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New selective acetylcholinesterase inhibitors designed from natural piperidine alkaloids

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Abstract—Five new piperidine alkaloids were designed from natural (−)-3-*O*-acetyl-spectaline and (−)-spectaline that were obtained from the flowers of *Senna spectabilis* (sin. *Cassia spectabilis*, Leguminosae). Two semi-synthetic analogues (**7** and **9**) inhibited rat brain acetylcholinesterase, showing IC_{50} of 7.32 and 15.1 μM, and were 21 and 9.5 times less potent against rat brain butyrylcholinesterase, respectively. Compound **9** (1 mg/kg, ip) was fully efficacious in reverting scopolamine-induced amnesia in mice. The two active compounds (**7** and **9**) did not show overt toxic effects at the doses tested in vivo.
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1. Introduction

Alzheimer's disease (AD) is a late-onset, progressive and eventually fatal neurodegenerative disorder whose prevalence is increasing together with the life expectancy throughout the world. Because cognitive deficits in AD are associated with the disruption of central cholinergic transmission, cholinergic-boosting drugs have been the mainstay of current therapy.[1] Tacrine (THA, Cognex®, **1**) was the first synthetic drug approved by the USA Food and Drug Administration for AD treatment. This substance is a centrally-acting cholinesterase inhibitor (ChEI) that shows moderate effect in relieving AD symptoms of mild and medium intensity. However, its application became limited due to serious side effects, like hepatotoxicity that forced patients to discontinue the treatment.[2] Besides THA, currently there are three other ChEI drugs available in USA and Europe for AD treatment: donepezil (**2**, Aricept®), rivastigmine (**3**, Exelon®), and galanthamine (**4**, Reminyl®) (Fig. 1). Of these, compounds **1**, **2**, and **4** are reversible inhibitors, while **3** acts pseudo-irreversibly. These compounds also differ with regard to selectivity for acetyl- (AChE) versus butyrylcholinesterase (BuChE) and central versus peripheral activity.[3] Galanthamine (**4**) is a natural product that has the additional effect of allosterically potentiating nicotinic acetylcholine receptors, and it has been used as a prototype in anti-cholinesterase drug development.[2,4]

The structural diversity of known ChEIs and the possibility to explore distinct modes of action have stimulated phytochemical studies with plant and microorganism species that can furnish new anti-cholinesterase structural motifs. In this context, various species have been investigated as possible sources of ChEIs based on ethnobotanic data or reports of their popular use.[5]

In the search for new bioactive agents, we have recently isolated the new piperidine alkaloid, (−)-3-*O*-acetyl-

Keywords: Piperidine alkaloids; Acetylcholinesterase inhibitors; Alzheimer's disease; *Senna spectabilis*.

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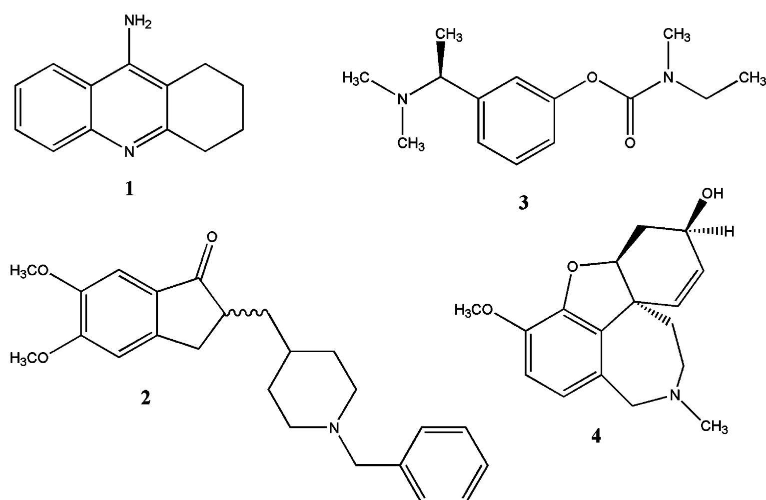


Figure 1. Marketed cholinesterase inhibitors tacrine (1), donepezil (2), rivastigmine (3), and galanthamine (4).

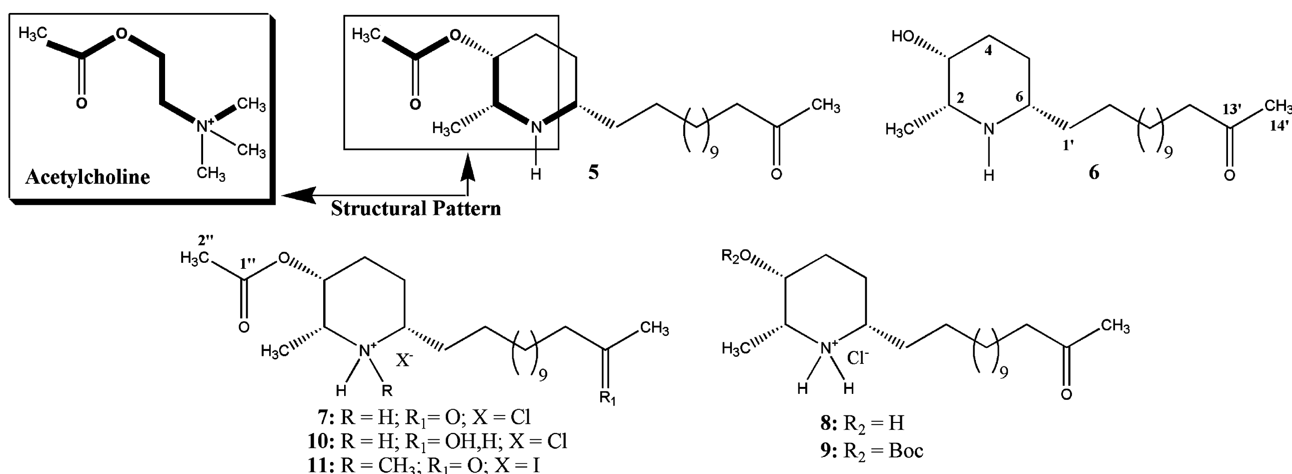


Figure 2. Structural design of new piperidine derivatives (**7**–**11**) from natural *Senna* sp. alkaloids (**5** and **6**).

spectaline (**5**) along with a large amount of (−)-spectaline (**6**) from *Senna spectabilis*.[6–8] A detailed analysis of the structure of **5** allows one to recognize an acetylcholine moiety internalized in the molecule (Fig. 2). This observation led us to design derivatives that could be active as ChE inhibitors. In this paper we report the semi-synthetic preparation of five piperidine alkaloid derivatives (compounds **7**–**11**) and the pharmacological study by using Ellman's assay,[9] tests of scopolamine-induced amnesia and acute toxicity.

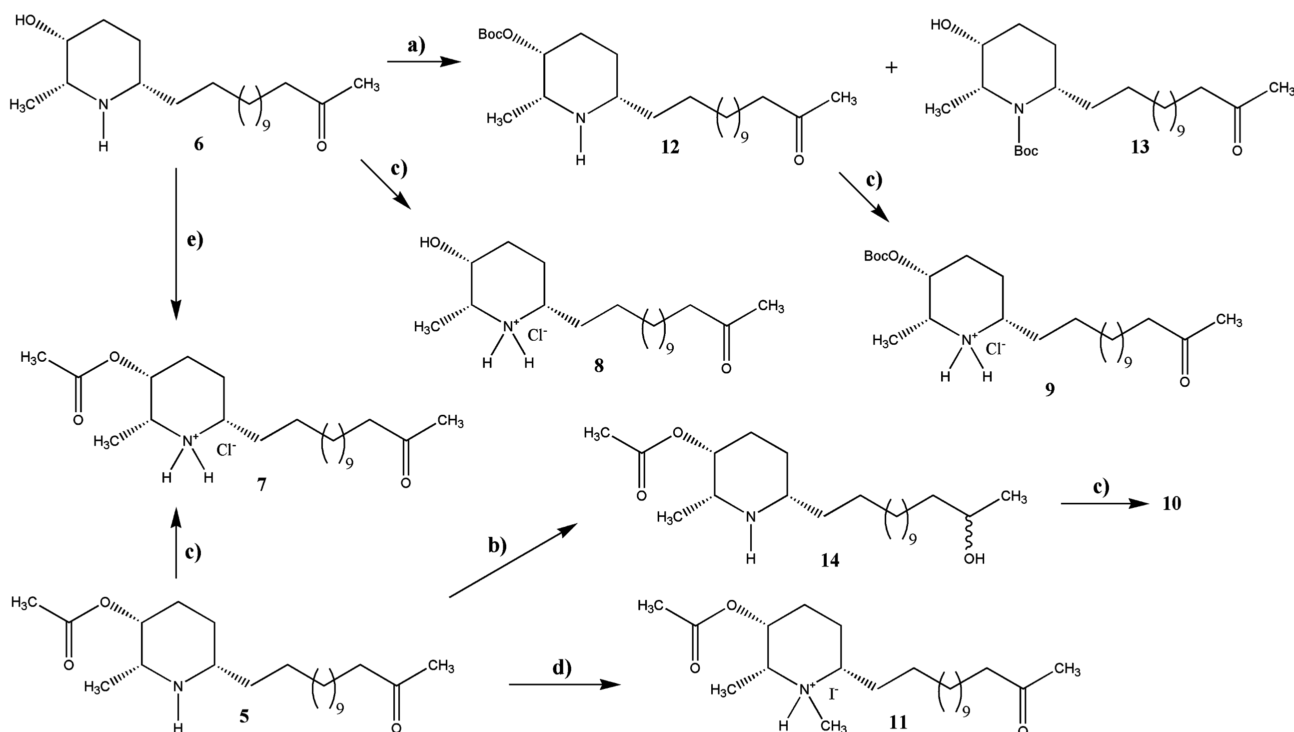
2. Results and discussion

2.1. Chemistry

For the preparation of the target derivatives **7**–**11** from *O*-acetyl-spectaline (**5**) and (−)-spectaline (**6**) we followed the synthetic route outlined in Scheme 1. Carbonate derivative **12** was obtained in 60% yield after its column chromatography purification from a 2:1 mixture with carbamate **13**, resulting from treatment of **6** with $(Boc)_2O/Et_3N$ in CH_2Cl_2.[10,11] On the other hand, chemoselective reduction of keto-carbonyl group of derivative **5** with $NaBH_4$ in MeOH furnished the diastereomeric mixture of alcohols **14**, in 97% yield. Next, treatment of the piperidine derivatives **5**, **6**, **12**, and **14** with HCl in CH_2Cl_2, under anhydrous conditions, yielded almost quantitatively the corresponding hydrochlorides **7**, **8**, **9**, and **10**. Additionally, tertiary ammonium derivative **11** was prepared in 50% yield by treatment of acetate **5** with methyl iodide in acetone at reflux. Alternatively, compound **7** could be prepared in 95% yield from more abundant natural (−)-spectaline[7] (**6**) through its direct treatment with acetyl chloride in anhydrous chloroform. All structures were in agreement with analytical and spectral data.[12]

2.2. Pharmacology

The bioactivity of compounds **7**–**11** as inhibitors of mammalian central nervous system cholinesterases was investigated in rat brain tissue, as previously described.[13] Concentration-response curves were obtained



Scheme 1. Synthesis of piperidine derivatives **7**–**11**. Reagents and conditions: (a) $(Boc)_2O$, Et_3N, 4-DMAP, CH_2Cl_2, rt, 72 h (60% yield of **12** after purification by column chromatography); (b) $NaBH_4$, MeOH, rt, 2 h, 97%; (c) HCl, anhydrous CH_2Cl_2, rt, 0.5–1 h, 98–100%; (d) MeI, acetone, reflux, 5 days, 50% yield; (e) AcCl, $CHCl_3$, 70 °C, 8 h, 95% yield.

Table 1. IC_{50} of new piperidine analogues (**7**–**11**), THA (**1**), and galanthamine (**4**) for inhibition of rat brain cholinesterases (ChE) (μM)[a]

Compound	Total ChE (μM)[b]		BuChE (μM)		SI[c]
THA (**1**)[d]	0.16 ± 0.03	(4)	0.24 ± 0.10	(2)	1.6
7	7.32 ± 1.02	(4)	150.1 ± 1.4	(2)	21
8	236 ± 42	(2)			
9	15.1 ± 3.1	(3)	143.2 ± 24.9	(3)	9.5
10	426 ± 141	(3)			
11	243 ± 44	(3)			
Gal. (**4**)[d]	3.10 ± 0.18	(2)			

[a] Values are means ± SEM of the IC_{50} from the indicated number of animals in parenthesis.
[b] Total cholinesterase (ChE) and butyrylcholinesterase (BuChE) activities were determined by Ellman's method,[9] as described in the Experimental section.
[c] SI = selectivity index = IC_{50} BuChE/IC_{50} total ChE ratio.
[d] Data from Ref. 13.

for all new compounds **7**–**11**, and their mean inhibitory concentration (IC_{50}) was estimated (Table 1). Compounds **7** and **9** were effective inhibitors of AChE, showing mean IC_{50}'s of 7.32 and 15.1 μM, respectively. On the other hand, compounds **8** and **10**–**11** achieved less than 50% inhibition at the highest concentration tested (200 μM), therefore their IC_{50}'s should be considered rough estimates. The presence of the acetyl group in **7** conferred a 30-times gain in potency with respect to (−)-spectaline hydrochloride (**8**), prepared from the more abundant natural alkaloid (**6**). The anti-cholinesterase activity of **7** is consistent with the hypothesis of an acetylcholine-mimicking property of the molecule (Fig. 2). Thus, the *N*-methyl substitution in the piperidine ring in **11** would be expected to increase potency, because of the greater similarity with the ammonium group in acetylcholine. However, the inhibitory potency was markedly reduced in **11**, suggesting that the methyl group introduced steric hindrance to the ionic interaction of the charged nitrogen.

This unfavorable behavior might occur with **11**, but not with acetylcholine, because of conformational constraints imposed by the ring structure. Addition of the *t*-Boc group to the acetyl moiety in **9** slightly reduced the potency in comparison with **7**. The difference between **7** and **9** may be analogous to that between acetylcholine and butyrylcholine with respect to AChE.[14] Compounds **7** and **9** were then chosen for testing in a rat brain BuChE assay, in order to assess their selectivity and to further explore the analogy with the choline esters. Compounds **7** and **8** showed similar IC_{50} in the BuChE assay, both being less potent than in the AChE assay. Their selectivity indices (SI) of 21 and 9.5 (for **7** and **9**, respectively) are higher than that of the standard centrally acting inhibitor, THA (**1**) (Table 1) or galanthamine (**4**).[15] The observation that brain BuChE did not distinguish the compounds with and without the *t*-Boc subunit is further evidence that the choline ester-like motif may interact with the enzyme by mimicking the substrates.

The selected compounds **7** and **9** were tested in vivo for their ability to prevent scopolamine-induced amnesia in

two tasks, passive avoidance and water maze.[16,17] In the passive-avoidance test, the step-down latency in the training session was around 5 s in all experimental groups (4.8 ± 0.3 s for all animals). Mice were tested for retention of the avoidance task 24 h after the training procedure. The control animals showed a 15-fold increase in the step-down latency in this test session, remaining on the wooden platform for 79.3 ± 10.6 s ($n = 22$). Animals given the centrally acting muscarinic receptor antagonist scopolamine showed amnesia, with a step-down latency of 16.8 ± 6.4 s ($n = 19$) in the test session. This amnesia was reverted by pre-treatment with the potent cholinesterase inhibitor THA (**1**) (5.6 mg/kg), and animals remained on the platform for 67.5 ± 13.9 s ($n = 13$). Compound **9** (1 mg/kg) was able to revert the amnesia induced by scopolamine, and the observed step-down latency was 41.6 ± 10.9 s ($n = 13$) ($p < 0.05$). Mice treated with compound **7** also showed increased step-down latency, but the effect did not reach significance at the dose tested (1 mg/kg; Table 2).

In the spatial memory test (water maze), in the fourth day of test, control animals were able to localize the hidden platform after 42.3 ± 13.2 s ($n = 10$), and this ability was disrupted by scopolamine. Treated animals could not remember the position of the platform, showing an escape latency of 115.8 ± 17.2 s ($n = 8$; maximum time: 150 s). Scopolamine-induced amnesia was antagonized by pre-treatment with THA (**1**) and compound **7**, and rats had an escape latency of 5.5 ± 4.5 s ($n = 4$) and 32.2 ± 6.3 s ($n = 4$), respectively ($p < 0.001$) (Table 2). Therefore, compound **7** and **9** are effective in antagonizing the amnesia-induced by scopolamine in the water maze and passive-avoidance test, respectively, suggesting that these compounds also act as potent AChE inhibitors in tests in vivo.

Interestingly, none of the animals treated with compound **7** or **9** (up to 10 mg/kg in mice) showed cholinergic side effects like tremor, salivation, diarrhea, or lacrimation. However, we observed diarrhea in 70% of rats and mice treated with THA (1 and 5.6 mg/kg), 15 min after the administration. THA (**1**) in concentrations of 30 and 50 mg/kg (up to 10 times the concentration effective in reversing the amnesia in mice) also produced splayed hindlimbs and lacrimation in 100% of mice, with moderate to severe tremor and salivation, after 30 min of administration. We also found that THA (**1**) (30 mg/kg) was lethal to 60% of mice, showing a narrow therapeutic window.

Table 2. Antagonism of amnesia induced by scopolamine[a]

	Passive avoidance step-down latency (s)	Water maze escape latency (s)
Saline + saline	79.2 ± 10.7	42.3 ± 13.8
Saline + scopol.	16.7 ± 6.4	115.7 ± 17.2
THA + scopol.	67.5 ± 13.9**	15.5 ± 4.5*
7 + scopol.	24.8 ± 8.8	32.2 ± 6.3*
9 + scopol.	41.6 ± 10.9*	123.2 ± 24.8

[a] Latencies (seconds) are expressed as mean ± SEM. Asterisks denote statistical significance between saline/scopolamine and treated groups (compound/scopolamine); *$p < 0.01$; **$p < 0.001$.

3. Conclusion

Our results show that semi-synthetic piperidine alkaloids designed from natural spectaline, such as compounds **7** and **9**, act as cholinesterase inhibitors in vivo with a significant selectivity toward brain acetylcholinesterase. Whether such selectivity is a desirable property in AD has been a matter of considerable debate, because of the evidence of a possible role of BuChE, particularly in late stages of the disease.[3] However, recent pre-clinical studies support the earlier belief that AChE-selective inhibitors produce less peripheral side effects.[18] The compounds described herein, which can be efficiently prepared from abundant plant material, seem worth developing as candidate drugs for treating patients with Alzheimer's disease.

4. Experimental

4.1. Chemistry

Melting points were determined with a Microquimica MQAPF-301 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were determined in dimethylsulfoxide-d_6 and methanol-d_4 with a Varian INOVA 500 spectrometer at 500 and 125 MHz, respectively. Splitting patterns were as follows: s, singlet; d, doublet; t, triplet; dq, double quadruplet; br, broad; m, multiplet. ^{1}H and ^{13}C NMR assignments were confirmed by gHMQC, gCOSY, and gHMBC experiments. IR spectra were obtained with a Nicolet-1400 FTIR spectrometer using KBr pellets. Prior to concentration under reduced pressure, all organic extracts were dried over anhydrous magnesium sulfate powder. The progress of all reactions was monitored by TLC performed on 2.0 × 6.0 aluminum sheets precoated with neutral aluminum oxide (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed by spraying with iodochloroplatinate reagent (Merck). For column chromatography Merck neutral aluminum oxide (70–230 mesh) was used. Solvents used in the reactions were dried, redistilled prior to use, and stored over +3–4 Å molecular sieves. Reaction mixtures were generally stirred under a dry nitrogen atmosphere.

4.2. (2*R*,3*R*,6*S*)-2-Methyl-6-(13-oxotetradecyl)piperidin-3-yl acetate (5) and 13-[(2*S*,5*R*,6*R*)-5-hydroxy-6-methylpiperidin-2-yl]tetradecan-2-one (6)

Natural alkaloids (−)-3-*O*-acetyl-spectaline (**5**) and (−)-spectaline (**6**) were obtained from the ethanolic extract of flowers of *S. spectabilis* (DC.) Irwin et Barn (Leguminosae), as previously described.[3–5]

4.3. General procedure for the preparation of hydrochloride derivatives 7–10

The corresponding piperidine alkaloid substrate (0.30 mmol) was dissolved in 3 mL of dried dichloromethane, and the reaction mixture was cooled to 0 °C with an ice/NaCl/H_2O bath. Then, dried gaseous HCl (generated from a keeper bottle by the mixture of con-

centrated H_2SO_4 and 37% aq HCl) was added. The addition of gaseous HCl was maintained for 30 min at room temperature. The solvent was evaporated under reduced pressure, and the crude product was recrystallized from chloroform.

4.3.1. (2*R*,3*R*,6*S*)-2-Methyl-6-(13-oxotetradecyl)piperidin-3-yl acetate hydrochloride (7). Hydrochloride **7** was prepared by the treatment of the natural (−)-3-*O*-acetyl-spectaline (**5**) (0.32 mmol) and anhydrous HCl. Compound **7** was obtained in quantitative yield, as a pale yellow solid, mp 143–145 °C: ^{1}H NMR (500 MHz, CD_3OD) δ 1.22 (d, 3H, H-7), 1.24–1.27 (m, 18H, H-2′ to H-10′), 1.49 (m, 4H, H-4$_{ax}$, H-5$_{ax}$, 2H-11′), 1.86 (m, 2H, H-1′), 1.98 (m, 1H, H-5$_{eq}$), 2.02 (m, 1H, H-4$_{eq}$), 2.06 (s, 3H, H-14′), 2.09 (s, 3H, H-2″), 2.41 (t, J = 7.5 Hz, 2H, H-12′), 3.12 (m, 1H, H-6), 3.45 (dq, J = 2.0, 6.5 Hz, 1H, H-3), 5.04 (m, 1H, H-3); ^{13}C NMR (125 MHz, CD_3OD) δ 15.5 (C-7), 20.7 (C-2″), 24.1 (C-11′), 24.8 (C-2′), 26.2 (C-5), 29.8 (C-14′, C-4), 30.2–30.5 (C-3″ to C-10″), 34.6 (C-1′), 44.3 (C-12′), 55.8 (C-2), 58.5 (C-6), 69.2 (C-3), 171.3 (C-1″), 209.5 (C-13′). IR (KBr) cm^{-1}: 3340 (NH_2), 1730 (C=O, ester), 1715 (C=O, ketone).

4.3.2. 14-[(2*S*,5*R*,6*R*)-5-Hydroxy-6-methylpiperidin-2-yl]-tetradecan-2-one hydrochloride (8). Treatment of natural (−)-spectaline (**6**) (0.30 mmol) with anhydrous HCl, yielded compound **8** (98%) as a white solid, mp 151–153 °C: ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.04 (d, J = 6.5, 3H, H-7); 1.19 (m, 18H, H-2′ to H-10′); 1.28 (m, 2H, H-1′); 1.41 (m, 2H, H-4$_{ax}$, H-5$_{ax}$); 1.50 (m, 2H, H-11′); 1.81 (m, 1H, H-5$_{eq}$); 1.84 (m, 1H, H-4$_{eq}$); 2.06 (s, 3H, H-14′); 2.34 (t, J = 7.5 Hz, 2H, H-12.′); 2.92 (m, 1H, H-6); 3.12 (dq, J = 1.0, 6.5 Hz, 1H, H-2); 3.69 (br s, 1H, H-3), 8.76 (br s, 1H, exchanges with D_2O, NH_2^+) 7.91 (br s, 1H, exchanges with D_2O, NH_2^+); ^{13}C NMR (125 MHz, DMSO-d_6) δ 16.0 (C-7), 23.9 (C-11′), 25.3 (C-5), 29.2 (C-14′), 29.4–29.7 (C-2′ to C-10′), 30.4 (C-4), 33.3 (C-1′), 43.4 (C-12′), 55.8 (C-2), 56.8 (C-6), 64.8 (C-3), 209.3 (C-13′); IR (KBr) cm^{-1}: 3348 (NH_2), 1725 (C=O).

4.3.3. *tert*-Butyl (2*R*,3*R*,6*S*)-2-methyl-6-(13-oxotetradecyl)-piperidin-3-yl carbonate hydrochloride (9). Compound **6** (1.54 mmol) was dissolved in 15 mL of dried CH_2Cl_2, under stirring and N_2 atmosphere. Then, Et_3N (2.156 mmol) and catalytic amount of 4-DMAP were added. After 5 min, a solution of $(Boc)_2O$ (1.694 mmol) in 15 mL of dried CH_2Cl_2 was added and the mixture was stirred for 72 h at room temperature. After total conversion, 10 mL of H_2O were added to the reaction mixture, which was next extracted with $CHCl_3$. The organic extract was washed with 2 N HCl, brine, dried over $MgSO_4$, and evaporated. The residue was purified by neutral alumina column chromatography using a chloroform/hexanes/methanol mixture as eluent. The piperidine carbonate derivative, 2-(*R*)-methyl-6-(*S*)-(tetradecyl-13′-one)-3-(*R*)-*O*-*tert*-butoxycarbonyl piperidine, was obtained in 60% yield (mp 58–60 °C). This carbonate was then reacted with anhydrous HCl to furnish the desired hydrochloride **9**, in 98% yield, as a white solid, mp 126–129 °C: ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.21 (d, J = 8.0 Hz, 3H, H-7), 1.22 (s, 9H, 3Me, Boc) 1.23–1.25 (m, 20H, H-2′ to H-10′), 1.44 (m, 2H, H-11′), 1.58 (m, 2H, H-4$_{ax}$, H-5$_{ax}$), 1.74 (m, 1H, H-5$_{eq}$), 1.77 (m, 1H, H-4$_{eq}$), 2.06 (s, 3H, H-14′), 2.39 (t, J = 7.0 Hz, 2H, H-12′), 2.91 (m, 1H, H-6), 3.70 (br s, 1H, H-2), 5.37 (br s, 1H, H-3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 15.7 (C-7), 23.9 (C-11′), 25.3 (C-5), 29.4 (3Me–Boc), 29.5 (C-1′ to C-10′), 29.5 (C-4), 30.3 (C-14′), 43.4 (C-12′), 55.8 (C-2), 56.7 (C-6), 65.0 (C-3), 82.0 (C, Boc), 153.6 (C=O, Boc), 208.6 (C-13′); IR (KBr) cm^{-1}: 3338 (NH_2), 1728 (C=O, Boc), 1715 (C=O, ketone).

4.3.4. (2*R*,3*R*,6*S*)-6-(13-Hydroxytetradecyl)-2-methylpiperidin-3-yl acetate hydrochloride (10). Natural 3-*O*-acetyl-spectaline (**5**) (0.136 mmol) was added to a stirred solution of $NaBH_4$ (0.272 mmol) in 2 mL of MeOH. The reaction mixture was stirred for 30 min at room temperature, and then was quenched by the addition of 2 mL of saturated NH_4Cl solution. After extraction with MeOH, the organic extract was dried over anhydrous magnesium sulfate and concentrated. The corresponding 3-*O*-acetyl-13′-hydroxy-spectaline was obtained as a pale yellow solid (mp 61–63 °C), in 97% yield. This compound was then reacted with anhydrous HCl to furnish the title hydrochloride **10** (100%), as a pale yellow solid, mp 134–136 °C: ^{1}H NMR (500 MHz, CD_3OD) δ 1.07 (d, J = 6.0 Hz, 3H, H-14′), 1.23 (d, J = 6.5 Hz, 3H, H-7), 1.26 (m, 20H, H-1′ to H-10′), 1.52 (m, 2H, H-4$_{ax}$, H-5$_{ax}$), 1.75 (m 2H, H-12′), 1.86 (m, 1, H-5$_{eq}$), 1.98 (m, 1H, H-4$_{eq}$), 2.09 (s, 3H, H-2″), 3.16 (m, 1H, H-6), 3.45 (m, 1H, H-2), 3.65 (m, 1H, H-13′), 5.03 (br s, 1H, H-3); ^{13}C NMR (125 MHz, CD_3OD) δ 15.6 (C-7), 20.8 (C-2″), 23.5 (C-14′), 24.1 (C-11′), 26.3 (C-2′), 26.9 (C-5), 28.1 (C-4), 30.4–30.8 (C-3′ to C-10′), 34.6 (C-1′), 55.8 (C-2), 58.5 (C-6), 68.6 (C-13′), 69.2 (C-3), 171.3 (C-1″); IR (KBr) cm^{-1} 3334, 1726 (C=O, ester).

4.3.5. (2*R*,3*R*,6*S*)-1,2-Dimethyl-6-(13-oxotetradecyl)piperidin-3-yl acetate hydroiodide (11). Methyl iodide (3 mL) was added to a solution of 3-*O*-acetyl-spectaline (**5**) (0.2 mmol) in 10 mL of dried acetone. The reaction mixture was refluxed for five days until complete consumption of the starting material. The solvent was evaporated and the crude product was purified by PTLC, furnishing the iodide **11**, in 50% yield, as brown oil. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.19 (d, J = 7.0 Hz, 3H, H-7), 1.20–1.25 (m, 18H, H-2′ to H-10′), 1.43 (m, 3H, H-4$_{ax}$, H-5$_{ax}$, H-11′), 1.80 (m, 4H, H-4$_{eq}$, H-5$_{eq}$, 2H-1′), 2.04 (s, 3H, H-14′), 2.09 (s, 3H, H-2″), 2.37 (t, J = 7.5 Hz, 2H, H-12′), 2.86 (s, 3H, Me–N), 3.07 (m, 1H, H-6), 3.80 (m, 1H, H-2), 4.98 (br s, 1H, H-3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 14.9 (C-7), 24.4 (C-11′), 26.2 (C-2″), 26.4 (C-5), 28.5–28.7 (C-2′ to – 10′), 28.8 (C-4), 29.7 (C-14′), 32.6 (C-1′), 42.7 (C-12′), 50.1 (CH_3–N), 53.3 (C-2), 55.8 (C-6), 69.5 (C-3), 169.3 (C-1″), 208.5 (C-13′); IR (KBr) cm^{-1}: 3338, 1725 (C=O, ester).

4.3.6. One-pot preparation of acetyl hydrochloride 7 from natural spectaline (6). Compound **6** (0.44 mmol) was dissolved in 10 mL of AcOEt and then, 0.6 mL of

concentrated HCl were added. The reaction mixture was stirred for 1 h at room temperature. The solvent was evaporated, the hydrochloride formed was dried under vacuum and dissolved in 8 mL of $CHCl_3$. An excess of acetyl chloride was added, and the reaction was kept at 60 °C overnight. The reaction mixture was concentrated, furnishing the acetyl hydrochloride **7** in 95% yield.

4.4. Pharmacology

4.4.1. Cholinesterase activity assays. Ellman's colorimetric method[9] was adapted for determination of total cholinesterase and butyrylcholinesterase activities in rat brain homogenates. Brain tissue from adult Wistar rats was homogenized at 8% w/v (or 40%, for butyrylcholinesterase) in 0.1 M sodium phosphate buffer, pH 7.4, with added NaCl 58.5 g/L and Triton X-100 0.05% v/v. Aliquots of homogenate (5–20 μL) were incubated with anti-cholinesterase compounds for 10 min in phosphate buffer pH 7.4 before addition of 5,5′-dithiobis(2-nitrobenzoic) acid and either acetylthiocholine iodide or butyrylthiocholine iodide (0.5 or 10 mM final, respectively). The reaction was run at room temperature (22–25 °C) in a final volume of 200 μL in 96-well microplates and was followed at 412 nm for 5 min with a plate reader (SpectraMAX 250, Molecular Devices, USA). In every experiment, cholinesterase-independent (nonspecific) substrate hydrolysis was determined by including one experimental group treated with THA 20 μM; appropriate tissue and reagent blanks were also included. Reaction velocities were determined in three replicates per condition; these were averaged and expressed as percent activity relative to control (solvent), after subtracting the rate of nonspecific hydrolysis. All compounds were tested in nine concentrations from 0.5 to 200 μM. The IC_{50} based on a single-site model was determined by nonlinear regression. Results are reported as mean ± SEM of IC_{50} obtained independently from 2 to 4 animals.

4.5. Behavioral studies

4.5.1. Animals and drugs. The study was performed on 80 adult male Swiss albino mice (25–30 g), and 30 adult three-month old Wistar rats (200–250 g) for the passive avoidance and water maze tests, respectively. Animals were obtained from our facility center following approved guidelines for animal care. Animals were allowed to acclimate to the behavioral experimental room for at least four days before procedures began, in a temperature-controlled environment (20 ± 2 °C), relative humidity, with lights on from 6 a.m. to 6 p.m. (12 h-diurnal light/12 h-dark cycles) and ad libitum access to food and water, 15 per cage for mice and 4 per cage for rats. Injection volumes were 10 mL/kg for mice and 1 mL/kg for rats. THA and (−)-scopolamine hydrobromide were purchased from Sigma Chemical Co. (Saint Louis, MO).

4.5.2. Passive-avoidance test. The methods used were a modification of those outlined by Cumin et al.[16] Briefly, in the first day of testing, mice were individually transferred from plastic cages to the passive avoidance box (20 × 20 cm), where they could freely explore it during 10 s and immediately removed to the cage (acclimation session). After 24 h, animals were injected ip with saline, THA (5.6 mg/kg), compound **7** (1 mg/kg), or compound **9** (1 mg/kg), and after 15 min, saline or scopolamine (1 mg/kg) was injected ip. After 30 min of the scopolamine administration, animals were placed at a wooden platform (4 × 4 × 4 cm) inside the passive avoidance box, and the step-down latency computed when touching a grid floor with their four paws and receiving an aversive stimulus, an electric shock of 0.6 mA/3 s. This session was called training session, and the animals, which had shown a step-down latency superior to 15 s were not included in the experiment. After 24 h of the training session, the pharmacological procedure was repeated with selected mice, and the step-down latency once more obtained (test session).

4.5.3. Spatial memory test. Spatial memory in rats was assessed using an adaptation of the Morris water maze,[17] carried out in the same behavioral room in which the rats were acclimated under the conditions of temperature, humidity, and light cycle described above. A circular pool (180 cm in diameter, 50 cm high) was filled to a depth of 30 cm with opaque water at a temperature of 20 ± 2 °C, and divided into four quadrants of equal area. A platform with 8 cm diameter was placed 1 cm below the water surface, midway between the center and the rim of the pool in one of the quadrants. The rat was placed into the pool facing the rim, and its escape latency, time taken by the rat to find the platform, measured by an observer. If the rat failed to find it within 150 s, the animal was placed on the platform for 20 s and then removed from the pool. The rat was given two trials a day for four days with an inter-trial interval of 15 min. The location of the escape platform remained unchanged, but the point of entry of the rat into the pool was different on each day. During the four days of spatial memory testing, rats were injected ip with saline, THA (1 mg/kg), compound **7** (1 mg/kg), or compound **9** (1 mg/kg), and after 15 min, were treated ip with saline or scopolamine (1 mg/kg). After 30 min of the last ip injection, animals were challenged to localize a hidden escape platform (first trial).

4.5.4. Evaluation of cholinergic side effects and toxicity. THA (**1**), compound **7**, or compound **9** was injected ip in doses up to 10 times higher than those capable of reversing the amnesia in rodents in our study. Animals were placed in novel individual cages, and the presence or absence of signs of cholinergic hyperactivity was observed 10, 30, and 60 min after injection of the cholinesterase inhibitors. At these times, the intensity of whole body tremor and salivation was scored from 0 to 3 (0 = absent; 1 = mild; 2 = moderate; 3 = severe). Splayed hindlimbs, diarrhea, and lacrimation were evaluated as percentage of animals showing those symptoms.

4.5.5. Statistical analysis. Results were expressed as mean ± SEM and differences in means were estimated using ANOVA followed by Dunnet's post-hoc test. Results were considered significant at $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.04.030.

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