European Journal of Medicinal Chemistry 45 (2010) 5594–5601

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis of novel chiral Δ^2-isoxazoline derivatives related to ABT-418 and estimation of their affinity at neuronal nicotinic acetylcholine receptor subtypes

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ARTICLE INFO

Article history:
Received 22 June 2010
Received in revised form
1 September 2010
Accepted 2 September 2010
Available online 17 September 2010

Keywords:
Neuronal nicotinic acetylcholine receptors
ABT-418
1,3-Dipolar cycloaddition
Nicotinic ligands
Binding affinity

ABSTRACT

The enantiopure diastereomeric Δ^2-isoxazoline derivatives (2*S*,5′*R*)-**5a**–**10a** and (2*S*,5′*S*)-**5b**, (2*S*,5′*S*)-**9b**, (2*S*,5′*S*)-**11b**, which are structural analogues of both ABT-418 **2** and oxyimino ethers (*S*)-**3** and (*Z*)-(*S*)-**4**, were synthesized through cycloaddition reactions involving nitrile oxides as 1,3-dipoles and (*S*)-*N*-Boc-2-vinylpyrrolidine-**13** as the dipolarophile. The absolute configuration was unequivocally assigned to target compounds by means of an X-ray analysis. The derivatives under study were assayed at neuronal acetylcholine nicotinic receptors (nAChRs), where they showed a meaningful reduction in affinity at the heteromeric α4β2 subtype when compared to the reference molecules. Conversely, *anti* (2*S*,5′*S*)-**5b** and *syn* (2*S*,5′*R*)-**10a** isomers showed an affinity for the α7 nAChRs comparable to that observed for the model compound ABT-418.

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs), which belong to the family of Cys-loop ligand-gated ion channels, are responsible for rapid excitatory cholinergic transmission. The various physiological functions regulated by nAChRs are due either to their synaptic activation or to their involvement in the modulation of neurotransmitter systems other than the cholinergic one [1,2]. Accordingly, nAChRs have been identified as major therapeutic targets mainly for pathological conditions of the central nervous system, such as Alzheimer's and Parkinson's diseases, Tourette's syndrome, schizophrenia, attention deficit/hyperactivity disorder (ADHD), pain, substance abuse, anxiety and depression [3–6]. In addition, some subtype selective radioligands with improved brain penetration are under development for mapping and recording the expression of central nAChRs [7,8]. Worth mentioning, an alternative approach to the design and synthesis of compounds selective for a specific nAChR subtype is to target its allosteric binding sites, which are usually less conserved than the acetylcholine recognition site (orthosteric site) [9,10].

The potential therapeutic impact of novel drug candidates targeting nAChRs is strictly reliant on their selectivity for a given subtype. Among the 12 different neuronal nAChRs characterized so far in the vertebrate brain [11,12], the most expressed are the heteromeric α4β2 channels and the homomeric α7 channels, which represent preferred biological targets for the design of new selective nicotinic agonists/partial agonists [1,5,11]. Among the various approaches involving the chemical manipulation of naturally occurring nicotinic ligands [1,11,13], structure activity studies on (*S*)-nicotine **1**, the active ingredient of tobacco, afforded ABT-418 **2**, a potent and rather selective α4β2 nAChR agonist, in which a 3-methylisoxazol-5-yl moiety bioisosterically replaced the pyridine ring of the parent compound (Fig. 1) [14]. Notably, ABT-418 has demonstrated signals of efficacy in adults with ADHD [15]. More recently, a group of ABT-418 analogues, in which an oxyimino ether moiety replaced the 3-methyl-5-isoxazolyl residue, was synthesized and tested [16]. In particular, the two-enantiopure oxime ethers (*S*)-**3** and (*Z*)-(*S*)-**4** (Fig. 1) displayed a moderate

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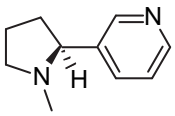
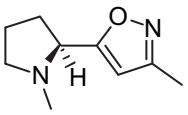
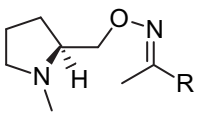
doi:10.1016/j.ejmech.2010.09.009



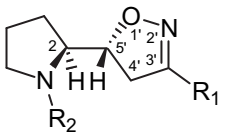
(*S*)-Nicotine **1**



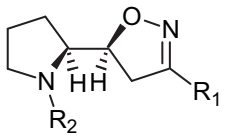
ABT-418 **2**



(*S*)-**3**: R = CH_3
(*Z*)-(*S*)-**4**: R = H



(2*S*,5′*R*)-(−)-**5a**: R_1 = CH_3, R_2 = H
(2*S*,5′*R*)-(−)-**6a**: R_1 = CH_3, R_2 = CH_3
(2*S*,5′*R*)-(−)-**7a**: R_1 = Br, R_2 = H
(2*S*,5′*R*)-(−)-**8a**: R_1 = Br, R_2 = CH_3
(2*S*,5′*R*)-(−)-**9a**: R_1 = OCH_3, R_2 = H
(2*S*,5′*R*)-(−)-**10a**: R_1 = OCH_3, R_2 = CH_3



(2*S*,5′*S*)-(+)-**5b**: R_1 = CH_3, R_2 = H
(2*S*,5′*S*)-(+)-**9b**: R_1 = OCH_3, R_2 = H
(2*S*,5′*S*)-(+)-**11b**: R_1 = O*t*-Bu, R_2 = H

Fig. 1. Structures of the heterocyclic derivatives investigated in this study.

submicromolar affinity for the α4β2 subtype coupled with an interesting α4β2 vs α7 selectivity profile [16].

Based on the above discussed results, as an extension of our studies on novel selective heterocyclic ligands targeting neuronal nAChR subtypes [17–19] we report in this paper the synthesis of Δ^2-isoxazolines (2*S*,5′*R*)-**5a**–**10a**, (2*S*,5′*S*)-**5b**, (2*S*,5′*S*)-**9b**, and (2*S*,5′*S*)-**11b**, which may be regarded as hybrid structural analogues of isoxazole **2** and oxime ethers **3** and **4** (Fig. 1). The 3-Br or 3-OMe substituted derivatives were prepared on the basis of recent results obtained by our research group with a series of spirocyclic Δ^2-isoxazolinyl nicotinic ligands [17]. The target compounds were evaluated for their binding affinity at both rat neuronal nAChR subtypes (α4β2 and α7) and human cloned muscarinic receptors (hm1-5).

2. Results and discussion

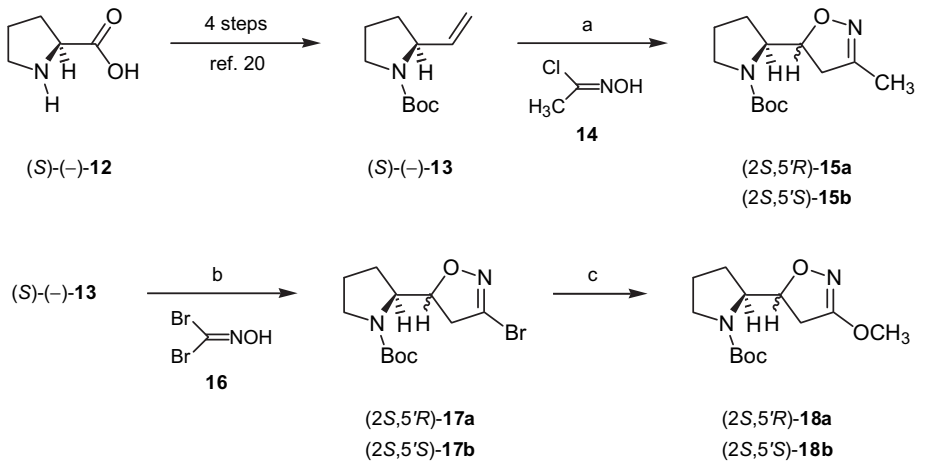
As a first step of the synthetic sequence, we converted commercially available (*S*)-(−)-proline **12** into (*S*)-(−)-*tert*-butyl-2-vinylpyrrolidine-1-carboxylate **13**, according to a published procedure [20] (Scheme 1). The latter alkene was utilized as dipolarophile in 1,3-dipolar cycloaddition reactions involving acetonitrile oxide and bromonitrile oxide, generated in situ in a basic medium from their stable halogenoxime precursors, i.e. cloroacetaldoxime **14** [21] and dibromoformaldoxime **16** [22], respectively (Scheme 1). The *syn*/*anti* diastereomeric mixtures of Δ^2-isoxazolines **15a**/**15b** and **17a**/**17b** were obtained in high yield but the cycloadducts in each couple turned out to be inseparable by means of a silica gel column chromatography. Replacement of the 3-bromo group of diastereomers **17a**/**b** with the 3-methoxy moiety to yield derivatives **18a**/**b** was smoothly accomplished by refluxing the mixture of compounds **17a**/**b** with a methanol suspension of potassium carbonate. Once again, the mixture of diastereomers **18a**/**b** could not be separated by a preparative column chromatography (Scheme 1).

In a further attempt to split the three diastereomeric mixtures **15a**/**b**, **17a**/**b** and **18a**/**b**, we removed the *N*-Boc protection by a standard treatment with trifluoracetic acid in dichloromethane. As illustrated in Scheme 2, the goal was finally achieved via functionalization of the pyrrolidine nitrogen with the trityl group. We separated (2*S*,5′*R*)-(−)-**19a** from (2*S*,5′*S*)-(+)-**19b** as well as (2*S*,5′*R*)-(−)-**22a** from (2*S*,5′*S*)-(+)-**22b**. Due to the lability of the trityl-protecting group on silica gel, the flash chromatography was performed on neutral alumina. Both pericyclic reactions produced a mixture of cycloadducts in about 3:2 *syn*/*anti* ratio. Worth noting, in the case of **17a** and **17b**, the above reported conversions and the related chromatographic purification gave the expected 3-bromo-substituted isomer *syn* (2*S*,5′*R*)-(−)-**20a** along with the unexpected 3-*tert*-butoxy-substituted counterpart *anti* (2*S*,5′*S*)-(−)-**21b**. Evidently, the 3-bromo group of **17b** was prone to the nucleophilic displacement by *tert*-butanol, most likely during the *N*-Boc cleavage step.

The relative stereochemistry to the *syn*/*anti* diastereomers, and hence the absolute configuration to the 5′-stereogenic center, was preliminarily assigned by comparing the values of the chemical shifts and coupling constants of protons H-2 and H-5′ in the two couples of *N*-trityl intermediates (−)-**19a**/(+)-**19b** and (−)-**22a**/(+)-**22b**. As previously observed with structurally related Δ^2-isoxazolines [23,24], the H-5′ of *syn* isomers (−)-**19a** and (−)-**22a** resonated at lower field strength (5.16 and 5.22 δ, respectively) than the H-5′ of the corresponding *anti* isomers (+)-**19b** and (+)-**22b** (4.89 and 4.97 δ, respectively). Additionally, $J_{2-5'}$ for the *syn* isomers (−)-**19a** and (−)-**22a** (J = 0.5 and 1.8 Hz) was found to be smaller than $J_{2-5'}$ for the *anti* isomers (+)-**19b** and (+)-**22b** (J = 5.7 and 5.7 Hz) [23,24]. The attribution was subsequently confirmed by an X-ray analysis carried out on derivative **7a** (see below).

The six *N*-trityl derivatives (−)-**19a**, (+)-**19b**, (−)-**20a**, (−)-**21b**, (−)-**22a**, and (+)-**22b**, depicted in Scheme 2, were then converted in a mild acidic medium into the corresponding final secondary amines (−)-**5a**, (+)-**5b**, (−)-**7a**, (+)-**11b**, (−)-**9a**, and (+)-**9b**. The most abundant *syn* isomers (−)-**5a**, (−)-**7a**, and (−)-**9a** were submitted to a reductive amination, performed with aqueous formaldehyde and sodium cyanoborohydride, to provide the corresponding tertiary amines (−)-**6a**, (−)-**8a**, and (−)-**10a**, respectively.

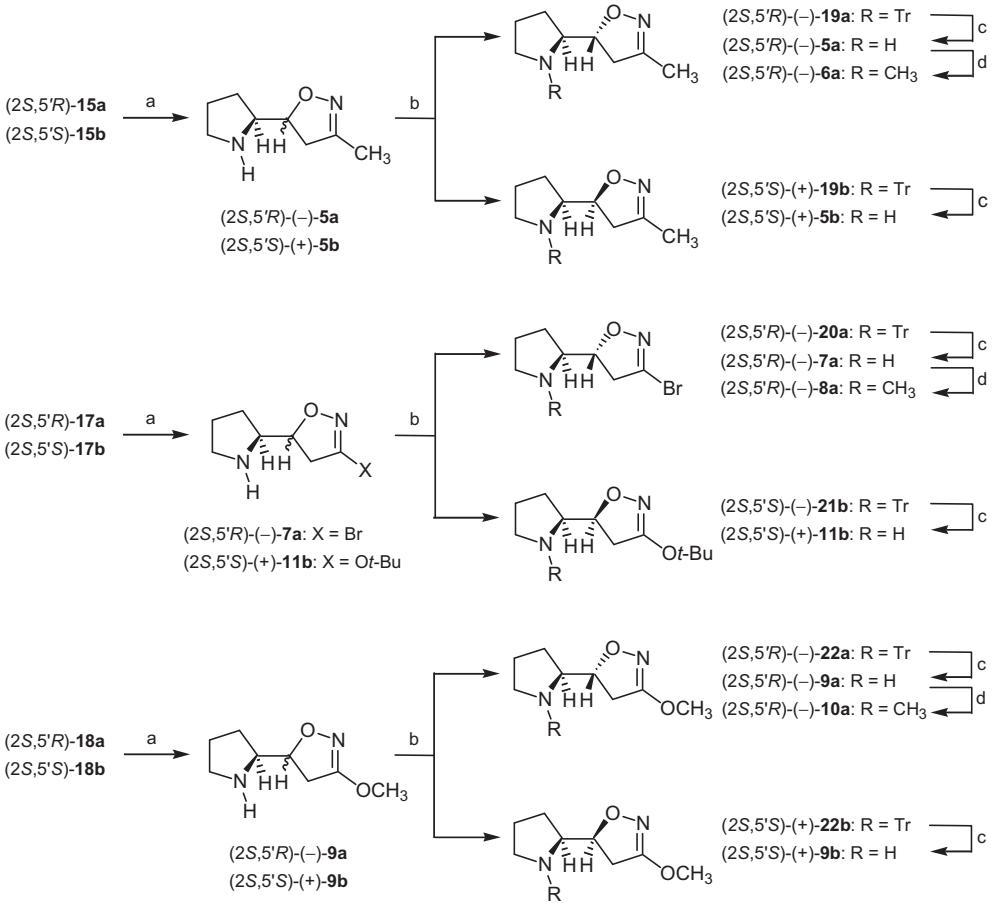
To confirm the *syn*/*anti* attribution, we decided to perform a single-crystal X-ray diffraction experiment on the fumarate of (−)-**7a**, since the presence of a bromine atom in this derivative



Scheme 1. Reagents and conditions: (a) TEA, CH_2Cl_2, r.t., 1 week; (b) $NaHCO_3$, EtOAc, r.t., 48 h; (c) K_2CO_3, MeOH, r.t., 12 h.

would allow the resolution of the absolute configuration by anomalous dispersion. After several fruitless attempts to grow single-crystals of adequate dimensions of (−)-**7a** fumarate, we finally succeeded to obtain a sample of racemic (±)-**7a** fumarate of suitable quality for the structure solution. We therefore determined the relative configuration of the two stereocenters in the racemic crystal since the molecules present in the crystallographic unit cell were found to have (2*S*,5′*R*) or (2*R*,5′*S*)-configuration (Fig. 2) [25].

The target derivatives (−)-**5a**-(−)-**10a**, (+)-**5b**, (+)-**9b**, and (+)-**11b** were assayed for binding affinity at rat α4β2 and α7 nAChRs, using [^{3}H]epibatidine and [^{125}I]α-bungarotoxin as radioligands, respectively. The K_i values, calculated from the competition curves of three separate experiments by means of the LIGAND program [26], are reported in Table 1 and compared with those previously obtained by some of us for reference compounds **1**–**4**.



Scheme 2. Reagents and conditions: (a) 30% CF_3COOH in CH_2Cl_2, 0 °C to r.t., 1 h; (b) $(C_6H_5)_3CCl$, TEA, CH_2Cl_2, r.t., 24 h; (c) CF_3COOH-H_2O (2:1), CH_2Cl_2, 0 °C, 1 h; (d) 37% aq. HCHO, $NaBH_3CN$, CH_3CN, r.t., 1 h.

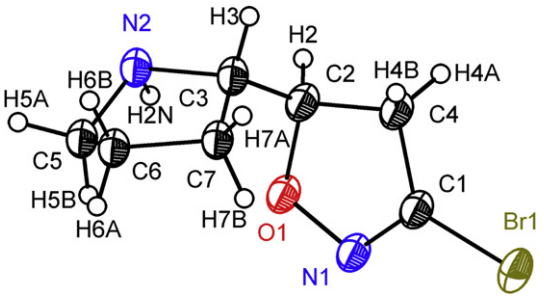


Fig. 2. Room temperature experimental X-ray geometry of enantiomer (2*S*,5′*R*)-**7a** in the **7a** fumarate racemic crystal, with the atom numbering scheme. Thermal ellipsoids are drawn at 25% probability level.

Inspection of the data listed in Table 1 clearly shows that insertion of the Δ^2-isoxazoline moiety sharply reduces the affinity at the α4β2 nAChR subtype. Indeed, the binding affinity of the *syn* isomer (−)-**6a** (K_i = 12 μM) is reduced by two orders of magnitude when compared to that of ABT-418 (K_i = 0.020 μM), its closest analogue. A similar trend can be noticed if the comparison is made with oxyimino ethers **3** and **4** (K_i = 0.52 and 0.33 μM, respectively). Worth noting, the binding affinity at the α7 nAChRs of the novel Δ^2-isoxazoline derivatives is similar or even better than that reported for oxyimino ethers **3** and **4**. In particular, derivative (+)-**5b** has a K_i value two orders of magnitude lower than that of (*S*)-**3** and roughly three fold lower than that reported for ABT-418 (K_i = 0.44 vs 1.20 μM) [16]. Furthermore, at the homomeric α7 receptor, a pronounced stereochemical effect among couples of diastereomers can be appreciated. In fact, the affinity of the *anti* (+)-**5b** derivative is two orders of magnitude higher than that of its related *syn* (−)-**5a** isomer (K_i = 0.44 vs 40 μM), and a comparable trend characterizes the *anti* (+)-**9b**/*syn* (−)-**9a** couple (K_i = 11.30 vs 277 μM). Finally, by comparing the data of (+)-**9b** with those of (+)-**11b**, we can deduce that an increase in the bulkiness of the 3-substituent is detrimental to the binding at both nAChRs, particularly at the α7 subtype (K_i = 11.30 vs > 500 μM).

The synthesized compounds were also assayed for binding affinity at human muscarinic acetylcholine receptor (mAChR) subtypes (hm1–5) in transfected Chinese Hamster Ovary (CHO) cells labeled with [^{3}H]quinuclidinyl benzylate. The compounds (10 μM) were preliminarily tested at hm2 and hm5, representative of the two subgroups (M_2, M_4 and M_1, M_3, M_5, respectively) of mAChRs. None of the investigated derivatives displayed a percent inhibition of the radioligand binding higher than 50%. As a consequence, they were not further assayed at all five mAChRs, and their K_i values may be considered higher than 10 μM at all the subtypes.

Table 1
Affinity of (*S*)-nicotine **1**, ABT-418 **2**, (*S*)-**3**, (*Z*)-(*S*)-**4**, and derivatives (−)-**5a**–**10a**, (+)-**5b**, (+)-**9b**, and (+)-**11b** for native α4β2 and α7 nAChR subtypes present in rat cortical membranes and labeled by [^{3}H]epibatidine and [^{125}I]α-bungarotoxin. The K_i values were derived from three competition–binding experiments. The numbers in brackets refer to the % CV.

Entry	α4β2	α7
	[^{3}H]Epi	[^{3}H]α-BgTx
	K_i (μM)	K_i (μM)
(2*S*,5′*R*)-(−)-**5a**	18 (34)	40 (37)
(2*S*,5′*S*)-(+)-**5b**	5.10 (21)	0.44 (28)
(2*S*,5′*R*)-(−)-**6a**	12 (17)	325 (24)
(2*S*,5′*R*)-(−)-**7a**	4.20 (19)	35 (22)
(2*S*,5′*R*)-(−)-**8a**	3.80 (15)	43 (18)
(2*S*,5′*R*)-(−)-**9a**	28 (20)	277 (42)
(2*S*,5′*S*)-(+)-**9b**	50 (24)	11.30 (14)
(2*S*,5′*R*)-(−)-**10a**	10.30 (23)	2.90 (12)
(2*S*,5′*S*)-(+)-**11b**	184 (32)	>500
(*S*)-Nicotine 1	0.002[a]	0.469[a]
ABT-418 2	0.020[a]	1.20[a]
(*S*)-3	0.52[a]	49[a]
(*Z*)–(*S*)-4	0.33[a]	>50[a]
(±)-Epibatidine	0.021 nM[b]	0.71 nM
α-BgTx	n.d.	0.90 nM[b]

[a] Ref. [16].
[b] K_d values.

3. Conclusion

We prepared a set of novel enantiopure chiral pyrrolidine-isoxazolinyl stereoisomers using as a key step the 1,3-dipolar cycloaddition of nitrile oxides to (*S*)-(−)-*N*-Boc-2-vinylpyrrolidine. The target derivatives were assayed for their binding affinity at nicotinic as well as muscarinic acetylcholine receptors. All the compounds displayed a drastic reduction of affinity at their key biological target, i. e. the α4β2 nAChR, which is rather selectively recognized by the model ligands ABT-418 and related oxyimino ethers. Among the investigated derivatives, the *anti* isomer (2*S*,5′*S*)-(+)-**5b** and the *syn* isomer (2*S*,5′*R*)-(−)-**10a** retained the affinity of ABT-418 at the α7 nAChR subtype. On the whole, replacement of the isoxazole ring with a Δ^2-isoxazoline moiety in the molecular skeleton of ABT-418 led to a group of minor nicotinic ligands, both in terms of affinity and subtype selectivity. This result further emphasizes the importance for high-affinity/selectivity nicotinic ligands of appropriate planar heteroaromatic rings acting as hydrogen bond acceptor-π moieties. Moreover, the derivatives under study did not show any appreciable affinity at the different muscarinic receptor subtypes.

4. Experimental protocols

4.1. Chemistry

The spectroscopic and polarimetric data for alkene (*S*)-(−)-**13**, prepared following a known procedure [20], matched those known from the literature. Chloroacetaldoxime [21] and dibromoformaldoxime [22] were obtained according to known experimental protocols. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (^{1}H, 300.063; ^{13}C, 75.451 MHz) spectrometer in $CDCl_3$ solutions (unless otherwise indicated) at 20 °C. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. TLC analyses were performed on commercial silica gel 60 F_{254} aluminium sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or the Dragendorff reagent. Rotary power determinations (sodium D line, 589 nm) were carried out with a Jasco P-1010 Polarimeter coupled with a Huber thermostat. Chiral HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975 using a KROMASIL 5-AmyCoat column (0.46 cm × 25 cm). Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within ±0.4%.

4.1.1. Mixture of tert-butyl-2-(3-methyl-4,5-dihydroisoxazol-5-yl) pyrrolidine-1-carboxylates syn (2*S*,5′*R*)-***15a*** and anti (2*S*,5′*S*)-***15b***

To a magnetically stirred solution of (*S*)-*tert*-butyl-2-vinylpyrrolidine-1-carboxylate (−)-**13** (1.50 g, 7.60 mmol) in dichloromethane (60 mL) were added chloroacetaldoxime **14** (1.07 g, 11.41 mmol) and triethylamine (1.60 mL, 11.41 mmol). The reaction mixture was stirred at r.t. for one week with addition of further amounts of **14** (1.07 g × 10) and TEA (1.60 mL × 10). After completion of the cycloaddition, the crude mixture was filtered, evaporated and the residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate 4:1) to afford the

mixture of *syn*/*anti* cycloadducts **15a** and **15b** (1.82 g, 94% overall yield) as a light yellow viscous oil [R_f = 0.26 (petroleum ether/ethyl acetate 4:1)]. ^{1}H NMR (toluene d_8, 100 °C): 1.61 (s, 9H), 1.67–1.95 (m, 4H), 2.07 (s, 3H), 2.57–2.73 (m, 1H), 2.77–2.90 (m, 1H), 3.35–3.63 (m, 2H), 3.92–4.17 (m, 1H), 4.64–4.80 (m, 1H).

4.1.2. Mixture of tert-butyl-2-(3-bromo-4,5-dihydroisoxazol-5-yl) pyrrolidine-1-carboxylates syn (2S,5′R)-**17a** and anti (2S,5′S)-**17b**

To a magnetically stirred suspension of (*S*)-*tert*-butyl-2-vinylpyrrolidine-1-carboxylate (−)-**13** (1.85 g, 9.34 mmol) and sodium hydrogen carbonate (10.24 g, 0.122 mol) in ethyl acetate (25 mL) was added dibromoformaldoxime **16** (1.90 g, 9.34 mmol). The reaction mixture was stirred at r.t. for 2 days with addition of a further amount (1 × 1.90 g) of **16**. After completion of the cycloaddition, Celite was added and the resulting slurry was filtered under vacuum and washed with ethyl acetate. The solvent was evaporated and the residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate 95:5) to give the mixture of *syn*/*anti* cycloadducts **17a** and **17b** (2.27 g, 76% overall yield) as a yellow viscous oil [R_f = 0.31 (petroleum ether/ethyl acetate 9:1)]. ^{1}H NMR (toluene d_8, 100 °C): 1.47 (s, 9H), 1.55–1.90 (m, 4H), 2.62–2.78 (m, 1H), 2.92–3.04 (m, 1H), 3.14–3.23 (m, 1H), 3.25–3.38 (m, 1H), 3.68–4.03 (m, 1H), 4.48–4.64 (m, 1H).

4.1.3. Mixture of tert-butyl-2-(3-methoxy-4,5-dihydroisoxazol-5-yl)pyrrolidine-1-carboxylates syn (2S,5′R)-**18a** and anti (2S,5′S)-**18b**

To a solution of the isomeric mixture of **17a** and **17b** (3.04 g, 9.52 mmol) in methanol (80 mL) was added potassium carbonate (13.15 g, 95 mmol), and the reaction mixture was magnetically stirred at r.t. for 12 h. After completion of the reaction, Celite was added and the resulting slurry was filtered under vacuum and washed with dichloromethane. The concentrated residue was diluted with water (50 mL) and extracted with dichloromethane (5 × 30 mL). The residue of the pooled organic phases was purified by silica gel column chromatography (petroleum ether/ethyl acetate 4:1), and afforded the mixture of isomers **18a** and **18b** (2.26 g, 88% overall yield) as a colorless viscous oil [R_f = 0.34 (petroleum ether/ethyl acetate 4:1)]. ^{1}H NMR: 1.45 (s, 9H), 1.85 (m, 2H), 1.99 (m, 2H), 2.80–3.03 (m, 2H), 3.27–3.48 (m, 2H), 3.82 (s, 3H), 3.84–4.12 (m, 1H), 4.52–4.87 (m, 1H).

4.1.4. 3-Methyl-5-pyrrolidin-2-yl-4,5-dihydroisoxazoles syn (2S,5′R)-(−)-**5a** and anti (2S,5′S)-(+)-**5b**

To the mixture of cycloadducts **15a** and **15b** (1.82 g, 7.16 mmol) dissolved in dichloromethane (15 mL) at 0 °C was added trifluoroacetic acid (2.76 mL, 35.8 mmol) dropwise. After 30 min the reaction mixture was further stirred at r.t. for 1 h. After evaporation under vacuum, the residue was diluted with 0.1 N HCl (50 mL, pH = 2–3) and extracted with diethyl ether (3 × 30 mL). The residual aqueous phase, made basic by portionwise addition of $NaHCO_3$, was extracted with dichloromethane (6 × 30 mL). The organic layers were dried over anhydrous sodium sulfate and the solvent was evaporated at reduced pressure. The crude residue was purified by silica gel column chromatography (dichloromethane/methanol 9:1) to obtain the *syn*/*anti* mixture of isomers (−)-**5a** and (+)-**5b** (904 mg, 82% overall yield) as a dark yellow oil [R_f = 0.38 (dichloromethane/methanol 9:1)].

To a solution of **5a** and **5b** (1.26 g, 8.18 mmol) in dichloromethane (80 mL), under N_2, were added triphenylchloromethane (2.28 g, 8.18 mmol) and triethylamine (3.41 mL, 24.54 mmol), and the reaction mixture was stirred at r.t. for 24 h. After evaporation of the solvent the residue was diluted with petroleum ether and filtered under vacuum. The crude filtrate was submitted to a neutral aluminium oxide flash chromatography (petroleum ether/ethyl acetate 99:1), which gave the *syn* (−)-**19a** (630 mg) and *anti* (+)-**19b** (314 mg) isomers, and an intermediate (1.52 g) fraction of their mixture (76% overall yield).

(2*S*,5′*R*)-(−)-**19a**: Crystallized from *n*-hexane/ethyl acetate as colorless prisms, mp 147.5–149.5 °C. R_f = 0.43 (petroleum ether/ethyl acetate 85:15); $[\alpha]_D^{20} = -125.4$ (c = 1.0, $CHCl_3$). ^{1}H NMR: 0.46 (m, 1H), 0.80 (m, 1H), 1.25 (m, 1H), 1.40 (m, 1H), 1.92 (s, 3H), 2.39 (dd, 1H, J = 9.1 and 17.2), 2.85 (dd, 1H, J = 11.0 and 17.2), 3.02 (m, 1H), 3.42 (m, 2H), 5.16 (ddd, 1H, J = 0.5, 11.0 and 11.0), 7.12–7.30 (m, 9H), 7.60 (d, 6H, J = 7.3). ^{13}C NMR: 13.6, 25.0, 25.4, 43.0, 51.4, 64.6, 78.4, 86.2, 126.2, 127.7, 129.8, 145.6, 155.0. Anal. Calcd for $C_{27}H_{28}N_2O$ (396.52): C, 81.78; H, 7.12; N, 7.06. Found: C, 82.09; H, 6.91; N, 7.31.

(2*S*,5′*S*)-(+)-**19b**: Yellow viscous oil, R_f = 0.33 (petroleum ether/ethyl acetate 85:15); $[\alpha]_D^{20} = +11.7$ (c = 1.0, $CHCl_3$). ^{1}H NMR: 0.35 (m, 1H), 1.13–1.30 (m, 3H), 1.99 (s, 3H), 2.82 (dd, 1H, J = 9.7 and 17.6), 3.02–3.20 (m, 3H), 3.75 (m, 1H), 4.89 (ddd, 1H, J = 5.7, 10.5 and 10.5), 7.14–7.34 (m, 9H), 7.58 (d, 6H, J = 7.3). ^{13}C NMR: 13.6, 24.6, 26.6, 41.0, 51.8, 62.0, 78.3, 83.1, 126.4, 127.8, 129.9, 145.2, 155.0. Anal. Calcd for $C_{27}H_{28}N_2O$ (396.52): C, 81.78; H, 7.12; N, 7.06. Found: C, 81.95; H, 6.80; N, 6.87.

To a solution of (−)-**19a** (395 mg, 1.0 mmol) in dichloromethane (3.7 mL) at 0 °C were added water (38 μL) and trifluoroacetic acid (77 μL, 0.99 mmol). The reaction mixture was stirred at r.t. for 1 h and evaporated under vacuum. The residue was added to 0.1 N HCl (15 mL) and extracted with diethyl ether (3 × 10 mL). The residual aqueous phase, made basic by portionwise addition of K_2CO_3, was extracted with dichloromethane (8 × 10 mL). The pooled organic extracts were dried over anhydrous sodium sulfate, the solvent was evaporated giving 98 mg of (−)-**5a** (64% yield).

(2*S*,5′*R*)-(−)-**5a**: Light yellow oil, $[\alpha]_D^{20} = -118.7$ (c = 1.0, $CHCl_3$). ^{1}H NMR: 1.51 (m, 1H), 1.78 (m, 2H), 1.88 (m, 1H), 1.98 (s, 3H), 2.16 (bs, 1H), 2.84 (dd, 1H, J = 16.9 and 8.1), 2.88–3.02 (m, 3H), 3.24 (m, 1H), 4.54 (ddd, 1H, J = 5.5, 7.7 and 8.1). ^{13}C NMR: 13.6, 25.8, 28.0, 41.4, 47.1, 61.2, 82.7, 155.5. Anal. Calcd for $C_8H_{14}N_2O$ (154.21): C, 62.31; H, 9.15; N, 18.17. Found: C, 62.58; H, 9.07; N, 17.95.

The same procedure, performed on (+)-**19b** (270 mg, 0.68 mmol), afforded 55 mg of (+)-**5b** (52% yield).

(2*S*,5′*S*)-(+)-**5b**: Colorless oil, $[\alpha]_D^{20} = +104.1$ (c = 1.05, $CHCl_3$). ^{1}H NMR: 1.44 (m, 1H), 1.81 (m, 3H), 1.99 (s, 3H), 2.20 (bs, 1H), 2.75 (dd, 1H, J = 7.7 and 17.2), 2.87 (m, 1H), 3.00 (dd, 1H, J = 10.3 and 17.2), 3.11 (m, 2H), 4.48 (ddd, 1H, J = 2.9, 7.7 and 10.3). ^{13}C NMR: 13.5, 25.3, 27.9, 42.0, 46.7, 62.3, 83.0, 155.7. Anal. Calcd for $C_8H_{14}N_2O$ (154.21): C, 62.31; H, 9.15; N, 18.17. Found: C, 62.09; H, 9.38; N, 17.92.

4.1.5. 3-Methyl-5-(1-methylpyrrolidin-2-yl)-4,5-dihydroisoxazole syn (2S,5′R)-(−)-**6a**

To a solution of (−)-**5a** (140 mg, 0.91 mmol) in acetonitrile (15 mL), under N_2, were added formaldehyde (0.34 mL, 4.54 mmol) and $NaBH_3CN$ (114 mg, 1.82 mmol). The reaction mixture was stirred for 1 h at r.t., then 2 N HCl (8 mL) was added. After stirring for 2 h, the mixture was extracted with diethyl ether (3 × 5 mL). The residual aqueous phase, made basic by portionwise addition of $NaHCO_3$, was extracted with dichloromethane (6 × 3 mL). After the usual work-up, the residue was purified by silica gel column chromatography (dichloromethane/methanol 95:5), providing (−)-**6a** (100 mg, 65% yield).

(2*S*,5′*R*)-(−)-**6a**: Colorless oil, R_f = 0.41 (dichloromethane/methanol 9:1); $[\alpha]_D^{20} = -102.8$ (c = 0.95, $CHCl_3$); ^{1}H NMR: 1.53 (m, 1H), 1.67 (m, 2H), 1.83 (m, 1H), 1.96 (s, 3H), 2.24 (m, 1H), 2.36 (s, 3H), 2.46 (m, 1H), 2.85 (m, 2H), 3.06 (m, 1H), 4.60 (ddd, 1H, J = 3.6, 9.9 and 9.9). ^{13}C NMR: 13.5, 23.0, 27.1, 40.5, 42.1, 58.2, 67.6, 81.7, 155.6.

Anal. Calcd for $C_9H_{16}N_2O$ (168.24): C, 64.25; H, 9.59; N, 16.65. Found: C, 64.52; H, 9.37; N, 16.48.

4.1.6. 3-Bromo-5-pyrrolidin-2-yl-4,5-dihydroisoxazole syn (2*S*,5′*R*)-(−)-**7a** and 3-tert-butoxy-5-pyrrolidin-2-yl-4,5-dihydroisoxazole anti (2*S*,5′*S*)-(+)-**11b**

The mixture of *syn*/*anti* cycloadducts **17a** and **17b** (2.27 g, 7.11 mmol) was reacted with trifluoroacetic acid following the procedure described for **15a** and **15b**. The crude residue was purified by silica gel column chromatography (dichloromethane/methanol 9:1) producing 1.04 g of yellow oil as a mixture of (−)-**7a** and (+)-**11b** [R_f = 0.42 (dichloromethane/methanol 9:1)].

A mixture of **7a** and **11b** (350 mg) was reacted with triphenylchloromethane according to the procedure applied to **5a** and **5b**. The crude reaction mixture underwent a neutral aluminium oxide flash chromatography (petroleum ether/ethyl acetate 99:1), which gave the trityl derivatives (−)-**20a** (513 mg) and (−)-**21b** (106 mg).

(2*S*,5′*R*)-(−)-**20a**: Crystallized from *n*-hexane as colorless prisms, mp 78.5–80.5 °C. R_f = 0.64 (petroleum ether/ethyl acetate 9:1); $[\alpha]_D^{20}$ = −83.2 (*c* = 0.36, $CHCl_3$). ^{1}H NMR: 0.46 (m, 1H), 0.83 (m, 1H), 1.25 (m, 1H), 1.40 (m, 1H), 2.70 (dd, 1H, *J* = 9.9 and 17.2), 3.03 (m, 1H), 3.12 (dd, 1H, *J* = 11.0 and 17.2), 3.44 (m, 2H), 5.27 (ddd, 1H, *J* = 2.2, 11.0 and 11.0), 7.14–7.33 (m, 9H), 7.58 (d, 6H, *J* = 7.5). ^{13}C NMR: 25.1, 29.9, 45.4, 54.5, 64.1, 78.3, 87.8, 126.4, 127.9, 129.8, 136.9, 146.4. Anal. Calcd for $C_{26}H_{25}BrN_2O$ (461.39): C, 67.68; H, 5.46; N, 6.07. Found: C, 67.87; H, 5.23; N, 6.29.

(2*S*,5′*S*)-(−)-**21b**: Crystallized from *n*-hexane/ethyl acetate as colorless prisms, mp 161.5–164.5 °C. R_f = 0.52 (petroleum ether/ethyl acetate 9:1); $[\alpha]_D^{20}$ = −46.6 (*c* = 1.0, $CHCl_3$). ^{1}H NMR: 0.56 (m, 1H), 0.81 (m, 1H), 1.24–1.41 (m, 2H), 1.46 (s, 9H), 2.42–2.58 (m, 2H), 3.07 (m, 1H), 3.27 (m, 1H), 3.46 (d, 1H, *J* = 8.8), 4.90 (ddd, 1H, *J* = 1.8, 7.5 and 7.5), 7.14–7.28 (m, 9H), 7.58 (m, 6H). ^{13}C NMR: 25.2, 25.4, 27.8, 39.5, 51.4, 59.3, 62.8, 78.3, 82.3, 126.4, 127.8, 129.7, 145.4, 169.7. Anal. Calcd for $C_{30}H_{34}N_2O_2$ (454.60): C, 79.29; H, 7.54; N, 6.16. Found: C, 78.91; H, 7.80; N, 5.85.

A solution of (−)-**20a** (490 mg, 1.06 mmol) was reacted as described for (−)-**19a**, and 104 mg of the free base (−)-**7a** were obtained (45% yield).

(2*S*,5′*R*)-(−)-**7a**: Light yellow viscous oil, $[\alpha]_D^{20}$ = −43.3 (*c* = 1.0, $CHCl_3$). ^{1}H NMR: 1.49 (m, 1H), 1.82 (m, 2H), 1.92 (m, 1H), 2.28 (bs, 1H), 2.98 (m, 2H), 3.20 (m, 2H), 3.38 (m, 1H), 4.67 (ddd, 1H, *J* = 5.5, 9.9 and 9.9). ^{13}C NMR: 25.7, 27.8, 44.0, 47.0, 60.5, 84.5, 137.6. Anal. Calcd for $C_7H_{11}BrN_2O$ (219.08): C, 38.38; H, 5.06; N, 12.79. Found: C, 38.70; H, 4.87; N, 12.56.

Similarly, the *N*-trityl derivative (+)-**21b** (250 mg, 0.55 mmol) produced 70 mg of the free base (+)-**11b** (60% yield).

(2*S*,5′*S*)-(+)-**11b**: Light yellow viscous oil, $[\alpha]_D^{20}$ = +17.4 (*c* = 1.12, $CHCl_3$). ^{1}H NMR: 1.34 (m, 1H), 1.40 (s, 9H), 1.65–1.88 (m, 3H), 2.22 (bs, 1H), 2.49 (dd, 1H, *J* = 8.5 and 16.2), 2.71 (dd, 1H, *J* = 7.7 and 16.2), 2.95 (m, 2H), 3.24 (dd, 1H, *J* = 7.4 and 7.4), 4.12 (dd, 1H, *J* = 8.5 and 8.5). ^{13}C NMR: 25.3, 27.4, 27.5, 38.9, 46.7, 59.2, 60.2, 80.6, 169.2. Anal. Calcd for $C_{11}H_{20}N_2O_2$ (212.29) C, 62.23; H, 9.50; N, 13.20. Found: C, 62.47; H, 9.58; N, 12.97.

4.1.7. Fumarates of 3-bromo-5-pyrrolidin-2-yl-4,5-dihydroisoxazole syn (2*S*,5′*R*)-(−)-**7a** and syn (2*S**,5′*R**)-(±)-**7a** for X-ray analysis

To a solution (−)-**7a** (180 mg, 0.82 mmol) in methanol (1.5 mL) was added a solution of fumaric acid (96 mg, 0.82 mmol) in methanol (1.5 mL), and the mixture was stirred at r.t. for 16 h. The corresponding salt was obtained quantitatively after removal of the solvent.

(2*S*,5′*R*)-(−)-**7a** × $C_4H_4O_4$: Crystallized from 2-propanol/ethyl acetate as a pale yellow powder, mp 120–122.5 °C $[\alpha]_D^{20}$ = −160.4 (*c* = 1.0, MeOH). Chiral HPLC analysis, mobile phase: *n*-hexane/2-propanol 4:1; flow rate: 0.8 mL/min; λ 220 nm; t_R 7.13 min. E.e. > 98%. ^{1}H NMR (D_2O): 1.61 (m, 1H), 1.77–1.96 (m, 2H), 2.01 (m, 1H), 3.07 (dd, 1H, *J* = 7.6 and 18.2), 3.19 (m, 2H), 3.49 (dd, 1H, *J* = 11.1 and 18.2), 3.72 (m, 1H), 5.02 (ddd, 1H, *J* = 3.2, 7.6 and 11.1), 6.53 (s, 1H). ^{13}C NMR (D_2O): 23.4, 23.5, 44.2, 46.2, 61.5, 79.1, 134.8, 140.3, 171.8. Anal. Calcd for $C_{11}H_{15}BrN_2O_5$ (335.15) C, 39.42; H, 4.51; N, 8.36. Found: C, 39.60; H, 4.32; N, 8.12.

The corresponding (±)-**7a** fumarate was obtained, with a comparable overall yield, through the reaction sequence described for the synthesis of the *syn* laevorotatory enantiomer. The intermediate derivatives and the final salt had the same spectroscopic features described above for the pure antipode. The resulting salt afforded crystals suitable to perform X-ray analysis (see below).

(2*S**,5′*R**)-(±)-**7a** × $C_4H_4O_4$: Crystallized from 2-propanol as colorless prisms, mp 128.5–130 °C. Anal. Calcd for $C_{11}H_{15}BrN_2O_5$ (335.15) C, 39.42; H, 4.51; N, 8.36. Found: C, 39.57; H, 4.67; N, 8.48.

4.1.8. 3-Bromo-5-(1-methylpyrrolidin-2-yl)-4,5-dihydroisoxazole syn (2*S*,5′*R*)-(−)-**8a**

The free base (−)-**7a** (200 mg, 0.91 mmol) was reacted following the protocol applied to the synthesis of (−)-**6a**. After the usual work-up, the residue was purified by silica gel column chromatography (dichloromethane/methanol 9:1), providing the tertiary amine (−)-**8a** (75 mg, 35% yield).

(2*S*,5′*R*)-(−)-**8a**: Light yellow oil, R_f = 0.68 (dichloromethane/methanol 9:1); $[\alpha]_D^{20}$ = −131.4 (*c* = 0.51, $CHCl_3$). ^{1}H NMR: 1.52 (m, 1H), 1.75 (m, 2H), 1.93 (m, 1H), 2.31 (m, 1H), 2.39 (s, 3H), 2.60 (m, 1H), 3.09 (m, 1H), 3.15 (dd, 1H, *J* = 10.8 and 17.0), 3.26 (dd, 1H, *J* = 9.4 and 17.0), 4.77 (ddd, 1H, *J* = 3.2, 9.7 and 9.7). ^{13}C NMR: 23.3, 27.5, 42.4, 42.9, 58.3, 66.7, 84.0, 137.6. Anal. Calcd for $C_8H_{13}BrN_2O$ (233.11): C, 41.22; H, 5.62; N, 12.02. Found: C, 40.87; H, 5.88; N, 12.35.

4.1.9. 3-Methoxy-5-pyrrolidin-2-yl-4,5-dihydroisoxazoles syn (2*S*,5′*R*)-(−)-**9a** and anti (2*S*,5′*S*)-(+)-**9b**

The mixture of *syn*/*anti* cycloadducts **18a** and **18b** (2.15 g, 7.95 mmol) was reacted with trifluoroacetic acid following the procedure described in Section 4.1.4 for **15a** and **15b**. The crude residue was purified by silica gel column chromatography (dichloromethane/methanol 9:1) affording 1.26 g of yellow oil (93% overall yield) as a mixture of (−)-**9a** and (+)-**9b** [R_f = 0.44 (dichloromethane/methanol 9:1)].

Derivatives (−)-**9a** and (+)-**9b** (1.25 g, 7.34 mmol) were reacted with triphenylchloromethane as described in Section 4.1.4 for **5a** and **5b**. The crude reaction mixture was submitted to a neutral aluminium oxide flash chromatography (petroleum ether/ethyl acetate 99:1), which afforded the *syn* (−)-**22a** (605 mg) and *anti* (+)-**22b** (170 mg) trityl isomers, and an intermediate (1.82 g) fraction of their mixture (86% overall yield).

(2*S*,5′*R*)-(−)-**22a**: Crystallized from ethyl acetate as colorless prisms, mp 165.5–166.5 °C. R_f = 0.47 (petroleum ether/ethyl acetate 85:15); $[\alpha]_D^{20}$ = −81.7 (*c* = 1.19, $CHCl_3$). ^{1}H NMR: 0.44 (m, 1H), 0.86 (m, 1H), 1.45 (m, 2H), 2.53 (dd, 1H, *J* = 10.3 and 16.7), 2.81 (dd, 1H, *J* = 10.3 and 16.7), 3.04 (m, 1H), 3.42 (m, 2H), 3.82 (s, 3H), 5.22 (ddd, 1H, *J* = 1.8, 9.4 and 9.4), 7.13–7.27 (m, 9H), 7.58 (d, 6H, *J* = 7.3). ^{13}C NMR: 25.0, 25.2, 36.5, 51.4, 57.3, 63.9, 78.3, 87.3, 126.3, 127.8, 129.8, 145.6, 167.5. Anal. Calcd for $C_{27}H_{28}N_2O_2$ (412.52): C, 78.61; H, 6.84; N, 6.79. Found: C, 78.49; H, 6.88; N, 6.97.

(2*S*,5′*S*)-(+)-**22b**: Colorless viscous oil, R_f = 0.40 (petroleum ether/ethyl acetate 85:15); $[\alpha]_D^{20}$ = +3.8 (*c* = 0.64, $CHCl_3$). ^{1}H NMR: 0.36 (m, 1H), 1.10–1.43 (m, 3H), 2.87 (dd, 1H, *J* = 10.1 and 16.3), 2.98–3.16 (m, 3H), 3.74 (m, 1H), 3.83 (s, 3H), 4.97 (ddd, 1H, *J* = 5.7, 10.1 and 10.1), 7.10–7.36 (m, 9H), 7.58 (d, 6H, *J* = 7.3). ^{13}C NMR: 24.6, 26.5, 34.9, 51.6, 57.4, 61.9, 78.0, 84.3, 126.4, 127.8, 129.9, 145.2,

168.0. Anal. Calcd for $C_{27}H_{28}N_2O_2$ (412.52): C, 78.61; H, 6.84; N, 6.79. Found: C, 78.92; H, 7.12; N, 6.50.

A solution of (−)-**22a** (595 mg, 1.44 mmol) was reacted as described for (−)-**19a**, giving the free base (−)-**9a** (101 mg, 41% yield).

(2*S*,5′*R*)-(−)-**9a**: Pale yellow oil, $[\alpha]_D^{20} = -52.3$ ($c = 1.28$, $CHCl_3$). ^{1}H NMR: 1.43 (m, 1H), 1.69 (m, 2H), 1.81 (m, 1H), 1.93 (bs, 1H), 2.79–2.93 (m, 4H), 3.21 (dd, 1H, $J = 7.1$ and 7.1), 3.75 (s, 3H), 4.47 (ddd, 1H, $J = 5.8$, 9.1 and 9.1). ^{13}C NMR: 25.7, 27.9, 35.0, 47.1, 57.4, 60.5, 84.5, 167.8. Anal. Calcd for $C_8H_{14}N_2O_2$ (170.21): C, 56.45; H, 8.29; N, 16.46. Found: C, 56.77; H, 8.01; N, 16.70.

Similarly, (+)-**22b** (320 mg, 0.78 mmol) provided 90 mg the free base (+)-**9b** (68% yield).

(2*S*,5′*S*)-(+)-**9b**: Pale yellow oil, $[\alpha]_D^{20} = +42.8$ ($c = 1.33$, $CHCl_3$). ^{1}H NMR: 1.40 (m, 1H), 1.67–1.88 (m, 3H), 2.07 (bs, 1H), 2.75 (dd, $J = 8.2$ and 16.2, 1H), 2.90 (m, 1H), 2.98 (m, 2H), 3.19 (m, 1H), 3.86 (s, 3H), 4.49 (ddd, 1H, $J = 1.4$, 7.1 and 8.2). ^{13}C NMR: 25.3, 27.7, 35.9, 46.7, 57.4, 61.8, 84.3, 167.8. Anal. Calcd for $C_8H_{14}N_2O_2$ (170.21): C, 56.45; H, 8.29; N, 16.46. Found: C, 56.17; H, 8.52; N, 16.27.

4.1.10. 3-Methoxy-5-(1-methylpyrrolidin-2-yl)-4,5-dihydroisoxazole syn (2*S*,5′*R*)-(−)-***10a***

The free base (−)-**9a** (500 mg, 2.94 mmol) was reacted following the protocol applied to the synthesis (−)-**6a**. After the usual work-up, the residue was purified by silica gel column chromatography (dichloromethane/methanol 95:5), providing the tertiary amine (−)-**10a** (260 mg, 48% yield).

(2*S*,5′*R*)-(−)-**10a**: Light yellow oil, $R_f = 0.40$ (dichloromethane/methanol 9:1); $[\alpha]_D^{20} = -73.4$ ($c = 1.16$, $CHCl_3$). ^{1}H NMR: 1.62 (m, 1H), 1.76 (m, 2H), 1.91 (m, 1H), 2.26 (m, 1H), 2.41 (s, 3H), 2.53 (m, 1H), 2.88 (dd, 1H, $J = 10.1$ and 16.3), 2.99 (dd, 1H, $J = 9.7$ and 16.3), 3.09 (m, 1H), 3.85 (s, 3H), 4.68 (ddd, 1H, $J = 4.0$, 10.1 and 10.1). ^{13}C NMR: 23.3, 27.4, 34.4, 42.4, 57.3, 58.3, 67.3, 83.4, 167.9. Anal. Calcd for $C_9H_{16}N_2O_2$ (184.24): C, 58.67; H, 8.75; N, 15.21. Found: C, 58.95; H, 8.51; N, 14.97.

4.2. Single-crystal X-ray diffraction analysis of (±)-***7a*** fumarate

A batch consisting of about 5 mg of (±)-**7a** fumarate was dissolved in methanol and then crystallized by slow evaporation of the solvent at $T = 4$ °C. After 5 days, a solid precipitate appeared, which was made up by some white, very thin needles together with a small quantity of colorless, larger prisms. After testing some useless specimens, one of the latter (dimensions: 0.100 × 0.050 × 0.025 mm^3) was selected for the X-ray analysis. Crystal data: title compound: $C_7H_{11}N_2OBr\cdots C_4H_4O_4$, *Mr* 335.15 a.m.u.; monoclinic; space group $P2_1/c$ (No 14), centrosymmetric; unit cell (Å, Å^3): $a = 5.6092$ (12), $b = 25.018$ (5), $c = 9.7897$ (19), $V = 1333.3$ (3); *Z*: 4; Dc: 1.670 g/cm^3; F(000): 680; μ: 3.101 mm^{-1}. The sample was twinned, with relative weight of the minor twin fraction being as large as 16.7(6) %. A full sphere of 20,099 diffraction data (both components) was collected within $\sin\vartheta/\lambda = 0.55$ Å^{-1} (Mo source); the data were subsequently corrected for X-ray absorption by an empirical procedure and merged, giving a final set of 1734 independent, observed reflections. It was impossible to obtain reliable data at greater resolution due to the very low sample dimensions. In the final least-square model, riding motion constraints were applied to the hydrogen atoms, whose isotropic thermal parameters U_{iso} were estimated as 1.2 U_{iso} of the heavy atom to which they were bonded. Moreover, the anisotropic thermal motion of the C, N and O atoms was constrained to be equal among the atoms belonging to the same ring. More information can be found on the deposited data [25].

4.3. Receptor binding assays

4.3.1. Membranes binding of [^{3}H]epibatidine and [^{125}I]α-bungarotoxin

The cortex tissues were dissected, immediately frozen on dry ice and stored at −80 °C for later use. In each experiment, the cortex tissues from two rats were homogenized in 10 mL of a buffer solution [50 mM Na_3PO_4, 1 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4] using a potter homogenizer; the homogenates were then diluted and centrifuged at 60,000*g* for 1.5 h. The total membrane homogenization, dilution and centrifugation procedures were performed twice, then the pellets were collected, rapidly rinsed with a buffer solution (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 2 mM PMSF, pH 7), and resuspended in the same buffer containing a mixture of 20 μg/mL of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin.

4.3.2. [^{3}H]Epibatidine binding

(±)-[^{3}H]Epibatidine with a specific activity of 56–60 Ci/mmol was purchased from Perkin–Elmer (Boston MA); the nonradioactive α-bungarotoxin, epibatidine, and nicotine were purchased from Sigma–Aldrich (Italy). It has been previously reported that [^{3}H]epibatidine also binds to α-bungarotoxin binding receptors with nM affinity [27]. In order to prevent the binding of [^{3}H]epibatidine to the α-bungarotoxin binding receptors, the membrane homogenates were pre-incubated with 2 μM α-bungarotoxin and then with [^{3}H]epibatidine. The saturation experiments were performed by incubating aliquots of cortex membrane homogenates with 0.01–2.5 nm concentrations of (±)-[^{3}H]epibatidine overnight at 4 °C. Nonspecific binding was determined in parallel by means of incubation in the presence of 100 nM unlabelled epibatidine. At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine and washed with 15 mL of a buffer solution (10 mM Na_3PO_4, 50 mM NaCl, pH 7.4), and the filters were counted in a β counter.

4.3.3. [^{125}I]α-Bungarotoxin binding

The saturation binding experiments were performed using aliquots of cortex membrane homogenates incubated overnight with 0.1–10 nm concentrations of [^{125}I]α-bungarotoxin (specific activity 200–213 Ci/mmol, Amersham) at r.t. Nonspecific binding was determined in parallel by means of incubation in the presence of 1 μM unlabelled α-bungarotoxin. After incubation, the samples were filtered as described above and the bound radioactivity was directly counted in a γ counter.

4.3.4. nACh Receptor affinity of compounds (−)-***5a***, (+)-***5b***, (−)-***6a***, (−)-***7a***, (−)-***8a***, (−)-***9a***, (+)-***9b***, (−)-***10a***, and (+)-***11b***

The inhibition of radioligand binding by epibatidine and the test compounds was measured by pre-incubating cortex homogenates with increasing doses (10 pM–10 mM) of the reference nicotinic agonists, epibatidine or nicotine, and the drug to be tested for 30 min at r.t., followed by overnight incubation with a final concentration of 0.075 nM [^{3}H]epibatidine or 1 nM [^{125}I]α-bungarotoxin at the same temperatures as those used for the saturation experiments. These ligand concentrations were used for the competition binding experiments because they are within the range of the K_D values of the ligands for the two different classes of nAChRs. For each compound, the experimental data obtained from the three saturation and three-competition binding experiments were analyzed by means of a non-linear least-square procedure, using the LIGAND program as described by Munson and Rodbard [26]. The binding parameters were calculated by simultaneously

fitting three independent saturation experiments and the K_i values were determined by fitting the data of three independent competition experiments. The errors in the K_D and K_i values of the simultaneous fits were calculated using the LIGAND software, and were expressed as percentage coefficients of variation (% CV). When final compound concentrations up to 500 μM did not inhibit radioligand binding, the K_i value was defined as being >500 μM based on the Cheng and Prusoff's equation [28].

Acknowledgments

This research was financially supported by the Italian FIRB grant # RBNE03FH5Y and PRIN grant # 20072BTSR2. We are indebted to Prof. P. Ernsberger, Case Western Reserve University, Cleveland, OH, USA, for the affinity data of the studied compounds at the human cloned muscarinic receptor subtypes.

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