dd, 1H), 3.00–3.50 (m, 3H), 3.55 (t, 1H), 3.80 (s, 2H), 5.00 (d, 2H), 6.50 (s, 2H). Anal. ($C_{10}H_{12}N_2OS{\cdot}C_4H_4O_4$) C, H, N.

(*S*)-(+)-7-Methyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(*S*)-10c] fumarate and (*R*)-(−)-7-methyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(*R*)-10c] fumarate. To a stirred solution of **10c** base (102.5 g, 0.492 mol) in EtOH (200 mL) was added a solution of D-(+)-dibenzoyltartaric acid (44.0 g, 0.123 mol) in EtOH (200 mL). The mixture was cooled to 10 °C, and the crystals (102.0 g) were filtered off. Three recrystallizations from 80% EtOH gave 57.6 g of a D-(+)-dibenzoyltartrate salt with mp 165–166 °C. The absolute configuration of this salt was determined for **10c** to be *S* by an X-ray crystallographic analysis. The base was set free with NaOH yielding 28.2 g, which was precipitated from EtOH with fumaric acid (17 g). Yield: 41.8 g (0.128 mol, 26%). Mp 169–170 °C. $[\alpha]_D^{25}$: +40.7 ($c = 0.1$, H_2O). Anal. ($C_{10}H_{12}N_2OS{\cdot}C_4H_4O_4$) C, H, N. Enantiomeric excess was estimated as described for (−)-**10d** to be >98.8%. To the crude free base of (*R*)-(−)-**10c**, isolated from the filtrate from the first crystallization of (*S*)-(+)-**10c** D-(+)-dibenzoyltartrate was added L-(−)-dibenzoyltartaric acid monohydrate (50 g), and the (−) enantiomer isolated as the fumarate salt as described above. Yield of (*R*)-**10c** fumarate: 35.1 g (0.108 mol, 22%). Mp 171–172 °C. $[\alpha]_D^{25}$: −40.4 ($c = 0.1$, H_2O). Anal. ($C_{10}H_{12}N_2OS{\cdot}C_4H_4O_4$) C, H, N. Enantiomeric excess was estimated, as described for (−)-**10d**, to be >98.6%.

(*RS*)-5,7-Dimethyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine (11c) oxalate. From **10c** as described for the preparation of **11d** in 57% yield. Mp 135–140 °C (from Me_2CO); ^1H NMR (DMSO-d_6) 1.30 (d, 3H), 2.75 (dd, 1H), 2.80 (s, 3H), 3.25–3.70 (m, 3H), 3.65 (t, 1H), 3.75 (d, 1H), 4.05 (d, 1H), 5.05 (d, 2H) Anal. ($C_{11}H_{14}N_2OS{\cdot}C_2H_2O_4$) C, H, N.

(*R*)-(−)-5,7-Dimethyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(R)-11c] oxalate. From (*R*)-**10c** as described for the preparation of **11d** in 56% yield. Mp 156–157 °C (from Me_2CO). $[\alpha]_D^{25}$: −41.6° ($c = 0.5$, H_2O). Anal. ($C_{11}H_{14}N_2OS{\cdot}C_2H_2O_4$) C, H, N.

(*S*)-(+)-5,7-Dimethyl-3-propargyloxy-4,5,6,7-tetrahydroisothiazolo[4,5-*c*]pyridine [(*S*)-11c] oxalate. From (*S*)-**10c** as described for the preparation of **11d** in 57% yield. Mp 151–3 °C (from Me_2CO). $[\alpha]_D^{25}$: +42.5 ($c = 0.5$, H_2O). Anal. ($C_{11}H_{14}N_2OS{\cdot}C_2H_2O_4$) C, H, N.

X-ray crystallographic analysis

Compound $C_{40}H_{44}N_4O_{11}S_2$; the asymmetric unit consists of two cations ($C_{10}H_{13}N_2SO^+$) with one doubly charged D-(+)-dibenzoyltartrate anion ($C_{18}H_{12}O_8^{2-}$) and an ethanol (C_2H_5OH) of solvation; $M_r = 820.94$, monoclinic, space group $P2_1$, $a = 8.7253(7)$, $b = 21.142(2)$, $c = 11.876(2)$ Å, $= 105.28(1)$, $V = 2113.3(8)$ Å^3, $Z = 2$, $D_x = 1.290\ g\,cm^{-3}$, monochromatized Cu K_a radiation ($\lambda = 1.541838$ Å), $\mu = 1.62\ mm^{-1}$, $F(000) = 864$, $T = 294$ K. Data were collected on a Enraf–Nonius CAD4 diffractometer to a θ limit of 75 which yielded 4757 measured (4489 unique) reflections. There are 3602 unique, observed reflections (with $I \geq 3\sigma(I)$ as the criterion for being observed) out of the total measured. The structure was solved by direct methods[40,41] and refined using full-matrix least-squares on *F* (SDP-PLUS).[42] The final model was refined using 434 parameters and 3602 observed reflections. The non-hydrogen atoms were refined with a mixture of anisotropic (41 atoms) and isotropic (16 atoms) displacement parameters. The hydrogen atoms were included at their calculated positions. The final agreement statistics are: $R = 0.057$, $wR = 0.073$, $S = 2.17$ with $(\Delta/\sigma)_{max} = 0.01$. The least-squares weights were defined using $1/\sigma^2(F)$. The maximum peak height in a final difference Fourier map is 0.38(5) eÅ^{-3} and this peak is without chemical significance. The atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre. The coordinates can be obtained on request from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK.

Receptor radioligand binding

The affinity of the compounds for muscarinic receptors were estimated by their ability to displace [^{3}H]Oxo-M (0.20 nM) from whole rat brain homogenate, [^{3}H]PZ (1.0 nM) from whole rat brain homogenate, and [^{3}H]QNB (0.12 nM) from whole rat brain homogenate and from rat brainstem homogenate, respectively. Details have been described previously.[43] Inhibitory constants (K_i values) were estimated from IC_{50} values using the Cheng–Prusoff[44] equation: $K_i = IC_{50}/(1 + s/K_D)$, where *s* is the fixed concentration and K_D the dissociation constant of the labeled ligand. The following K_D constants were derived from computer-assisted Scatchard analyses of binding experiments: [^{3}H]Oxo-M, 0.48 ± 0.03 nM; [^{3}H]PZ, 1.8 ± 1.0 nM; [^{3}H]QNB to homogenate brain, 13.7 ± 0.9 pM; [^{3}H]QNB to homogenate brain stem, 9.7 ± 0.8 pM. Two complete concentration–response curves were determined using five concentrations of test drug in triplicate (covering 3

decades). IC_{50} values were estimated from handdrawn log concentration–response curves.

In a series of n determinations the variance of the log ratio (Var_R) between the double determinations was determined according to: $Var_R = (1/2n)\Sigma(\log R_i)^2$, 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_R2). The following standard deviations were obtained: [^{3}H]Oxo-M, 1.5 ($n = 100$); [^{3}H]PZ, 1.6 ($n = 100$); [^{3}H]QNB, 1.5 ($n = 100$).

Effects on cloned human mAChRs: receptor selection and amplification technology

R-SAT was performed as described earlier.[23,30–32] 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, Utah, USA).

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.[45] using 10 μg receptor DNA,[46,47] 10 μg p-SV-ß-galactosidase DNA (Promega, WI, USA) and 40 μg salmon sperm DNA (Sigma, MO, USA). Cells transfected with m2 or m4 receptor DNA were further cotransfected with 10 μg of the chimeric G-protein, Gq-i5.[48]

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

Agonist data from R-SAT experiments were fitted to the four parameter equation:[49,50]

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

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.[51] and Craig.[52]

Antagonists that were unable to fully inhibit a carbachol response were subjected to Schild analysis.[34] 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.[53]

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 0.05 was considered statistically significant.

Acknowledgements

This work was supported by grants from the Danish Medical (PharmaBiotec Neuroscience Center) and Technical Research Councils and the Lundbeck Foundation. The secretarial assistance of Mrs. A. Nordly and the technical assistance of Ms. M. Glarø are gratefully acknowledged. Finally we wish to acknowledge Dr. Bruce R. Conklin for providing the Gq-i5 construct. R-SAT™ is patented by Acadia Pharmaceuticals Inc., which we acknowledge for allowing us to use this assay.

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