The Enantiomers of Epiboxidine and of Two Related Analogs: Synthesis and Estimation of their Binding Affinity at α4β2 and α7 Neuronal Nicotinic Acetylcholine Receptors

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ABSTRACT Epiboxidine hydrochlorides (+)-**2** and (−)-**2**, which are the structural analogs of the antipodes of epibatidine (±)-**1**, as well as the enantiomeric pairs (+)-**3**/(−)-**3** and (+)-**4**/(−)-**4** were synthesized and tested for binding affinity at α4β2 and α7 nicotinic acetylcholine receptor (nAChR) subtypes. Final derivatives were prepared through the condensation of racemic *N*-Boc-7-azabicyclo[2.2.1]heptane-2-one (±)-**5** with the resolving agent (*R*)-(+)-2-methyl-2-propanesulfinamide. The pharmacological analysis carried out on the three new enantiomeric pairs evidenced an overall negligible degree of enantioselectivity at both nAChRs subtypes, a result similar to that reported for both natural and unnatural epibatidine enantiomers at the same investigated receptor subtypes. *Chirality 00:000–000, 2012.* © 2012 Wiley Periodicals, Inc.

KEY WORDS: epibatidine; epiboxidine and analogs; neuronal nicotinic acetylcholine receptors; chiral resolution; enantiopure nicotinic ligands; binding affinity

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop family of ligand-gated pentameric ion channels, which includes also $GABA_A$, glycine, and 5-HT_3 receptors.[1,2] If the clinical application of selective nicotinic antagonists at the muscular nAChRs has now well settled, research lines from private companies as well as public institutions are focused on the full physiological and pharmacological characterization of each of the subtypes expressed in the central nervous system (CNS), aiming at the identification of specific and innovative therapeutic targets.[3–5] One major issue of the research on compounds acting at neuronal nAChRs is the development of functionally selective ligands, mainly agonists or partial agonists, which could be of potential therapeutic significance for application to CNS pathologic conditions. Among them are Alzheimer's and Parkinson's diseases, epilepsy, schizophrenia, Tourette's syndrome, attention deficit hyperactivity disorder, pain, depression, and tobacco addiction.[5–7]

In addition to subtype selectivity, a further major aspect of the ligand/receptor interactions is the degree of stereoselectivity shown by chiral derivatives in their molecular recognition of receptor proteins. Within the superfamily of cholinergic receptors, the structure/activity relationships of chiral nicotinic ligands suggest an overall lower impact of stereoselectivity and enantioselectivity when compared with chiral muscarinic ligands. In this respect, (−)-epibatidine **1** (Figure 1), the alkaloid isolated in trace amounts from the Ecuadorian poison frog *Epipedobates tricolor*,[8] is a meaningful example. This natural toxin was initially tested for its antinociceptive activity, and epibatidine-induced analgesia, markedly higher than that of morphine, was found to be mediated by nAChRs.[8,9] Next, several synthetic approaches to (±)-epibatidine and its enantiomers[10] allowed an accurate analysis of their pharmacological profile at the various nAChR subtypes. Overall, epibatidine was characterized as an exceptionally potent, unselective nicotinic agonist with a marginal enantioselectivity at all the tested nAChRs, among them the heteromeric α4β2 and homomeric α7 receptors, which are the subtypes with the highest expression levels in the mammalian CNS.[11–14]

Among the various epibatidine-related compounds reported in the literature,[13] epiboxidine (±)-**2**[15] (Fig. 1), in which the 3-methylisoxazole ring bioisosterically replaced the 2-chloropyridine moiety of the reference compound, behaved as a potent α4β2 nicotinic agonist, retaining almost entirely the antinociceptive potency of epibatidine but with a lower toxicity.[15] Recently, we have expanded the study of epiboxidine structure/activity relationships through the elaboration of an alternative synthetic approach to (±)-**2**[16] as well as the preparation and biological screening of new epiboxidine-related compounds.[17,18] Among them, the close analog of epiboxidine (±)-**3**[16] and the permanently charged derivative (±)-**4**[18] emerged as novel interesting nicotinic ligands. As part of a research line focused on the design, synthesis, and pharmacological investigation of ligands acting at neuronal nAChR subtypes,[19,20] we aimed at extending our study to the enantiomers of (±)-**2**, (±)-**3**, and (±)-**4**. In this article, the preparation of the three target enantiomeric pairs (+)-**2**/(−)-**2**, (+)-**3**/(−)-**3**, and (+)-**4**/(−)-**4**, depicted in

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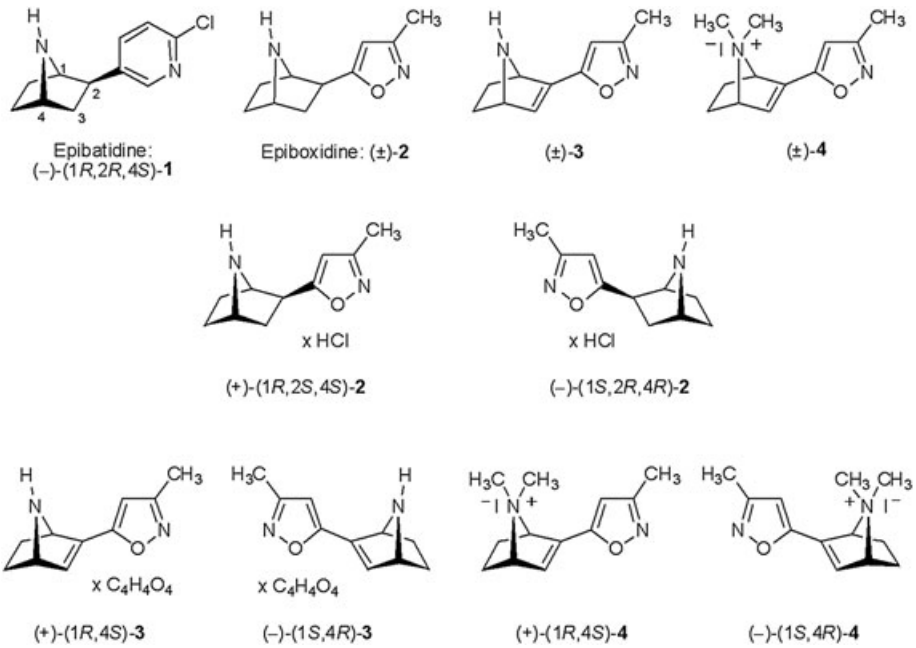


Fig. 1. Structure of natural epibatidine (–)-**1**, epiboxidine (±)-**2**, its analogs (±)-**3** and (±)-**4**, and the three couples of enantiomers (+)-**2**/(–)-**2**, (+)-**3**/(–)-**3**, and (+)-**4**/(–)-**4** examined in this study.

Figure 1, and the results of their binding affinity at neuronal α4β2 and α7 nAChR subtypes will be presented and discussed.

EXPERIMENTAL

Materials and Methods

Racemic *N*-Boc-7-azabicyclo[2.2.1]heptan-2-one (±)-**5** was prepared according to a literature method.[21,16] (*R*)-(+)-2-Methyl-2-propanesulfinamide was purchased from Aldrich. ^{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. Thin layer chromatography (TLC) analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or a phosphomolybdic acid solution and, for amines, with 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. High performance liquid chromatography (HPLC) analyses were performed with a Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975. Melting points were determined on a model B-540 Büchi apparatus and are uncorrected. Electrospray ionization (ESI) mass spectra were obtained on a Varian 320 LC-MS/MS instrument. Data are reported as mass-to-charge ratio (m/z) of the corresponding positively charged molecular ions. Microanalyses (C, H, and N) of new compounds agreed with the theoretical value within ±0.4%.

Synthetic Chemistry

(–)-(1*R*,4*S*)-*tert*-Butyl-2-{(*R*)-*tert*-butylsulfinylimino}-7-azabicyclo[2.2.1]heptane-7-carboxylate 6 and (–)-(1*S*,4*R*)-*tert*-butyl-2-{(*R*)-*tert*-butylsulfinylimino}-7-azabicyclo[2.2.1]heptane-7-carboxylate 7. (*R*)-(+)-2-Methyl-2-propanesulfinamide (1.58 g, 13.06 mmol) and $Ti(OEt)_4$ (6.07 ml, 24.2 mmol) were added to a stirred solution of (±)-**5** (2.76 g, 13.06 mmol) in dry THF (40 ml), and the reaction was heated at reflux under nitrogen, monitoring the disappearance of the starting material by TLC (petroleum ether/ethyl acetate 4:1). After 2 h, the mixture was cooled at room temperature, concentrated in vacuo, and the residue was dissolved in ethyl acetate (150 ml) and treated with brine (40 ml). After stirring for 15 min followed by filtration over a Celite® pad, the organic phase was separated and treated with water (2 × 40 ml), then dried over anhydrous Na_2SO_4, filtered, and the solvent was evaporated at reduced pressure. The residue underwent two sequential silica gel flash chromatographies (petroleum ether/ethyl acetate 9:1 → 3:1), which allowed complete separation of the two diastereomers (–)-**6** (1.68 g, 41% yield) and (–)-**7** (2.19 g, 53% yield).

(–)-(1*R*,4*S*,9*R*)-**6**: Colorless prisms (from diisopropyl ether), mp 63–64 °C. R_f = 0.25 (petroleum ether/ethyl acetate 4:1). HPLC analysis, MERCK LiChrospher Si 60 column (15 cm × 4.6 mm, 5 μm), mobile phase: *n*-hexane/ethyl acetate 3:2; flow rate: 0.5 ml/min; λ 254 nm; retention time: 7.04 min. $[\alpha]_D^{25} = -261.2$ (*c* 1.01, $CHCl_3$). ^{1}H NMR: 1.18 (s, 9H), 1.39 (s, 9H), 1.42–1.49 (m, 1H), 1.56–1.64 (m, 1H), 1.84–1.91 (m, 1H), 1.94–2.03 (m, 1H), 2.57–2.68 (m, 1H), 2.93–3.00 (m, 1H), 4.35–4.37 (m, 1H), 4.47–4.49 (m, 1H). ^{13}C NMR: 22.61, 27.75, 28.35, 41.54, 44.50, 57.09, 57.34, 65.00, 80.77, 155.41, 183.86. MS (ESI) m/z $[M+H]^+$ Calcd for $C_{15}H_{26}N_2O_3S$: 314.4. Found: 315.5. Anal. Calcd for $C_{15}H_{26}N_2O_3S$ (314.44): C, 57.30; H, 8.33; N, 8.91. Found: C, 57.63; H, 8.47; N, 9.11.

(–)-(1*S*,4*R*,9*R*)-**7**: Colorless prisms (from diisopropyl ether), mp 96–97 °C. R_f = 0.18 (petroleum ether/ethyl acetate 4:1). HPLC analysis, MERCK LiChrospher Si 60 column (15 cm × 4.6 mm, 5 μm), mobile phase: *n*-hexane/ethyl acetate 3:2; flow rate: 0.5 ml/min; λ 254 nm; retention time: 8.45 min. $[\alpha]_D^{25} = -269.1$ (*c* 1.01, $CHCl_3$). ^{1}H NMR: 1.22 (s, 9H), 1.43 (s, 9H), 1.47–1.65 (m, 2H), 1.85–1.94 (m, 1H), 1.98–2.07 (m, 1H), 2.57–2.69 (m, 1H), 2.88 (d, J = 18.2 Hz, 1H), 4.44–4.47 (m, 1H), 4.55–4.57 (m, 1H). ^{13}C NMR: 22.46, 27.90, 28.41, 40.27, 45.18, 56.94, 57.33, 64.69, 80.85, 155.17, 184.68. MS (ESI) m/z $[M+H]^+$ Calcd for $C_{15}H_{26}N_2O_3S$: 314.4. Found: 315.2. Anal. Calcd for $C_{15}H_{26}N_2O_3S$ (314.44): C, 57.30; H, 8.33; N, 8.91. Found: C, 57.42; H, 8.55; N, 8.72.

(–)-(1*R*,4*S*)-*tert*-Butyl-2-oxo-7-azabicyclo[2.2.1]heptane-7-carboxylate 5. 4N AcOH (50 ml) was added portionwise to a solution of (–)-**6** (775 mg; 2.46 mmol) in MeOH (50 ml), and the reaction mixture was stirred and heated at 40 °C per 18 h. The solution was concentrated at reduced pressure, and the aqueous acidic phase was extracted with Et_2O (3 × 20 ml). The pooled organic phases were sequentially treated with H_2O (20 ml), saturated aqueous $NaHCO_3$ solution (2 × 20 ml), brine (20 ml), and H_2O (20 ml). After standard workup, the crude solid (510 mg, 98% yield) was purified by crystallization.

(1*R*,4*S*)-(−)-**5**: Colorless powder (from diisopropyl ether), mp 44–45 °C. R_f = 0.20 (petroleum ether/ethyl acetate 95:5). Chiral HPLC analysis, DAICEL Chiralpak AD column (25 cm × 4.6 mm, 10 μm), mobile phase: *n*-hexane/2-propanol 98:2; flow rate: 1.0 ml/min; λ 254 nm; retention time: 13.08 min. E.e. 98%. $[\alpha]_D^{25} = -73.1$ (*c* 1.0, $CHCl_3$) {lit.[22]: $[\alpha]_D^{22} = -73.6$ (*c* 1.10, $CHCl_3$)}. ^{1}H NMR: 1.40 (s, 9H), 1.50–1.65 (m, 2H), 1.88–2.07 (m, 3H), 2.43 (dd, *J* = 17.3 and 5.2 Hz, 1H), 4.21 (m, 1H), 4.53 (m, 1H). ^{13}C NMR: 24.61, 27.74, 28.39, 45.42, 56.24, 64.13, 81.02, 155.26, 209.76. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{11}H_{17}NO_3$: 211.3. Found: 212.1. Anal. Calcd for $C_{11}H_{17}NO_3$ (211.26): C, 62.54; H, 8.11; N, 6.63. Found: C, 62.63; H, 7.98; N, 6.72.

(+)-(1*S*,4*R*)-*tert*-Butyl-2-oxo-7-azabicyclo[2.2.1]heptane-7-carboxylate 5. The title compound was prepared from diastereomer (−)-**7** (820 mg, 2.61 mmol) following the aforementioned procedure described for (−)-**5**. The crude ketone (518 mg, 94% yield) was purified by crystallization.

(1*S*,4*R*)-(+)-**5**: Colorless powder (from diisopropyl ether), mp 43–44 °C. Chiral HPLC analysis was performed in the same conditions as those reported for (−)-**5**. Retention time: 12.06 min. E.e. 98%. $[\alpha]_D^{25} = +75.7$ (*c* 1.0, $CHCl_3$) {lit.[22]: $[\alpha]_D^{22} = +73.5$ (*c* 1.0, $CHCl_3$)}. The NMR spectroscopic data matched those of (−)-**5**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{11}H_{17}NO_3$: 211.3. Found: 212.1. Anal. Calcd for $C_{11}H_{17}NO_3$ (211.26): C, 62.54; H, 8.11; N, 6.63. Found: C, 62.72; H, 8.03; N, 6.84.

(−)-(1*R*,4*S*)-*tert*-Butyl-2-(trifluoromethylsulfonyloxy)-7-azabicyclo[2.2.1]hept-2-ene-7 carboxylate 8. A solution of ketone (−)-**5** (467 mg, 2.21 mmol) in anhydrous THF (5 ml) was slowly added dropwise to a stirred solution of KHMDS (5.52 ml of a 0.5 M KHMDS solution in toluene) in anhydrous 1,2-dimethoxyethane (DME, 15 ml) cooled at −78 °C under argon. After 40 min, a solution of *N*-(5-chloro-2-pyridyl)bis(trifluoromethanesulfonimide) (1.04 g, 2.65 mmol) in anhydrous DME (5 ml) was dropped, and the reaction was kept under stirring for further 2 h at −78 °C, then for 3 days at room temperature, monitoring the disappearance of the starting material (TLC, petroleum ether/ethyl acetate 9:1). The solvent was then evaporated in vacuo, and the brown oily residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate 9:1) to provide 387 mg (51% yield) of the desired methanesulfonate.

(−)-(1*R*,4*S*)-**8**: Colorless oil. R_f = 0.62 (petroleum ether/ethyl acetate 9:1). $[\alpha]_D^{25} = -21.1$ (*c* 1.02, $CHCl_3$). ^{1}H NMR: 1.13–1.50 (m, 2H), 1.40 (s, 9H), 1.95–2.10 (m, 2H), 4.65 (m, 1H), 4.73 (m, 1H), 5.92 (bs, 1H). ^{13}C NMR: 25.40, 28.23, 29.91, 60.51, 81.21, 116.60, 120.86, 155.02. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{12}H_{16}F_3NO_5S$: 343.3. Found: 344.1. Anal. Calcd for $C_{12}H_{16}F_3NO_5S$ (343.32): C, 41.98; H, 4.70; N, 4.08. Found: C, 42.10; H, 4.52; N, 4.22.

(+)-(1*S*,4*R*)-*tert*-Butyl-2-(trifluoromethylsulfonyloxy)-7-azabicyclo[2.2.1]hept-2-ene-7-carboxylate 8. The title compound was prepared from ketone (+)-**5** (639 mg, 3.02 mmol) following the aforementioned procedure described for (−)-**8**. The crude reaction mixture was purified by silica gel column chromatography (petroleum ether/ethyl acetate 9:1) to afford (+)-**8** (592 mg, 57% yield).

(+)-(1*S*,4*R*)-**8**: Colorless oil. $[\alpha]_D^{25} = +21.5$ (*c* 0.88, $CHCl_3$). The NMR spectroscopic data matched those of (−)-**8**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{12}H_{16}F_3NO_5S$: 343.3. Found: 344.2. Anal. Calcd for $C_{12}H_{16}F_3NO_5S$ (343.32): C, 41.98; H, 4.70; N, 4.08. Found: C, 41.85; H, 4.63; N, 4.15.

(−)-(1*R*,4*S*)-*tert*-Butyl-2-(3-methylisoxazol-5-yl)-7-azabicyclo[2.2.1]hept-2-ene-7-carboxylate 9. To a magnetically stirred solution of enoltriflate (−)-**8** (270 mg, 0.79 mmol) in anhydrous THF (15 ml), kept at −78 °C under argon, were sequentially added $[Pd_2(dba)_3]\cdot CHCl_3$ (70 mg, 0.06 mmol), triphenylphosphine (15 mg, 0.02 mmol), then a solution of 3-methyl-5-tributylstannylisoxazole[16] (880 mg, 2.40 mmol) in anhydrous THF (5 ml) and anhydrous $ZnCl_2$ (107 mg, 0.79 mmol). The mixture was quickly warmed at room temperature and stirred overnight under argon until disappearance of the starting material (TLC, petroleum ether/ethyl acetate 9:1). After addition of brine (20 ml), the crude reaction was repeatedly extracted with ethyl acetate (4 × 20 ml). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate 9:1) to afford (−)-**9** (201 mg, 92%).

(−)-(1*R*,4*S*)-**9**: Colorless powder (from diisopropyl ether/ethyl acetate 1:1), mp 147–148 °C. R_f = 0.46 (petroleum ether/ethyl acetate 4:1). Chiral HPLC analysis, DAICEL Chiralcel OD-H column (25 cm × 4.6 mm, 5 μm), mobile phase: *n*-hexane/2-propanol 99:1; flow rate: 0.8 ml/min; λ 254 nm; retention time: 12.13 min. E.e. 99%. $[\alpha]_D^{25} = -16.9$ (*c* 1.05, $CHCl_3$). ^{1}H NMR: 1.24 (m, 2H), 1.41 (s, 9H), 2.00 (m, 2H), 2.30 (s, 3H), 4.82 (bs, 1H), 4.94 (m, 1H), 6.10 (s, 1H), 6.68 (bs, 1H). ^{13}C NMR: 11.62, 24.53, 25.49, 28.40, 60.89, 61.14, 102.22, 133.35, 135.17, 155.19, 160.24, 163.95. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{15}H_{20}N_2O_3$: 276.3. Found: 277.1. Anal. Calcd for $C_{15}H_{20}N_2O_3$ (276.33): C, 65.20; H, 7.30; N, 10.14. Found: C, 65.49; H, 7.14; N, 10.39.

(+)-(1*S*,4*R*)-*tert*-Butyl-2-(3-methylisoxazol-5-yl)-7-azabicyclo[2.2.1]hept-2-ene-7-carboxylate 9. The 7-azabicyclo[2.2.1]hept-2-ene derivative (+)-**9** was prepared from enoltriflate (+)-**8** (508 mg, 1.48 mmol) following the aforementioned procedure described for (−)-**9**. The crude reaction mixture was purified by silica gel column chromatography (petroleum ether/ethyl acetate 9:1) to afford (+)-**9** (364 mg, 89% yield).

(+)-(1*S*,4*R*)-**9**: Colorless powder (from diisopropyl ether/ethyl acetate 1:1), mp 147–148 °C. Chiral HPLC analysis was performed in the same conditions as those reported for (−)-**9**. Retention time: 13.11 min. E.e. 99%. $[\alpha]_D^{25} = +16.1$ (*c* 0.88, $CHCl_3$). The NMR spectroscopic data matched those of (−)-**9**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{15}H_{20}N_2O_3$: 276.3. Found: 277.4. Anal. Calcd for $C_{15}H_{20}N_2O_3$ (276.33): C, 65.20; H, 7.30; N, 10.14. Found: C, 65.48; H, 7.22; N, 10.41.

(−)-(1*R*,2*R*,4*S*)-*tert*-Butyl-2-(3-methylisoxazol-5-yl)-7-azabicyclo[2.2.1]heptane-7-carboxylate 10. A suspension of (−)-**9** (260 mg, 0.94 mmol) and 10% Pd/C (35 mg) in MeOH (25 ml) was stirred at room temperature for 5 h under hydrogen at atmospheric pressure, monitoring the disappearance of the starting material (TLC, petroleum ether/ethyl acetate 85:15). The reaction mixture was filtered over a Celite® pad, and the filtrate was concentrated to dryness at reduced pressure affording (−)-**10** (241 mg, 92%).

(−)-(1*R*,2*R*,4*S*)-**10**: Colorless powder (from diisopropyl ether/ethyl acetate 1:1), mp 86–87 °C. R_f = 0.51 (petroleum ether/ethyl acetate 7:3). Chiral HPLC analysis, DAICEL Chiralpak AD column (25 cm × 4.6 mm, 10 μm), mobile phase: *n*-hexane/2-propanol 98:2; flow rate: 1 ml/min; λ 254 nm; retention time: 12.72 min. E.e. 99%. $[\alpha]_D^{25} = -4.0$ (*c* 1.0, $CHCl_3$). ^{1}H NMR: 1.30–1.40 (m, 2H), 1.42 (s, 9H), 1.50–1.65 (m, 2H), 1.67–1.80 (m, 1H), 2.13–2.28 (m, 1H), 2.27 (s, 3H), 3.35 (m, 1H), 4.20 (m, 1H), 4.35 (m, 1H), 5.82 (s, 1H). ^{13}C NMR: 11.57, 24.62, 28.43, 29.84, 34.71, 39.31, 57.03, 59.61, 80.13, 102.93, 155.59, 159.90, 172.61. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{15}H_{22}N_2O_3$: 278.4. Found: 279.2. Anal. Calcd for $C_{15}H_{22}N_2O_3$ (278.35): C, 64.73; H, 7.97; N, 10.06. Found: C, 64.85; H, 8.14; N, 10.15.

(+)-(1*S*,2*S*,4*R*)-*tert*-Butyl-2-(3-methylisoxazol-5-yl)-7-azabicyclo[2.2.1]heptane-7-carboxylate 10. Alkene (+)-**9** (215 mg, 0.78 mmol), which was reacted following the aforementioned described procedure, gave 206 mg (95% yield) of the 7-azabicyclo[2.2.1]heptane derivative (+)-**10**.

(+)-(1*S*,2*S*,4*R*)-**10**: Colorless powder (from diisopropyl ether/ethyl acetate 1:1), mp 82–84 °C. Chiral HPLC analysis was performed in the same conditions as those reported for (−)-**10**. Retention time: 11.80 min. E.e. 99%. $[\alpha]_D^{25} = +4.4$ (*c* 1.0, $CHCl_3$). The NMR spectroscopic data matched those of (−)-**10**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{15}H_{22}N_2O_3$: 278.4. Found: 279.2. Anal. Calcd for $C_{15}H_{22}N_2O_3$ (278.35): C, 64.73; H, 7.97; N, 10.06. Found: C, 64.96; H, 7.63; N, 10.35.

(−)-(1*R*,2*S*,4*S*)-*tert*-Butyl-2-(3-methylisoxazol-5-yl)-7-azabicyclo [2.2.1]heptane-7- carboxylate 11. Potassium *tert*-butoxide (438 mg, 3.90 mmol) was added to a stirred solution of the *endo*-epimer (−)-**10** (182 mg, 0.65 mmol) in *tert*-butanol (50 ml). The reaction was heated at reflux for 30 h until a maximum conversion of about 60% (TLC monitoring, petroleum ether/ethyl acetate 4:1). The crude mixture was concentrated in vacuo, and the two *endo*/*exo*-epimers were separated by silica gel column chromatography (petroleum ether/ethyl acetate 95:5 → 3:1), which provided 106 mg (58% yield) of pure (−)-**11**.

(−)-(1*R*,2*S*,4*S*)-**11**: Pale yellow viscous oil. $R_f = 0.25$ (petroleum ether/ethyl acetate 85:15). Chiral HPLC analysis, DAICEL Chiralpak AD column (25 cm × 4.6 mm, 10 μm), mobile phase: *n*-hexane/2-propanol 98:2; flow rate: 1 ml/min; λ 254 nm; retention time: 13.62 min. E.e. 99%. $[\alpha]_D^{25} = -13.9$ (*c* 1.0, $CHCl_3$). ^{1}H NMR: 1.33 (s, 9H), 1.40–1.57 (m, 2H), 1.70–1.85 (m, 2H), 1.90 (d, *J* = 6.6 Hz, 2H), 2.20 (s, 3H), 2.99 (t, *J* = 6.8 Hz, 1H), 4.32 (bs, 1H), 4.34 (bs, 1H), 5.82 (s, 1H). ^{13}C NMR: 11.64, 28.35, 28.99, 29.10, 36.90, 41.23, 55.52, 60.36, 79.91, 101.40, 155.02, 159.80, 175.36. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{15}H_{22}N_2O_3$: 278.4. Found: 279.5. Anal. Calcd for $C_{15}H_{22}N_2O_3$ (278.35): C, 64.73; H, 7.97; N, 10.06. Found: C, 64.92; H, 7.77; N, 10.34.

(+)-(1*S*,2*R*,4*R*)-*tert*-Butyl-2-(3-methylisoxazol-5-yl)-7-azabicyclo [2.2.1]heptane-7-carboxylate 11. The *endo*-derivative (+)-**10** (145 mg, 0.52 mmol), which was reacted following the aforementioned described procedure, gave 81 mg (56% yield) of the *exo*-epimer (+)-**11**.

(+)-(1*S*,2*R*,4*R*)-**11**: Pale yellow viscous oil. Chiral HPLC analysis was performed in the same conditions as those reported for (−)-**11**. Retention time: 12.70 min. E.e. 99%. $[\alpha]_D^{25} = +13.4$ (*c* 1.0, $CHCl_3$). The NMR spectroscopic data matched those of (−)-**11**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{15}H_{22}N_2O_3$: 278.4. Found: 279.3. Anal. Calcd for $C_{15}H_{22}N_2O_3$ (278.35): C, 64.73; H, 7.97; N, 10.06. Found: C, 64.65; H, 8.22; N, 10.19.

(+)-5-{(1*R*,2*S*,4*S*)-7-Azabicyclo[2.2.1]heptan-2-yl}-3-methylisoxazole hydrochloride 2 × HCl. The *N*-Boc protected derivative (−)-**11** (120 mg, 0.43 mmol) was dissolved in 4N HCl in anhydrous dioxane (2 ml) at 0 °C. The reaction mixture was allowed to warm at room temperature, and then it was stirred for about 1 h monitoring the disappearance of the starting material (TLC, petroleum ether/ethyl acetate 85:15). The reaction mixture was then concentrated to dryness under reduced pressure, and the residue was purified by crystallization to afford 63 mg (73% yield) of (+)-**2** hydrochloride.

(+)-(1*R*,2*S*,4*S*)-**2** × HCl: Colorless prisms (from 2-propanol), mp 184–192 °C, dec. $[\alpha]_D^{25} = +19.5$ (*c* 0.50, MeOH). ^{1}H NMR (D_2O): 1.65–1.95 (m, 4H), 2.01–2.08 (m, 1H), 2.13 (s, 3H), 2.24–2.29 (m, 1H), 3.42 (dd, *J* = 5.5 and 9.1 Hz, 1H), 4.26 (bs, 1H), 4.38 (bs, 1H), 6.10 (s, 1H). ^{13}C NMR (D_2O): 11.64, 28.35, 28.99, 29.10, 36.90, 41.23, 55.52, 60.36, 79.91, 101.40, 155.02, 159.80, 175.36. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{10}H_{15}ClN_2O$: 214.7. Found: 179.1. Anal. Calcd for $C_{10}H_{15}ClN_2O$ (214.69): C, 55.94; H, 7.04; N, 13.05. Found: C, 56.14; H, 6.95; N, 13.36.

(−)-5-{(1*S*,2*R*,4*R*)-7-Azabicyclo[2.2.1]heptan-2-yl}-3-methylisoxazole hydrochloride 2 × HCl. Reaction of (+)-**11** (75 mg, 0.27 mmol), according to the aforementioned described procedure, gave (−)-**2** hydrochloride (35 mg, 61% yield), which was purified by crystallization.

(−)-(1*S*,2*R*,4*R*)-**2** × HCl: Colorless prisms (from 2-propanol), mp 186–190 °C, dec. $[\alpha]_D^{25} = -18.8$ (*c* 0.50, MeOH). The NMR spectroscopic data matched those of (+)-**2** hydrochloride. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{10}H_{15}ClN_2O$: 214.7. Found: 179.2. Anal. Calcd for $C_{10}H_{15}ClN_2O$ (214.69): C, 55.94; H, 7.04; N, 13.05. Found: C, 55.79; H, 7.11; N, 13.17.

(+)-5-{(1*R*,4*S*)-7-Azabicyclo[2.2.1]hept-2-en-2-yl}-3-methylisoxazole 3. The *N*-Boc protected derivative (−)-**9** (122 mg, 0.44 mmol) was dissolved in 4N HCl in anhydrous dioxane (2 ml) at a 0 °C. The reaction mixture was allowed to warm at room temperature, and then it was stirred for about 1 h monitoring the disappearance of the starting material (TLC, petroleum ether/ethyl acetate 85:15). The reaction mixture was concentrated under reduced pressure, then dissolved in water (5 ml), and treated with diethyl ether (3 × 5 ml). The residual aqueous phase was made basic by addition of solid Na_2CO_3 and extracted with dichloromethane (3 × 5 ml). After standard workup, the pooled organic phases provided the free secondary base (+)-**3** (57 mg, 73% yield).

(+)-(1*R*,4*S*)-**3**: Pale yellow viscous oil. $R_f = 0.57$ (dichloromethane/methanol 9:1). $[\alpha]_D^{25} = +63.8$ (*c* 0.85, $CHCl_3$). ^{1}H NMR: 1.16–1.27 (m, 2H), 1.93–2.10 (m, 3H), 2.30 (s, 3H), 4.34 (bs, 1H), 4.47 (bs, 1H), 6.05 (s, 1H), 6.72 (bs, 1H). ^{13}C NMR: 11.67, 23.79, 24.74, 61.17, 61.38, 102.20, 135.41, 136.48, 160.29, 164.02. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{10}H_{12}N_2O$: 176.2. Found: 177.1. Anal. Calcd for $C_{10}H_{12}N_2O$ (176.22): C, 68.16; H, 6.86; N, 15.90. Found: C, 68.45; H, 6.73; N, 16.12.

(−)-5-{(1*S*,4*R*)-7-Azabicyclo[2.2.1]hept-2-en-2-yl}-3-methylisoxazole 3. Reaction of (+)-**9** (225 mg, 0.81 mmol), according to the aforementioned described procedure, gave (−)-**3** (113 mg, 79% yield).

(1*S*,4*R*)-(−)-**3**: Pale yellow viscous oil. $[\alpha]_D^{25} = -65.9$ (*c* 1.0, $CHCl_3$). The NMR spectroscopic data matched those of the enantiomer (+)-**3**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{10}H_{12}N_2O$: 176.2. Found: 177.0. Anal. Calcd for $C_{10}H_{12}N_2O$ (176.22): C, 68.16; H, 6.86; N, 15.90. Found: C, 68.53; H, 6.51; N, 15.68.

(+)-5-{(1*R*,4*S*)-7-Azabicyclo[2.2.1]hept-2-en-2-yl}-3-methylisoxazole fumarate 3 × $C_4H_4O_4$ and (−)-5-{(1*S*,4*R*)-7-azabicyclo[2.2.1]hept-2-en-2-yl}-3-methylisoxazole fumarate 3 × $C_4H_4O_4$. A solution of fumaric acid (33 mg, 0.28 mmol) in MeOH was added to a solution of the free base (+)-**3** or (−)-**3** (50 mg, 0.28 mmol) in MeOH (2 ml). After stirring for 3 h at room temperature, the solvent was removed at reduced pressure, and the crude salt was obtained quantitatively.

(+)-(1*R*,4*S*)-**3** × $C_4H_4O_4$: Colorless prisms (from 2-propanol/diisopropyl ether 2:1), mp 172–174 °C, dec. $[\alpha]_D^{25} = +26.6$ (*c* 0.50, MeOH). ^{1}H NMR (CD_3OD): 1.49–1.67 (m, 2H), 2.19–2.25 (m, 2H), 2.30 (s, 3H), 4.86 (bs, 1H), 5.09 (bs, 1H), 6.57 (s, 1H), 6.67 (s, 2H), 6.83 (bs, 1H). ^{13}C NMR (CD_3OD): 9.94, 21.46, 22.32, 60.53, 61.22, 103.84, 129.77, 132.66, 134.97, 160.86, 161.75, 170.04. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{14}H_{16}N_2O_5$: 292.3. Found: 177.1. Anal. Calcd for $C_{14}H_{16}N_2O_5$ (292.29): C, 57.53; H, 5.52; N, 9.58. Found: C, 57.78; H, 5.46; N, 9.71.

(−)-(1*S*,4*R*)-**3** × $C_4H_4O_4$: Colorless prisms (from 2-propanol/diisopropyl ether 2:1), mp 174–175 °C, dec. $[\alpha]_D^{25} = -29.5$ (*c* 0.50, MeOH). The NMR spectroscopic data matched those of the enantiomer (+)-**3** fumarate. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{14}H_{16}N_2O_5$: 292.3. Found: 177.2. Anal. Calcd for $C_{14}H_{16}N_2O_5$ (292.29): C, 57.53; H, 5.52; N, 9.58. Found: C, 57.64; H, 5.78; N, 9.61.

(+)-3-Methyl-5-{(1*R*,4*S*)-7-methyl-7-azabicyclo[2.2.1]hept-2-en-2-yl}isoxazole 12. Sodium cyanoborohydride (137 mg, 2.2 mmol) was added in portions to a mixture of (+)-**3** (180 mg, 1.02 mmol), a 37% aqueous solution of formaldehyde (0.4 ml) and acetonitrile (10 ml) at 0 °C. The reaction mixture was stirred at room temperature for 1 h, then after concentration in vacuo, ethyl acetate (5 ml) and water (5 ml) were added. The two layers were separated and the residual aqueous phase was extracted with ethyl acetate (2 × 5 ml). After standard workup, the crude reaction mixture

underwent a silica gel column chromatography (dichloromethane/methanol 9:1), which afforded the *N*-methylated compound (+)-**12** (130 mg, 67% yield).

(+)-(1*R*,4*S*)-**12**: Yellow oil. R_f = 0.56 (dichloromethane/methanol 9:1). $[\alpha]_D^{25}$ = +29.7 (*c* 1.0, $CHCl_3$). ^{1}H NMR: 1.08–1.25 (m, 2H), 1.93–2.03 (m, 2H), 2.15 (s, 3H), 2.30 (s, 3H), 3.91 (m, 1H), 4.02 (m, 1H), 6.04 (s, 1H), 6.51 (bs, 1H). ^{13}C NMR: 11.59, 24.90, 25.74, 34.52, 67.73, 101.94, 131.17, 160.18, 165.15, 175.78. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{11}H_{14}N_2O$: 190.2. Found: 191.1. Anal. Calcd for $C_{11}H_{14}N_2O$ (190.24): C, 69.45; H, 7.42; N, 14.73. Found: C, 69.87; H, 7.12; N, 14.92.

(−)-3-Methyl-5-{(1*S*,4*R*)-7-methyl-7-azabicyclo[2.2.1]hept-2-en-2-yl}isoxazole 12. Reaction of (−)-**3** (115 mg, 0.65 mmol), according to the aforementioned described procedure, gave (−)-**12** (68 mg, 55% yield).

(−)-(1*R*,4*S*)-**12**: Light yellow oil. $[\alpha]_D^{25}$ = −31.8 (*c* 1.0, $CHCl_3$). The NMR spectroscopic data matched those of the enantiomer (+)-**12**. MS (ESI) *m/z* $[M+H]^+$ Calcd for $C_{11}H_{14}N_2O$: 190.2. Found: 191.1. Anal. Calcd for $C_{11}H_{14}N_2O$ (190.24): C, 69.45; H, 7.42; N, 14.73. Found: C, 69.79; H, 7.24; N, 14.55.

(+)-3-Methyl-5-{(1*R*,4*S*)-7-methyl-7-azabicyclo[2.2.1]hept-2-en-2-yl}isoxazole methyl iodide 4 and (−)-3-methyl-5-{(1*S*,4*R*)-7-methyl-7-azabicyclo[2.2.1]hept-2-en-2-yl}isoxazole methyl iodide 4. Iodomethane (156 µl, 2.52 mmol) was added to a solution of the tertiary base (+)-**12** or (−)-**12** (65 mg, 0.34 mmol) in methanol (2 ml), and the resulting mixture was stirred for 3 h at room temperature. The solvent was removed under reduced pressure to quantitatively yield the crude quaternary salt as a yellow solid residue, which was washed three times with small amounts of diethyl ether.

(+)-(1*R*,4*S*)-**4**: Colorless prisms (from abs. ethanol), mp 150–155 °C, dec. $[\alpha]_D^{25}$ = +62.3 (*c* 0.50, MeOH). ^{1}H NMR (D_2O): 1.48–1.64 (m, 2H), 2.12 (s, 3H), 2.28–2.41 (m, 2H), 2.93 (s, 3H), 2.96 (s, 3H), 4.62 (bs, 1H), 4.83 (bs, 1H), 6.37 (s, 1H), 6.73 (bs, 1H). ^{13}C NMR (D_2O): 10.52, 21.09, 21.79, 44.99, 45.36, 74.54, 75.77, 104.85, 129.73, 131.04, 161.31, 161.95. MS (ESI) *m/z* $[M]^+$ Calcd for $C_{12}H_{17}IN_2O$: 332.2. Found: 205.3. Anal. Calcd for $C_{12}H_{17}IN_2O$ (332.18): C, 43.39; H, 5.16; N, 8.43. Found: C, 43.52; H, 5.37; N, 8.34.

(−)-(1*S*,4*R*)-**4**: Colorless prisms (from abs. ethanol), mp 150–155 °C, dec. $[\alpha]_D^{25}$ = −64.5 (*c* 0.50, MeOH). The NMR spectroscopic data matched those of the enantiomer (+)-**4**. MS (ESI) *m/z* $[M]^+$ Calcd for $C_{12}H_{17}IN_2O$: 332.2. Found: 205.2. Anal. Calcd for $C_{12}H_{17}IN_2O$ (332.18): C, 43.39; H, 5.16; N, 8.43. Found: C, 43.48; H, 5.01; N, 8.56.

Receptor Binding Assays

Tissue preparation. 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. 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.

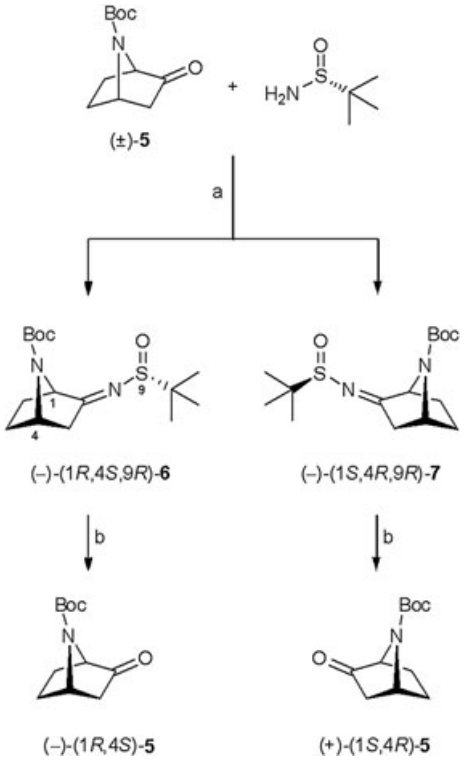
[^{3}H]Epibatidine binding. (±)-[^{3}H]Epibatidine with a specific activity of 56–60 Ci/mmol was purchased from Perkin Elmer (Boston MA); the nonradioactive α-bungarotoxin, epibatidine, and nicotine were purchased from Sigma Aldrich (Italy). It has been previously reported that [^{3}H]epibatidine also binds to α-bungarotoxin binding receptors with nM affinity.[11] To prevent the binding of [^{3}H]epibatidine to the α-bungarotoxin binding receptors, the membrane homogenates were preincubated with 2 µM α-bungarotoxin and then with [^{3}H]epibatidine. 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. After incubation, the samples were filtered through 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.

[^{125}I]α-Bungarotoxin binding. 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 room temperature. Nonspecific binding was determined in parallel by incubation in the presence of 1 µM unlabeled α-bungarotoxin. After incubation, the samples were filtered as described earlier, and the bound radioactivity was directly counted in a γ counter.

Affinity of derivatives (+)-2/(−)-2, (+)-3/(−)-3, and (+)-4/(−)-4 for nAChRs. Inhibition of radioligand binding by epibatidine and the test compounds was measured by preincubating cortex homogenates with increasing doses (10 pM–10 mM) of the reference nicotinic agonist epibatidine or nicotine, or the drugs to be tested, for 30 min at room temperature. This was followed by overnight incubation with a final concentration of 0.075 nM [^{3}H]epibatidine (for the α4β2 subtype) or 1 nM [^{125}I]α-bungarotoxin (for the α7 subtype) 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 ligand K_D values of the ligands for the two different classes of nAChRs. For each compound, experimental data obtained from three saturation and three competition binding experiments were analyzed by means of a nonlinear least square procedure, using the LIGAND program as described by Munson and Rodbard.[23] 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. 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).

RESULTS AND DISCUSSION

As illustrated in Scheme 1, the synthetic approach to the enantiomerically pure final derivatives started with the resolution of 7-*tert*-butoxycarbonyl-7-azabicyclo[2.2.1]heptan-2-one (±)-**5**,[21,16] an advanced key intermediate in a few syntheses of (±)-epibatidine.[24] Moreover, the two enantiomers of (±)-**5**, that is, (1*R*,4*S*)-(−)-**5** and (1*S*,4*R*)-(+)-**5**, were obtained by enantiospecific synthesis,[22] through enantiotopic discrimination of a suitable *meso*-intermediate[25] or exploiting the formation of diastereomeric aminales with (*R*,*R*)-1,2-diphenylehylenediamine.[24] In this study, we made use of commercial (*R*)-(+)-2-methyl-2-propanesulfinamide[26–28] which, in the presence of the Lewis acid $Ti(OEt)_4$,[29] efficiently condensed with (±)-**5** providing a mixture of the two epimeric *tert*-butanesulfinyl ketimines (−)-(1*R*,4*S*,9*R*)-**6** and (1*S*,4*R*,9*R*)-(−)-**7** in comparable amounts [(−)-**6**/(−)-**7** about 43:57] (Scheme 1). After separation by silica gel flash chromatography, the two isomers were individually treated with 4N acetic acid in methanol, which allowed to isolate the enantiomers (1*R*,4*S*)-(−)-**5** (from (−)-**6**) and (1*S*,4*R*)-(+)-**5** (from (−)-**7**) and, accordingly, to assign the absolute configuration to the precursor ketimines. Both



Scheme 1. Reagents and conditions: (a) $Ti(OEt)_4$, THF, 65 °C, 2 h and (b) 4N CH_3COOH, MeOH, 40 °C, 18 h.

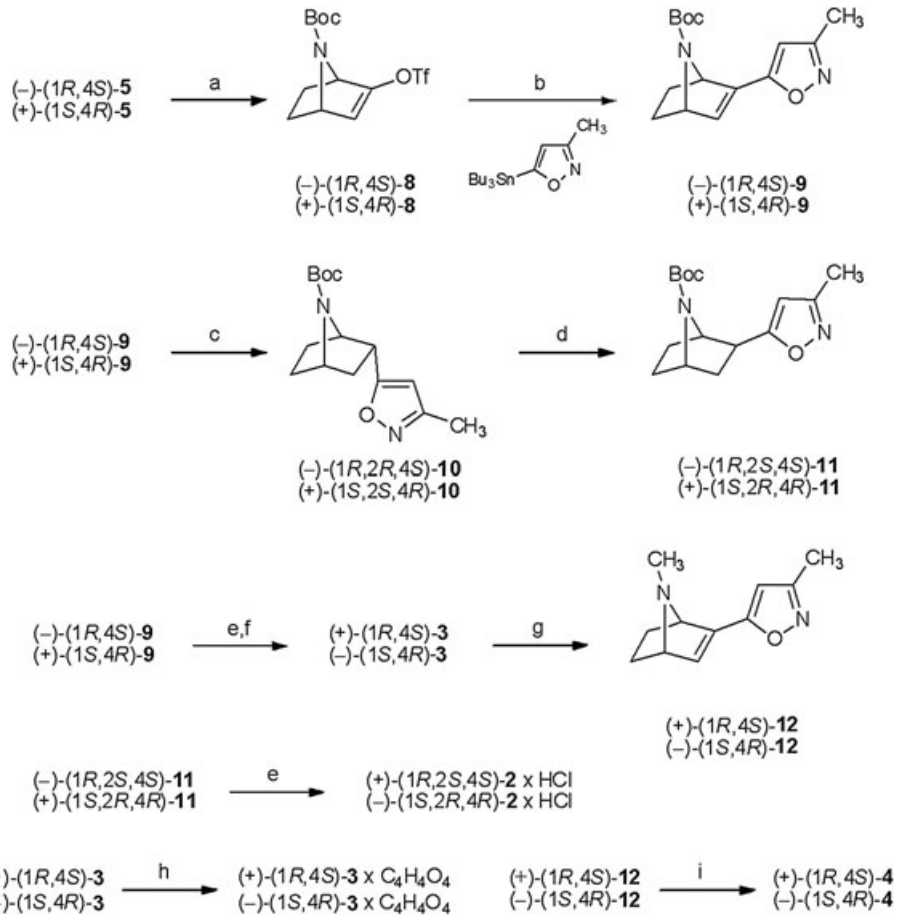
enantiomeric ketones were characterized by high enantiomeric purity (e.e. = 98%) and spectroscopic/polarimetric features in line with those reported in the literature.[22] Moreover, the lack of diastereoselection associated to the initial addition provided ketones (−)-**5** and (+)-**5** in comparable yields, and we could carry out the overall synthetic sequence in a parallel fashion.

The *N*-Boc protected enantiomeric ketones were then converted into their corresponding trifluoromethanesulfonates (1*R*,4*S*)-(−)-**8** and (1*S*,4*R*)-(+)-**8**, respectively, by sequential treatment with potassium bis(trimethylsilyl)amide, to form the enolate, and *N*-(5-chloro-2-pyridyl)bis(trifluoromethanesulfonimide).[30,31] Next, an efficient Stille–Miyaura cross-coupling protocol was carried out by reacting the enantiomers (1*R*,4*S*)-(−)-**8** and (1*S*,4*R*)-(+)-**8** with 3-methyl-5-tributylstannylisoxazole in the presence of the tris(dibenzylideneacetone) dipalladium(0)-chloroform adduct, triphenylphosphine, and anhydrous $ZnCl_2$.[16] The two enantiomeric isoxazole derivatives (1*R*,4*S*)-(−)-**9** and (1*S*,4*R*)-(+)-**9** (Scheme 2), obtained in 92% and 89% yield, were then submitted to a standard catalytic hydrogenation over 10% Pd/C, which gave the two *N*-Boc-*endo*-epiboxidines (1*R*,2*R*,4*S*)-(−)-**10** and (1*S*,2*S*,4*R*)-(+)-**10** as the sole isomers, as already found for the reduction of (±)-**9**[16] and similarly to what observed in a parallel preparation of the corresponding intermediate in the synthesis of (±)-epibatidine.[32] The subsequent epimerization reaction to the desired *exo*-isomers was achieved, with a conversion of about 60%, by prolonged heating of the two *endo*-isomers with potassium *tert*-butoxide in refluxing *tert*-butanol,[21] followed by a column chromatography purification. The two enantiomeric *N*-Boc-*exo*-epiboxidines (1*R*,2*S*,4*S*)-(−)-**11** and (1*S*,2*R*,4*R*)-(+)-**11** underwent removal of the *N*-Boc protection with a 4N solution of HCl in dioxane, which produced the corresponding epiboxidine antipodes as crystalline hydrochlorides (1*R*,2*S*,4*S*)-(+)-**2** and (1*S*,2*R*,4*R*)-(−)-**2**, respectively. The same protocol was applied to the two intermediate olefins (1*R*,4*S*)-(−)-**9** and (1*S*,4*R*)-(+)-**9**, but owing to the inherent hygroscopicity of the two hydrochlorides, we isolated the free bases (1*R*,4*S*)-(+)-**3** and (1*S*,4*R*)-(−)-**3**, which were then transformed into their crystalline 1:1 fumarates (Scheme 2). Alternatively, (1*R*,4*S*)-(+)-**3** and (1*S*,4*R*)-(−)-**3** were reacted with aqueous formaldehyde, followed by reduction of the intermediate aldimines with sodium cyanoborohydride, which provided the corresponding tertiary amines (1*R*,4*S*)-(+)-**12** and (1*S*,4*R*)-(−)-**12**. These enantiomers were treated with excess iodomethane to give the dimethylammonium salts (1*R*,4*S*)-(+)-**4** and (1*S*,4*R*)-(−)-**4** (Scheme 2).

The three couples of derivatives under study (+)-**2**/(−)-**2**, (+)-**3**/(−)-**3**, and (+)-**4**/(−)-**4** 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,[23] are reported in Table 1 and compared with the known K_i values of (±)-**1** and its enantiomers and those previously obtained by us for (±)-**2**, (±)-**3**, and (±)-**4**.

Inspection of the binding data at the explored nAChRs evidences the identity of the absolute configuration at the common stereogenic centers for the antipodes with the highest affinity. Indeed, within each enantiomeric pair, hydrochloride (1*R*,2*S*,4*S*)-(+)-**2**, fumarate (1*R*,4*S*)-(+)-**3**, and iodomethylate (1*R*,4*S*)-(+)-**4** were found to be the eutomers at both α4β2 and α7 nAChRs. Worth noting, the free base natural epibatidine (1*R*,2*R*,4*S*)-(−)-**1** and hydrochloride (1*R*,2*S*,4*S*)-(+)-**2** share the same spatial arrangement around the stereogenic centers and have opposite polarimetric rotation angles. The two enantiomeric epibatidines (1*R*,2*R*,4*S*)-(−)-**1** and (1*S*,2*S*,4*R*)-(+)-**1** displayed very high affinity at the α4β2 nAChRs (K_i values equal to 0.020 and 0.019 nM,[12] respectively), but their molecular interaction was characterized by the absence of enantioselectivity. Similarly, our data obtained at the same subtype for the enantiomeric epiboxidine hydrochlorides (1*R*,2*S*,4*S*)-(+)-**2** and (1*S*,2*R*,4*R*)-(−)-**2** (K_i values equal to 0.21 and 0.45 nM, respectively) and their structural analogs (1*R*,4*S*)-(+)-**3** and (1*S*,4*R*)-(−)-**3** (K_i values equal to 29 and 73 nM, respectively) revealed a lack of enantioselectivity (E.R. values of 2 and 2.5 for the two pairs) in addition to a progressive reduction in affinity. A lower affinity was detected at the α7 subtype for the epiboxidine enantiomers (K_i values of 5.2 and 15.5 nM), an outcome comparable with that reported for the epibatidine enantiomers[12] (K_i values of 7.0 and 4.9 nM), both pairs showing a negligible enantioselectivity (E.R. values of 3 and 0.7, respectively). On the other hand, the two antipodes of the "unsaturated" epiboxidine analog (±)-**3** evidenced a marked reduction in affinity (K_i values of 0.17 and 2.8 μM) at the α7 subtype together with a gain of the enantioselectivity (E.R. value of 16.5).

In terms of preferred recognition of ligands by the assayed nAChRs, the exceptional affinity shown by racemic epibatidine for the α4β2 subtype determines the high degree of α4β2 versus α7 subtype selectivity (Table 1), which is drastically reduced on passing from (±)-**1** (291)[33] to (±)-**2** (30)[18] and (±)-**3** (32).[16] Such selectivity profiles are retained when the affinity data obtained on the enantiomeric pairs (−)-**1**/(+)-**1** (350/258), (+)-**2**/(−)-**2** (25/34), and (+)-**3**/(−)-**3** (5.5/38) are taken into account, none of the tested individual isomers is able to substantially alter the nAChR subtype discrimination



Scheme 2. Reagents and conditions: (a) KHMDS, 0.5 M in toluene, *N*-(5-chloro-2-pyridyl)bis(trifluoromethanesulfonimide), DME, −78 °C to room temperature (rt), 3 days; (b) $[Pd_2(dba)_3] \cdot CHCl_3$, PPh_3, anhydrous $ZnCl_2$, THF, −78 °C to rt, 3 days; (c) H_2, 10% Pd/C, MeOH, rt, 5 h; (d) *tert*-BuOK/*tert*-BuOH, reflux, 30 h (about 60% conversion), flash chromatography; (e) 4N HCl, dioxane, rt, 1 h; (f) aq. Na_2CO_3, solvent extraction; (g) HCHO (37% aq.), $NaBH_3CN$, CH_3CN, rt, 1 h; (h) $C_4H_4O_4$,MeOH, rt, 3 h; (i) CH_3I, MeOH, rt, 3 h.

TABLE 1. Affinity of enantiomeric pairs (+)-2/(−)-2, (+)-3/(−)-3, and (+)-4/(−)-4 for native α4β2 and α7 nAChR subtypes present in rat cortical membranes labeled, respectively, by [^{3}H]epibatidine and [^{125}I]α-bungarotoxin

Entry	α4β2 [^{3}H]Epi K_i[a] (nM)	α7 [^{125}I]α-BgTx K_i[a] (nM)	Selectivity α4β2/α7
(±)-**1**	0.035[b]	10.2[b]	291
(−)-(1*R*,2*R*,4*S*)-**1**	0.020[c]	7.0[c]	350
(+)-(1*S*,2*S*,4*R*)-**1**	0.019[c]	4.9[c]	258
E.R.[d]	1	0.7	
(±)-**2**	0.24[e]	7.3[e]	30
(+)-(1*R*,2*S*,4*S*)-**2** × HCl	0.21 (17)	5.2 (22)	25
(−)-(1*S*,2*R*,4*R*)-**2** × HCl	0.45 (19)	15.5 (18)	34
E.R.[d]	2	3	
(±)-**3**	50[f]	1600[f]	32
(+)-(1*R*,4*S*)-**3** × $C_4H_4O_4$	29 (22)	170 (34)	5.5
(−)-(1*S*,4*R*)-**3** × $C_4H_4O_4$	73 (17)	2800 (38)	38
E.R.[d]	2.5	16.5	
(±)-**4**	13.3[e]	1.6[e]	0.12
(+)-(1*R*,4*S*)-**4**	10.2 (28)	1.1 (30)	0.11
(−)-(1*S*,4*R*)-**4**	67 (24)	23 (29)	0.34
E.R.[d]	6.6	21	

[a]K_i values were derived from three competition binding experiments. Numbers in brackets refer to the percent coefficients of variation. Literature data available for (±)-**1**, (+)-**1**, (−)-**1**, (±)-**2**, (±)-**3**, and (±)-**4** have been added for comparison.
[b]Ref. 33.
[c]Ref. 12.
[d]E.R., eudismic ratio; ratio between the K_i value of distomer and the K_i value of eutomer.
[e]Ref. 18.
[f]Ref. 16.

detected for the corresponding racemate. Worth pointing out, the permanently charged ammonium salt (±)-**4** showed an opposite affinity trend compared with that of the other investigated ligands because it preferentially bound the α7 subtype.[18] Both individual enantiomers of (±)-**4** preserved the affinity profile of the racemate, and the eutomer (1*R*,4*S*)-(+)-**4** [K_i = 10.2 nM (α4β2) and 1.1 nM (α7)] should be included in the number of high affinity unselective nicotinic ligands at α4β2 and α7 receptors. In addition, the (+)-**4**/(−)-**4** couple exhibited an increase of enantioselectivity at both receptor subtypes [E. R. = 6.6 (α4β2) and 21 (α7)] when compared with the other investigated enantiomeric pairs.

CONCLUSIONS

In this study, we prepared hydrochlorides (−)-**2** and (+)-**2**, the two enantiomers of epiboxidine, whose racemate is one of the most interesting synthetic analogs of natural (−)-epibatidine. Resolution of the racemic *N*-Boc-7-azabicyclo[2.2.1] heptane-2-one coupled with a Suzuki cross-coupling reaction was the key step of the applied synthetic sequence. With the same approach, we prepared two further epiboxidine-related nicotinic ligands, that is, the "unsaturated" fumarates of the secondary bases (−)-**3** and (+)-**3** as well as the dimethylammonium iodides (−)-**4** and (+)-**4**. The three enantiomeric pairs were assayed in binding experiments to evaluate their affinity for the neuronal α4β2 and α7 nAChR subtypes. On the whole, the degree of the observed enantioselectivity was poor for the three pairs of enantiomers at both investigated receptors, a result which to a large extent matches that known for the antipodes of epibatidine. Interestingly, both antipodes of (±)-**4**, at variance with those of (±)-**2** and (±)-**3**, preferentially recognized the α7 receptor, although with a low subtype selectivity.

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LITERATURE CITED

1. Sine SM, Engel AG. Recent advances in Cys-loop receptor structure and function. Nature 2006;440:448–455.
2. Tsetlin V, Kuzmin D, Kasheverov I. Assembly of nicotinic and other Cys-loop receptors. J Neurochem 2011;116:734–741.
3. Albuquerque EX, Pereira EFR, Alkondon M, Rogers, SW. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 2009;89:73–120.
4. Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol Toxicol 2007;47:699–729.
5. Jensen AA, Frølund B, Liljefors T, Krogsgaard-Larsen P. Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. J Med Chem 2005;48:4705–4745.
6. Cincotta SL, Yorek MS, Moschak TM, Lewis SR, Rodefer JS. Selective nicotinic acetylcholine receptor agonists: potential therapies for neuropsychiatric disorders with cognitive dysfunction. Curr Opin Investig Drugs 2008;9:47–56.
7. Taly A, Corringer PJ, Guedin D, Lestage P, Changeux JP. Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. Nat Rev Drug Discov 2009;8:733–750.
8. Spande TF, Garraffo HM, Edwards MW, Yeh JC, Pannell L, Daly JW. Epibatidine: a novel (chloropyridyl)azabicyloheptane with potent analgesic activity from an Ecuadorian poison frog. J Am Chem Soc 1992;114:3475–3478.
9. Badio B, Garraffo HM, Spande TF, Daly JW. Epibatidine: discovery and definition as a potent analgesic and nicotinic agonist. Med Chem Res 1994;4:440–448.
10. Broka C. Synthetic approaches to epibatidine. Med Chem Res 1994;4:449–460.
11. Gerzanich V, Peng X, Wang F, Wells G, Anand R, Fletcher S, Lindstrom J. Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. Mol Pharmacol 1995;48:774–782, and references cited therein.
12. Cox CD, Malpass JR, Gordon J, Rosen A. Synthesis of epibatidine isomers: *endo*-5- and 6-(6'-chloro-3'-pyridyl)-2-azabicyclo[2.2.1]heptanes. J Chem Soc Perkin Trans 1 2001;2372–2379.
13. Carroll FI. Epibatidine structure-activity relationships. Bioorg Med Chem Lett 2004;14:1889–1996, and references cited therein.
14. White R, Malpass JR, Handa S, Baker SR, Broad LM, Folly L, Mogg A. Epibatidine isomers and analogues: structure-activity relationships. Bioorg Med Chem Lett 2006;16:5493–5497.
15. Badio B, Garraffo HM, Plummer CV, Padgett WL, Daly JW. Synthesis and nicotinic activity of epiboxidine: an isoxazole analogue of epibatidine. Eur J Pharmacol 1997;321:189–194.
16. Rizzi L, Dallanoce C, Matera C, Magrone P, Pucci L, Gotti C, Clementi F, De Amici M. Epiboxidine and novel-related analogues: a convenient synthetic approach and estimation of their affinity at neuronal nicotinic acetylcholine receptor subtypes. Bioorg Med Chem Lett 2008;18:4651–4654,and references cited therein.
17. Dallanoce C, Magrone P, Bazza P, Grazioso G, Rizzi L, Riganti L, Gotti C, Clementi F, Frydenvang K, De Amici M. New analogues of epiboxidine incorporating the 4,5-dihydroisoxazole nucleus: synthesis, binding affinity at neuronal nicotinic acetylcholine receptors, and molecular modeling investigations. Chem Biodivers 2009;6:244–259.
18. Dallanoce C, Matera C, Pucci L, Gotti C, Clementi F, De Amici M, De Micheli C. Synthesis and binding affinity at α4β2 and α7 nicotinic acetylcholine receptors of new analogues of epibatidine and epiboxidine containing the 7-azabicyclo[2.2.1]hept-2-ene ring system. Bioorg Med Chem Lett, 2012;22: 829–832.
19. Dallanoce C, Magrone P, Matera C, Frigerio F, Grazioso G, De Amici M, Fucile S, Piccari V, Frydenvang K, Pucci L, Gotti C, Clementi F, De Micheli C. Design, synthesis, and pharmacological characterization of novel spirocyclic quinuclidinyl-^{2}-isoxazoline derivatives as potent and selective agonists of α7 nicotinic acetylcholine receptors. ChemMedChem 2011;6:889–903.
20. Pucci L, Grazioso G, Dallanoce C, Rizzi L, De Micheli C, Clementi F, Bertrand S, Bertrand D, Longhi R, De Amici M, Gotti C. Engineering of α-conotoxin MII-derived peptides with increased selectivity for native α6β2* nicotinic acetylcholine receptors. FASEB J 2011;25:3775–3789.
21. Zhang C, Trudell ML. A short and efficient total synthesis of (±)-epibatidine. J Org Chem 1996;61:7189–7191.
22. Hernández A, Marcos M, Rapoport H. Synthesis of (+) and (−)-*N*-BOC-7-azabicyclo[2.2.1]heptan-2-ones, versatile intermediates for the enantiospecific synthesis of (+)- and (−)-epibatidine and analogues. J Org Chem 1995;60:2683–2691.
23. Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 1980;107:220–239.
24. Moreno-Vargas AJ, Vogel P. Efficient resolution of *N*-Boc-7-azabicyclo [2.2.1]hept-5-en-2-one: formal syntheses of natural epibatidine and its enantiomer. Tetrahedron: Asymmetry 2003;14:3173–3176, and references cited therein.
25. Pandey G, Tiwari SK, Singh RS, Mali RS. A short and efficient synthesis of enantiopure (+)-*N*-Boc-7-azabicyclo[2.2.1]heptan-2-one utilizing an asymmetric desymmetrization protocol: formal total synthesis of (−)-epibatidine. Tetrahedron Lett 2001;42:3947–3949.
26. Ellman JA, Owens TD, Tang TP. *N-tert*-butanesulfinyl imines: versatile intermediates for the asymmetric synthesis of amines. Acc Chem Res 2002;35:984–995.
27. McMahon JP, Ellman JA. High stereoselective addition of organometallic reagents to *N-tert*-butanesulfinyl imines derived from 3- and 4-substituted cyclohexanones. Org Lett 2004;6:1645–1647.
28. Morton D, Stockman RA. Chiral non-racemic sulfinimines: versatile reagents for asymmetric synthesis. Tetrahedron 2006;62:8869–8905.
29. Chelucci G, Baldino S, Chessa S, Pinna GA, Soccolini F. An easy route to optically active 1-substituted-1-pyridyl-methylamines by diastereoselective

reduction of enantipoure *N-tert*-butanesulfinyl ketimines. Tetraedron: Asymmetry 2006;17:3163–3169.

30. Carroll FI, Liang F, Navarro HA, Brieaddy LE, Abraham P, Damaj MI, Martin BR. Synthesis, nicotinic acetylcholine receptor binding, and antinociceptive properties of 2-*exo*-2-(2'-substituted-5'-pyridinyl)-7-azabicyclo [2.2.1]heptanes, epibatidine analogues. J Med Chem 2001;44:2229–2237.
31. Choi Y, White JD. Intramolecular photocycloaddition of unsaturated isoquinuclidines. Synthesis of 2-azatetracyclo[4.0.0.^{4,9}0^{7,10}]decanes and 3-azatetracyclo[6.1.1.0.^{2,7}0^{5,19}] decanes. J Org Chem 2004;69: 3758–3764.
32. Fletcher SR, Baker R, Chambers MS, Herbert RH, Hobbs SC, Thomas SR, Verrier HM, Watt AP, Ball RG. Total synthesis and determination of the absolute configuration of epibatidine. J Org Chem 1994;59:1771–1778.
33. Zhang N, Tomizawa M, Casida JE. 5-Azidoepibatidine: an exceptionally potent photoaffinity ligand for neuronal α4β2 and α7 nicotinic acetylcholine receptors. Bioorg Med Chem Lett 2003;13:525–527.