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Synthesis, activity and theoretical study of ABT-418 analogues

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Abstract—This report shows the synthesis and biological evaluation of two new conformationally restricted ABT-418 analogues. This restriction is introduced by the incorporation of the 7-azabicyclo[2.2.1]heptane skeleton. Furthermore, we report here a high-level quantum mechanical study of their conformations in the gas phase. © 2002 Published by Elsevier Science Ltd.

1. Introduction

The alkaloid epibatidine **1** is a very specific agonist of the neuronal nicotinic acetylcholine receptor (nAChR)[1] that acts as a powerful analgesic through a non-opioid mechanism. Since the discovery of this alkaloid[2] by Daly and co-workers in 1992, several synthetic approaches have been reported by numerous research groups.[3] Its therapeutic potential as a nicotinic receptor agonist is interesting to treat the pain and other neurological disorders, including Alzheimer's and Parkinson's diseases.[4]

However, the epibatidine has a high toxicity and is not suitable for clinical development. In this context, several groups have begun to study epibatidine analogues by combining structural features of the known alkaloids. One of these known alkaloids is the nicotine **2**, and since it has strong undesired effects on cardiovascular and digestive systems, several nicotine analogues have been synthesized, particularly, analogues with rigid structures (7-azabicyclo-[2.2.1]heptane skeleton) have become interesting targets[5] (**3**) (Fig. 1).

The replacement of the pyridinyl ring in nicotine by a methylisoxazolyl ring represents a relative alteration. This compound, ABT-418 **4**, has been reported to have antinociceptive effects on mice.[6] Since the synthesis and evaluation of bridged analogues of nicotine have provided an insight into the pharmacophore for the nAChR,[7] we envisioned that similar studies directed toward ABT-418 analogues would also provide useful information.

ABT-418 was obtained from L-proline **5** with an efficient route that involved the conversion of the ester function into the corresponding β-keto oxime[8] (Scheme 1). Recently, we have developed a method to obtain a type of constrained proline **6** (Ahc)[9] and on the basis of our experience in the epibatidine field,[3b,10] we report here the synthesis and the biological activity of two new 3-methylisoxazol-5-yl derivatives **7a** and **8a**, both including a 7-azabicyclo[2.2.1]heptane system. These compounds can be regarded as conformationally restricted analogues of ABT-418.

2. Results and discussion

2.1. Chemistry

We used the benzamide **9** (precursor of the amino acid Ahc

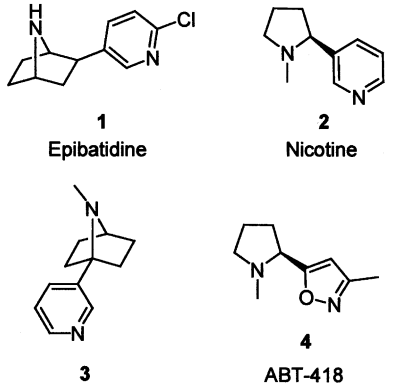

Figure 1. Structures of interesting nicotinic receptor agonists.

Keywords: amino acids and derivatives; bicyclic heterocyclic compounds; biologically active compounds; isoxazoles; molecular modeling/mechanics.

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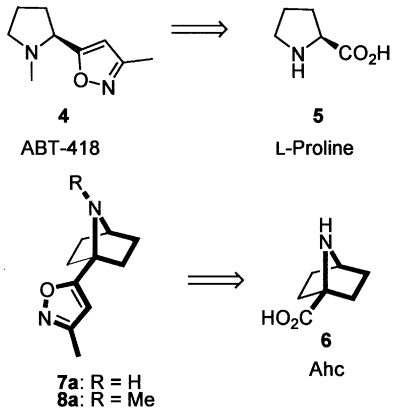
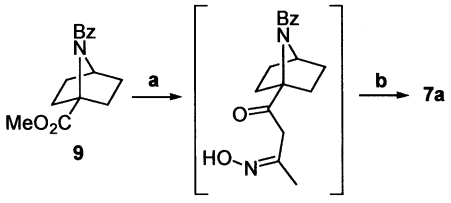


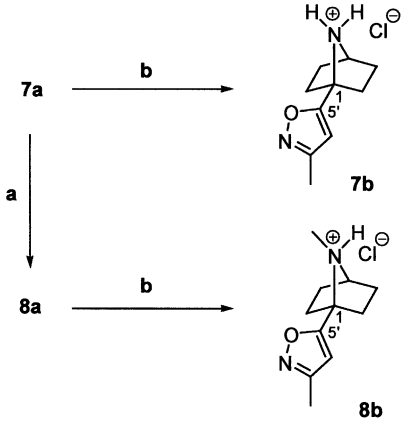
Scheme 1. Retrosynthetic analysis of ABT-418 from L-proline and of its analogues **7a** and **8a** from Ahc.

6) as starting material in the synthesis. It was obtained from methyl 2-benzamidoacrylate in five steps.[9] In a first attempt, we tried the reaction of methyl ester with dianion oxime using benzamide group as *N*-protected group. We employed two methods, one with the isolation of the intermediate β-keto oxime,[11] and another without isolation, but with immediately cyclization, dehydration and deprotection,[12] obtaining better yields in this second case. In this way, compound **7a** was obtained from **9** in a 25% yield (Scheme 2).

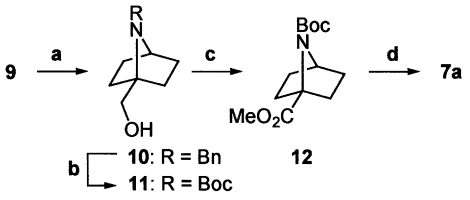
To obtain the other ABT-418 analogue, the amine **7a** was methylated with formic acid–formaldehyde[4] giving a good yield of **8a**. Taking into consideration that some ligands interact with their corresponding receptor in the protonated form, both isoxazolyl compounds **7a** and **8a** were transformed into the subsequent hydrochloride salts **7b** and **8b** by treatment with 2N HCl (Scheme 3).



Scheme 2. (a) 2 equiv. *n*-BuLi, acetone oxime, THF, 0°C→rt→reflux; (b) HCl conc., 80°C, 25% two steps.



Scheme 3. (a) H_2O, HCO_2H, HCHO, 14 h reflux, 70%; (b) 2N HCl, 100%.



Scheme 4. (a) $LiAlH_4$, THF 16 h, reflux, 99%; (b) $(Boc)_2O$, $Pd(OH)_2$, MeOH, 16 h, 98%; (c) (i) Jones' reagent, acetone, 4 h; (ii) CH_2N_2, diethyl ether, 97%; (d) (i) 2 equiv. *n*-BuLi, acetone oxime, THF, 0°C→rt→reflux; (ii) HCl conc. 80°C, 41% two steps.

In order to improve the yield of the target molecules, we changed the benzamide group of the compound **9** by another suitable group as Boc. With this aim, compound **9** was treated with $LiAlH_4$ and amide and ester functions were transformed into amino and alcohol groups by the corresponding reduction. The hydrogenolysis of **10** in the presence of $(Boc)_2O$ in MeOH gave the *N*-Boc alcohol **11** in an excellent yield. A subsequent oxidation with Jones' reagent and methylation with addition of diazomethane allowed to obtain the necessary compound **12**. The introduction of the methylisoxazolyl ring was carried out using the reaction with the oxime above described in only one step to give the key compound **7a** in a 41% yield (Scheme 4).

2.2. Biology

Affinity data for both ABT-418 analogues **7b** and **8b** towards nicotinic and muscarinic receptors are shown in Table 1, together with comparison data for the epibatidine, nicotine, citisine, atropine and pirenzepine. The two analogues do not show affinity for the muscarine receptor but differ in affinity for the nicotinic receptor; while **8b** do not show affinity for this receptor, the IC_{50}-value for compound **7b** demonstrates that the affinity is approximately 100 times lower than in the case of nicotine.

Even though the two analogues possess structures very similar to that of ABT-418 but less flexible due to the rigidity introduced by the 7-azabicyclic framework, they did not show a high affinity with the nAChR. This information provides an important insight into the design of new nAChR ligands.

Further evaluation of analgesic effects in vivo revealed that

Table 1. Nicotinic and muscarinic receptors affinities

Compound	Nicotinic receptor[a]		Muscarinic receptor[b]	
	%Shift (10^{-6} M)	IC_{50} (nM)	%Shift (10^{-6} M)	IC_{50} (nM)
Epibatidine	–	K_i=0.20	–	–
Nicotine	87.6	K_i=41.3	–	–
Citisine	[10^{-7} M]=92	K_i=3.3	–	–
Atropine	–	–	[10^{-7} M]=95	K_i=0.4
Pirenzepine	–	–	78	K_i=24.5
7b	62.4	K_i=386	−14.8	>10,000
8b	1.9	>10,000	−3.2	>10,000

[a] Inhibition of [^{3}H]-(±)-epibatidine in rat brain.
[b] Inhibition of [^{3}H]-QNB in rat cortex.

Table 2. Calculated energy, N–N distance and dihedral angle in nicotine and its analogues

	E (hartree)	N–N distance (Å)	Dihedral angle (°)
7a (HF/3-21G*)	−567.408642	4.32	53.9
	−567.412828	4.25	306.0
	−567.413777	4.75	190.8
8a (HF/3-21G*)	−606.225556	4.31	51.3
	−606.226988	4.76	206.7
	−606.226378	4.65	235.3
	−606.226236	4.49	266.5
	−606.226260	4.40	297.6
7b (HF/3-21G*)	−567.802556	4.13	42.6
	−567.802556	4.13	317.4
8b (HF/3-21G*)	−606.622452	4.33	53.8
	−606.626458	4.12	325.7
7b (B3LYP/6-31G**)	−574.573132	4.17	51.8
8b (B3LYP/6-31G**)	−613.888463	4.18	311.6
Epipatidine H^+ (B3LYP/6-31G*)	−997.061430	4.50	85.1[a]
Nicotine H^+ (B3LYP/6-31G**)	−496.130949	4.65	117.1[a]

[a] Aromatic heterocycle ring rotation angle.

8b was ineffective while **7b** needs high concentrations to show effects in the hot-plate assay.

2.3. Molecular modeling

The rigidity of our ABT-418 analogues allowed us to apply systematic bond rotations around C1–C5′. We achieved ab initio methods for compounds **7a** and **8a** and their protonated species **7b** and **8b**.

The calculations were carried our with the Gaussian-98 program[13] using HF/3-21G*. The step size for the rotation in **7a**, **8a**, **7b** and **8b** was 30°. In addition, the structures with minimum energy were re-optimized with the hybrid HF-density functional method B3LYP and the 6-31G** basis set.

The total and relative energies of the minima found are displayed in Table 2, together with the N–N distance and the dihedral angles, comparing these data with the information from the ab initio calculations contributed for epibatidine[14a] and nicotine.[14b] ABT-418 has not been compared with our compounds because its data arise from semi-empirical calculations.[15]

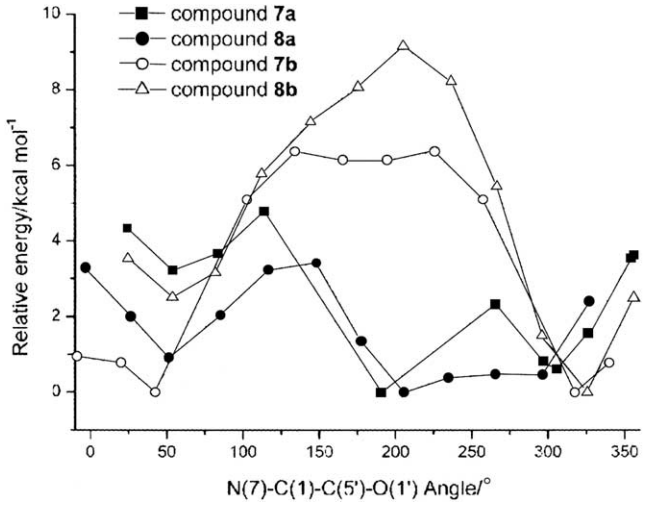


Figure 2. Rotational profiles of ABT-418 analogues **7a**, **8a**, **7b** and **8b**, determined using HF/3-21G*.

In general, the geometry of the different conformers of compounds **7a** and **8a** and its protonated forms **7b** and **8b** can be defined with the parameter corresponding to the isoxazol ring rotation angle. In this sense, the study of the conformation profile of the rotation of the isoxazol ring is shown in Fig. 2.

The low-energy conformers of the two non-protonated compounds **7a** and **8a** are gathered in Table 2 and can be divided in two groups, which differ in the disposition of the isoxazol ring that is rotated 137–252° and in energy 3.22–0.44 kcal/mol. Similar features have been observed in the protonated compounds **7b** and **8b**, with the difference that the two minima observed in **7b** have the same energy due to the symmetry of the molecule and in this case, the isoxazol ring is rotated 275°. The two minima observed in **8b** differ in 2.51 kcal/mol and in a rotation of isoxazol ring of 272°.

The N–N distance, that has been reported as an important parameter to predict affinity for the nAChRs in different pharmacophoric models,[14–16] shows close values in the protonated compounds (4.12–4.33 Å) and smaller than those described for epibatidine[14a] or nicotine[14b] (Table 2). We think that this feature is important since, until now, it has not been described active compound with internitrogen distances minor than 4.30 Å.[16b] In fact, the optimal internitrogen distance in the currently accepted model used to identify nicotinic geometries (Beers-Reich/Sheridan nicotinic pharmacophore) is a question that remain unanswered.

Taking into account the results of the biological assays of **7b** (few active) and **8b** (inactive), the structural similarities of the protonated nicotine with **8b** and the same N–N distance observed in both compounds (**7b** and **8b**), it seems that the N–N-distance is not alone the only parameter to keep in mind in order to establish the affinity for the nAChRs. In fact, the only difference between **7b** and **8b** is the *N*-methyl substituent and it is well-documented the drastic effect that the *N*-methylation in some cases has on the affinity.[16a] Moreover, it is not known if the *N*-methyl group directly

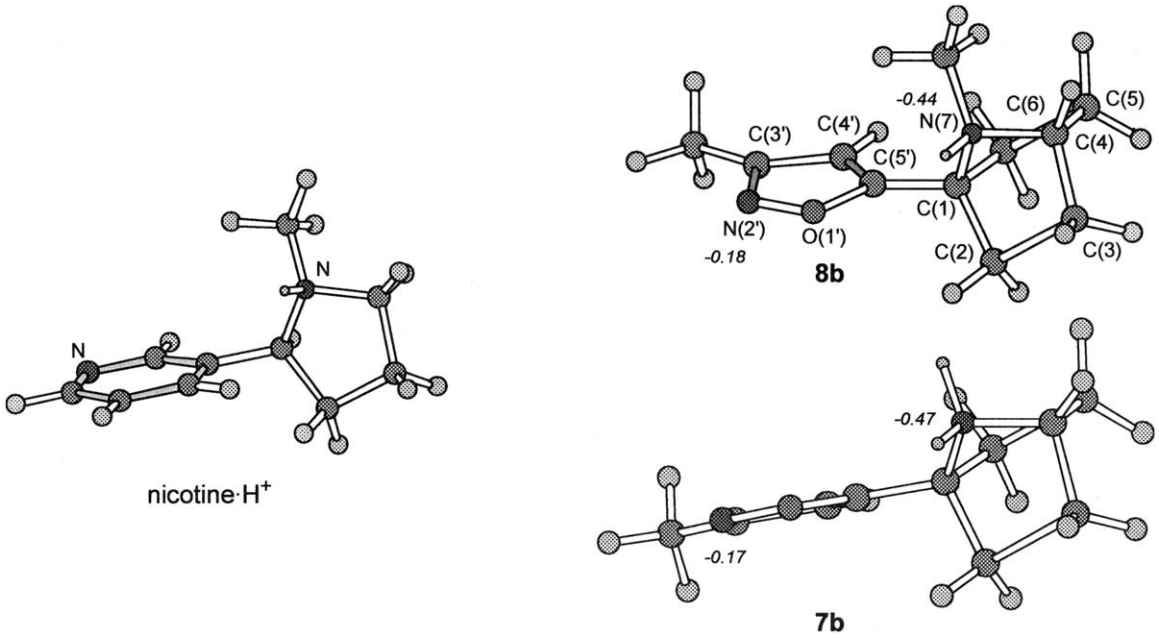


Figure 3. Three-dimensional structures of singly protonated nicotine, **7b** and **8b** optimized at the B3LYP/6-31G** level and the total atomic charges of nitrogens.

participates in a ligand–receptor interaction or whether its major role is to influence the conformation of the molecule.[16b] On the other hand, it has been described that the replacement of the *N*-methyl group by hydrogen (i.e. nornicotine) reduces affinity for the nAChRs[16b] and we observed the contrary effect in **7b** and **8b**.

In order to verify if the decrease of biological activity in **8b** in comparison with **7b** could be due to a change in the relative basicity of the nitrogens, we have calculated the Mulliken charge distribution,[13] showing similar total atomic charges for the two nitrogens in both compounds. These total atomic charges are depicted in cursive in Fig. 3.

The three-dimensional structure of the minima corresponding to the protonated nicotine and the closely related structures **7b** and/or **8b** are displayed in Fig. 3 and shows three important differences: (i) the relative orientation of the N atom of the heterocycle respect to the methyl substituent in **8b** is contrary to the observed in the nicotine, (ii) the N–N distance is minor in **7b** and **8b** than in nicotine and (iii) the number of conformers of low-energy is much smaller in the case of **7b** and **8b** that in the case of the nicotine, due to the presence of the $-CH_2-CH_2-$ bridge in **7b** and **8b**.

3. Conclusion

Starting from a precursor of the amino acid Ahc, we have synthesized two analogues of ABT-418, **7a** and **8a**, in which the flexibility of the pyrrolidine ring is limited by the incorporation of the rigid 7-azabicyclo[2.2.1]heptane skeleton. These analogues have been tested as agonists of nAChR and we have also evaluated the analgesic activity of their protonated species **7b** and **8b**, which have shown to be few active and inactive, respectively, moreover, a theoretical ab initio study of their conformations is reported. These features offer information on the investigation of the structure–activity relationship of simple ligands in their interaction with nAChR.

4. Experimental

4.1. General

Melting points are uncorrected. All the manipulations with air-sensitive reagents were carried out under a dry argon atmosphere using standard Schlenk techniques. Solvents were purified according to standard procedures. The chemical reagents were purchased from Aldrich Chemical Co. Analytical TLC was performed using Polychrom SI F_{254} plates. Column chromatography was performed using Kieselgel 60 (230–400 mesh). Organic solutions were dried over anhydrous Na_2SO_4 and, when necessary, concentrated under reduced pressure using a rotary evaporator. The ν_{max} (cm^{-1}) of IR spectra are given for the main absorption bands. NMR spectra were recorded at 300 MHz (^1H) and at 75 MHz (^{13}C) and are reported in ppm downfield from TMS. Mass spectra were obtained by electrospray ionization (ESI).

4.1.1. 1-(3-Methylisoxazol-5-yl)-7-azabicyclo[2.2.1]heptane (7a). *Method A*: A solution of acetone oxime (80 mg, 1.10 mmol) in 5 mL of dry THF at 0°C was treated dropwise with *n*-BuLi (1.1 mL, 2 M in hexane), and the reaction mixture was stirred at 0°C for 45 min. A solution of **9** (185 mg, 0.71 mmol) in 10 mL of THF was added dropwise while the reaction mixture was stirred at 0°C. After refluxing for 1 h, the reaction was allowed to occur at room temperature for another 16 h. The stirring was stopped and 2 mL of 2N HCl were added carefully after cooling the reaction mixture in an ice bath. The mixture was diluted with water and washed with ethyl acetate (3×10 mL). The aqueous layer was basified with solid $NaHCO_3/Na_2CO_3$

mixture and was extracted with $CHCl_3$/2-propanol 4:1 (3×20 mL). The combined organic layer was dried over Na_2SO_4, filtered and evaporated. The β-keto oxime was dissolved in 5 mL of 6N HCl and heated at reflux. After stirring at the same temperature for 7 h, the reaction mixture was cooled in an ice bath. It was neutralized with solid $NaHCO_3/Na_2CO_3$ mixture and extracted with $CHCl_3$/2-propanol 4:1 (3×20 mL). The organic layers were dried over Na_2SO_4, filtered and evaporated. The residue was chromatographed on silica gel using CH_2Cl_2/MeOH/Et_3N (89:10:1) as eluent to give 12 mg of **7a** (10% yield). *Method B*: A solution of acetone oxime (102 mg, 1.4 mmol) in 10 mL of dry THF at 0°C was treated dropwise with *n*-BuLi (1.5 mL, 2 M in hexane), and the reaction mixture was allowed to warm to room temperature over 30 min. A solution of **6** (259 mg, 1.0 mmol) in 20 mL of THF was added dropwise while the reaction mixture was stirred at room temperature. After the reaction mixture was refluxed for 45 min, the THF was removed under argon atmosphere to give a crude residue that was dissolved in 8 mL of concentrated HCl and heated at 80°C for 4 h. The mixture was cooled, diluted with water and washed with ethyl acetate (2×10 mL). The aqueous layer was basified with solid $NaHCO_3/Na_2CO_3$ mixture and was extracted with $CHCl_3$/2-propanol 4:1 (3×20 mL). The combined organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was chromatographed on silica gel using CH_2Cl_2/MeOH/Et_3N (89:10:1) as eluent to give 45 mg of **7a** (25% yield). *Method C*: A solution of acetone oxime (102 mg, 1.4 mmol) in 10 mL of dry THF at 0°C was treated dropwise with *n*-BuLi (1.5 mL, 2 M in hexane), and the reaction mixture was allowed to warm to room temperature over 30 min. A solution of **11** (259 mg, 1.0 mmol) in 20 mL of THF was added dropwise while the reaction mixture was stirred at room temperature. After the reaction mixture was refluxed for 45 min, the THF was removed under an argon atmosphere to give a crude residue that was dissolved in 8 mL of concentrated HCl and heated at 80°C for 4 h. The mixture was cooled, diluted with water and washed with ethyl acetate (2×10 mL). The aqueous layer was basified with saturated $NaHCO_3$ solution and was extracted with $CHCl_3$/2-propanol 4:1 (3×20 mL). The combined organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was chromatographed on silica gel using CH_2Cl_2/MeOH/Et_3N (89:10:1) as eluent to give 73 mg of **7a** (41% yield). Anal. calcd for $C_{10}H_{14}N_2O$; C, 67.39; H, 7.92; N, 15.72; found C, 67.48; H, 7.81; N, 15.90; IR (CH_2Cl_2, cm^{-1}): 3625, 2956, 2877, 1609, 1270, 1018; ^1H NMR ($CDCl_3$): δ 1.54–1.62 (m, 2H); 1.76–1.93 (m, 6H); 2.23 (br s, 1H); 2.28 (s, 3H); 3.76–3.83 (m, 1H); 6.05 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 11.4, 31.2, 35.1, 57.8, 63.4, 101.4, 159.6, 173.5.

4.1.2. Hydrochloride salt of 7a (7b). The compound **7a** was dissolved in 5 mL of 2N HCl and stirred for 10 min. The solvent was evaporated and the residue was eluted through a C_{18} reverse-phase Sep-pak cartridge which, after the removal of the water, gave the salt **7b** in 100% yield. ^1H NMR (D_2O): δ 1.86–1.99 (m, 2H); 2.10–2.35 (m, 9H); 4.34–4.41 (m, 1H); 6.52 (s, 1H).

4.1.3. 7-Methyl-1-(3-methylisoxazol-5-yl)-7-azabicyclo-[2.2.1]heptane (8a). A mixture of **7a** (61 mg, 0.34 mmol) in water (0.5 mL), formic acid (0.25 mL), and formaldehyde (0.25 mL) was heated at reflux for 14 h. The solution was evaporated, the residue was partitioned between ethyl acetate and 2 M K_2CO_3, and the aqueous layer was extracted with ethyl acetate (2×20 mL). The combined organic layer was dried, filtered and evaporated and the residue was chromatographed on silica gel using CH_2Cl_2/MeOH (90:10) as eluent to give **8a** in 70% yield. Anal. calcd for $C_{11}H_{16}N_2O$; C, 68.72; H, 8.39; N, 14.57; found C, 68.59; H, 8.48; N, 14.40; IR (CH_2Cl_2, cm^{-1}): 1735, 1620; ^1H NMR ($CDCl_3$): δ 1.42–1.52 (m, 2H); 1.70–1.81 (m, 2H); 1.90–2.15 (m, 7H); 2.29 (s, 3H); 3.37–3.44 (m, 1H); 6.05 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 11.4, 32.2, 35.1, 40.2, 57.9, 62.1, 102.3, 158.3, 174.2.

4.1.4. Hydrochloride salt of 8a (8b). The compound **8a** was dissolved in 5 mL of 2N HCl and stirred for 10 min. The solvent was evaporated and the residue was eluted through a C_{18} reverse-phase Sep-pak cartridge which, after the removal of the water, gave the salt **8b** in 100% yield. ^1H NMR (D_2O): δ 1.89–2.08 (m, 2H); 2.18–2.40 (m, 7H); 2.45–2.60 (m, 5H); 4.18–4.22 (m, 1H); 6.63 (s, 1H).

4.1.5. 7-Benzyl-1-hydroxymethyl-7-azabicyclo[2.2.1]-heptane (10). To a solution at 0°C containing $LiAlH_4$ (351 mg, 9.25 mmol) in THF (35 mL), methyl ester **9** (600 mg, 2.3 mmol) was added as a solution in THF (10 mL). The suspension was refluxed overnight under an inert atmosphere. The suspension was cooled and water (10 mL), NaOH 10% (10 mL) and again water (10 mL) were added. This mixture was stirred for 30 min and then extracted with ethyl acetate (30 mL). The aqueous layer was washed with ethyl acetate (2×20 mL). The combined organic layers were dried, filtered and evaporated to give 494 mg of the pure compound **10** (99%). Anal. calcd for $C_{14}H_{19}NO$; C, 77.38; H, 8.81; N, 6.45; found C, 77.45; H, 8.71; N, 6.61; IR (CH_2Cl_2, cm^{-1}): 3610, 2960, 1605, 1453, 1024; ^1H NMR ($CDCl_3$): δ 1.24–1.31 (m, 4H); 1.69–1.75 (m, 4H); 3.08–3.13 (m, 1H); 3.37 (br s, 2H); 3.65 (br s, 2H); 7.12–7.33 (m, 5H); ^{13}C NMR ($CDCl_3$): δ 27.9, 30.3, 49.0, 59.8, 62.1, 69.4, 126.7, 128.2, 128.5, 140.0.

4.1.6. 7-(*tert*-Butoxycarbonyl)-1-hydroxymethyl-7-aza-bicyclo[2.2.1]heptane (11). A solution of **10** (492 mg, 2.26 mmol) in degassed MeOH (10 mL) was added over a suspension of $(Boc)_2O$ (494 mg, 2.26 mmol) and 10% $Pd(OH)_2/C$ (321 mg) in degassed MeOH (20 mL). The reaction was hydrogenated at atmospheric pressure overnight. The reaction mixture was filtered, over Celite and evaporated to obtain 503 mg of a clean oil, corresponding to compound **11** (98%). Anal. calcd for $C_{12}H_{21}NO_3$; C, 64.41; H, 9.31; N, 6.16; found C, 64.53; H, 9.23; N, 6.30; IR (CH_2Cl_2, cm^{-1}): 3180–3520 (br), 1648; ^1H NMR ($CDCl_3$): δ 1.34–1.52 (m, 13H); 1.73–1.87 (m, 4H); 3.87–3.90 (m, 2H); 4.20–4.25 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 28.3, 29.2, 31.7, 58.3, 61.9, 69.0, 80.1, 155.1.

4.1.7. Methyl 7-(*tert*-butoxycarbonyl)-7-azabicyclo [2.2.1]-heptane-1-carboxylate (12). A 1.5-fold excess of Jones reagent was dropwise added to a solution of **11** (200 mg, 0.98 mmol) in acetone (10 mL) at 0°C over 5 min. The mixture was stirred at 0°C for 4 h. The excess of Jones reagent was destroyed with 2-propanol. The mixture was

then diluted with water (10 mL) and extracted with ethyl acetate (4×20 mL). The combined organic extracts were dried and concentrated. The residual white solid (187 mg) was identified by NMR. IR (CH_2Cl_2, cm^{-1}): 3679, 3500, 2981, 1691; ^1H NMR ($CDCl_3$): δ 1.34–1.50 (m, 11H); 1.72–1.86 (m, 4H); 2.05–2.13 (m, 2H); 4.26–4.30 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 27.9, 29.0, 33.8, 59.7, 69.1, 81.6, 156.9, 178.4. The residue was dissolved in diethyl ether (50 mL) and esterified with an excess of diazomethane in diethyl ether for 30 min. The solvent was removed under vacuum and the required compound was used without further purification.

4.2. Biological assays

4.2.1. Muscarinic binding assays. The method used was similar to the previously described.[17] Membranes were prepared from cerebral cortices of male Wistar rats. The tissues were homogenized at 4°C with an Ultra-Turrax (13,500 rpm) for 15 s in 50 mM sodium potassium phosphate buffer, pH 7.4 (10 vols. w/v). The homogenate were centrifuged for 10 min at 1000*g* at 4°C, the resulting supernatant recentrifuged twice for 10 min at 48,000*g* and the pellet resuspended in the homogenizing buffer to a dilution of 250 times. The homogenates were incubated with 0.2 nM [^{3}H]-QNB for 90 min at 25°C and the reactions terminated by filtration. Non-specific binding was defined as that in the presence of 1 μM atropine. IC_{50} values of displacement were determined by computer-assisted curve fitting (EBDA/LIGAND).[18] K_i values were calculated from IC_{50} values by the equation derived by Cheng and Prusoff.[19]

4.2.2. Nicotinic binding assays. The method used was similar to the previously described.[20] Brain homogenate was prepared from male Wistar rats. The tissues were homogenized at 4°C with a Polytron (14,500 rpm) for 30 s in 50 mM Tris–HCl buffer, pH 7.4 (10 vols. w/v). The homogenate was centrifuged twice for 10 min at 48,000*g* and the pellet resuspended in the same buffer to a dilution of 150 times. The homogenates were incubated with 0.1 nM [^{3}H]-Epibatidine for 180 min at 24°C and the reactions terminated by filtration. Non-specific binding was defined as that in the presence of 300 μM nicotine. IC_{50} values of displacement were determined by computer-assisted curve fitting (EBDA/LIGAND).[18] K_i values were calculated from IC_{50} values by the equation derived by Cheng and Prusoff.[19]

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