Brief Articles

Hexahydrochromeno[4,3-*b*]pyrrole Derivatives as Acetylcholinesterase Inhibitors

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In a search for less flexible analogues of caproctamine (**1**), a diamine diamide endowed with an interesting AChE affinity profile, we discovered compound **2**, in which the terminal 2-methoxybenzyl groups of **1** have been incorporated into a tricyclic system. Since this compound retains good AChE inhibitory activity and its hexahydrochromeno[4,3-*b*]pyrrole moiety is reminiscent of the hexahydropyrrolo[2,3-*b*]indole of physostigmine (**3**), we have designed and synthesized carbamates **4**−**6**, and their biological evaluation has been assessed in vitro against human AChE and BChE. The 6-carbamate **4** was almost as potent as physostigmine and was 60- and 550-fold more potent than the 7-carbamate **5** and the 8-carbamate **6**, respectively. The two enantiomers of **4**, (−)-**4** and (+)-**4**, did not show a marked enantioselectivity. Finally, a similar time-dependent pattern of inhibition of AChE was observed for **3** and **4**.

Introduction

Acetylcholinesterase (AChE) is the enzyme involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses in the central and peripheral nervous systems. Inhibitors of AChE activity promote an increase in the concentration and the duration of action of synaptic ACh, thus causing an enhancement of the cholinergic transmission through activation of the synaptic nicotinic and muscarinic receptors.

In a previous study[1] we were interested in developing noncovalent inhibitors of AChE based on a polyamine backbone for potential use against Alzheimer's disease because this type of inhibitor, through binding at a peripheral anionic site, might affect amyloid formation,[2] which appears to be accelerated by AChE during an early step in the development of the senile plaques. This research led to the discovery of caproctamine (**1**), a diamine diamide endowed with an interesting affinity profile toward AChE.[1] In a related study on polyamines as muscular nicotinic receptor noncompetitive antagonists in order to determine whether flexibility is an important determinant of potency with respect to cholinergic receptors, we designed polyamines with less flexible moieties.[3] Among other modifications, the terminal 2-methoxybenzyl groups of polyamines were incorporated into a hexahydrochromeno[4,3-*b*]pyrrole system to verify whether the spatial relationship of the methoxy moiety relative to the amine function differentially affects affinity for nicotinic and muscarinic receptors. Since the hexahydrochromeno[4,3-*b*]pyrrole moiety is reminiscent of the hexahydropyrrolo[2,3-*b*]-indole system of physostigmine, a classical inhibitor of AChE that is used widely in medicine,[4] it stimulated our interest in designing new physostigmine-related compounds to verify whether the new ring system may act as a suitable molecular scaffold in AChE recognition. To this end, as a preliminary step, we synthesized compound **2** and tested it for AChE activity. Interestingly, it turned out to be as active as benextramine, a tetraamine disulfide which served as the basis for the design of the diamine diamide caproctamine (**1**).[1] This finding suggests clearly that the terminal ring system contributes to the affinity for AChE owing to the observation that polyamines lacking substituents on the terminal nitrogen atoms only very weakly inhibited AChE.[1] Since it was known that removal of the carbamate group from the physostigmine structure leads to a dramatic decrease in the affinity for AChE,[5] we reached the conclusion that the inclusion, at an appropriate position, of a carbamate moiety into the hexahydrochromeno[4,3-*b*]pyrrole system might produce potent AChE inhibitors. To this end, we synthesized carbamates **4**−**6**. Furthermore, the enantiomers of the most potent derivative **4** were investigated to verify the importance, if any, of stereochemistry on the affinity for AChE. The quaternary derivative **7** has been included in this study to verify further the role of a permanent positive charge on affinity.

Chemistry

The design strategy for our compounds is shown in Figure 1. All compounds were synthesized by standard procedures (Scheme 1) and were characterized by IR, ^{1}H NMR, mass spectra, and elemental analysis. Diamine diamide **2** was synthesized as previously reported.[3]

The substituted hexahydrochromeno[4,3-*b*]pyrrole system required for the synthesis of physostigmine-related

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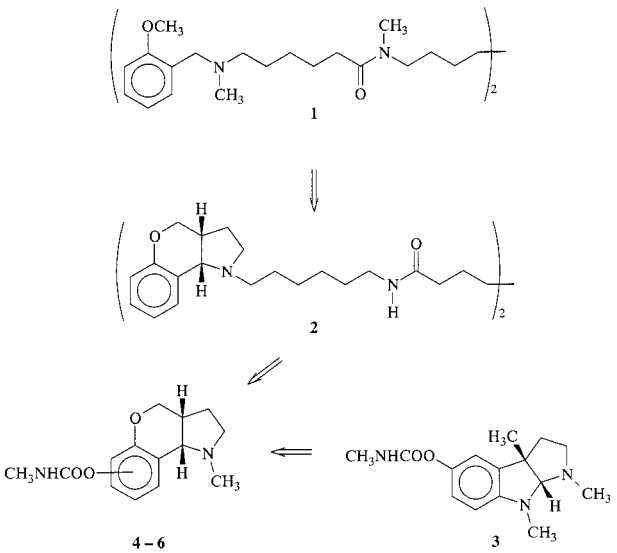
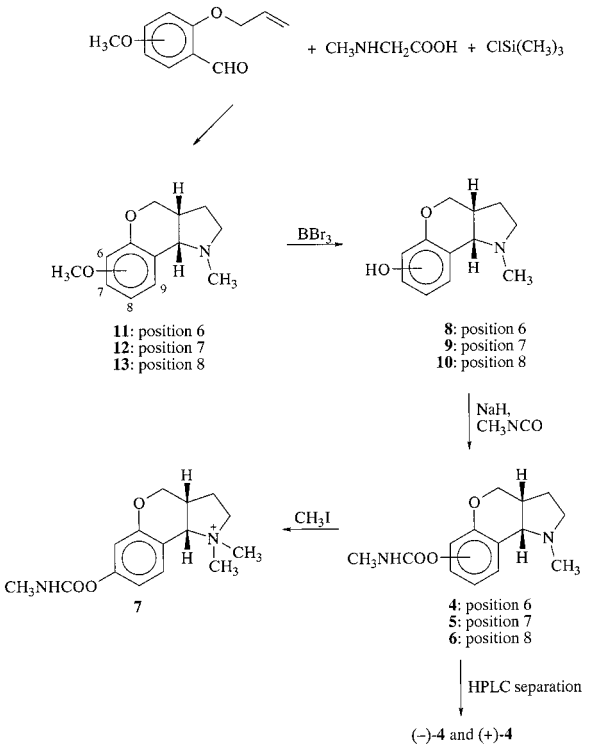


Figure 1. Design strategy for the synthesis of compounds **4**−**6** by replacing the pyrroline ring of physostigmine (**3**) with the dihydropyran ring of **2**.

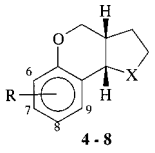
Scheme 1



compounds was synthesized by following an adapted procedure reported for related unsubstituted compounds.[3,6] Thus, starting from *N*-methylglycine and the appropriate substituted 2-allyloxybenzaldehyde, compounds **11**−**13** were obtained, which, upon treatment with BBr_3, afforded phenols **8**−**10**. The carbamate functionality was introduced by reaction of methyl isocyanate with **8**−**10**, affording **4**−**6**. Quaternary derivative **7** was obtained by treatment of **5** with methyl iodide.

The stereochemistry of the ring fusion in compounds **11**−**13**, and as a consequence in the compounds that were obtained starting from them, was deduced from the coupling constant of hydrogens at the ring junction. Thus, in agreement with similar assignments for other

Table 1. Inhibition of AChE and BChE Activities by Physostigmine-Related Compounds



			pIC_{50}[a]	
no.	R	X	AChE	BChE
1			6.77 ± 0.01	4.93 ± 0.04
2			5.01 ± 0.01	6.04 ± 0.05
3			7.75 ± 0.03	7.64 ± 0.02
4	6-$OCONHCH_3$	NCH_3	7.53 ± 0.02	7.23 ± 0.03
(−)-**4**	6-$OCONHCH_3$	NCH_3	7.64 ± 0.03	7.27 ± 0.04
(+)-**4**	6-$OCONHCH_3$	NCH_3	7.13 ± 0.04	6.11 ± 0.06
5	7-$OCONHCH_3$	NCH_3	5.71 ± 0.01	5.45 ± 0.02
6	8-$OCONHCH_3$	NCH_3	4.79 ± 0.01	4.99 ± 0.01
7	7-$OCONHCH_3$	$N^+(CH_3)_2$	5.74 ± 0.01	5.53 ± 0.03
8	6-OH	NCH_3	4.40 ± 0.03	3.10 ± 0.04

[a] AChE and BChE were from human erythrocytes. pIC_{50} values [$-\log IC_{50}$ (μM)] represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements.

hexahydrochromeno[4,3-*b*]pyrroles,[6] a *cis* fusion ring was assigned to **11**−**13** since the benzylic methine proton exhibited a coupling constant in the range 5−7 Hz, which is a typical constant value for a *cis* relationship.

Enantiomers (+)-**4** and (−)-**4** were obtained by HPLC chromatographic resolution of racemic **4**. They were separated in 20 min (t_R = 8.5 and 19 min for (+)-**4** and (−)-**4**, respectively) with a resolution factor of 6.2. The enantiomeric excess (ee) was higher than 99.0% for both enantiomers.

Results and Discussion

Physostigmine (**3**) has been utilized widely in medicine for the treatment of several disorders, including paralytic ileum and atony of the bladder, glaucoma, myasthenia gravis, termination of the actions of competitive neuromuscular nicotinic antagonists, and overcoming intoxication with atropine and other drugs such as tricyclic antidepressants and phenothiazines.[4] More recently, it is being used also in the experimental treatment of Alzheimer's disease.[4] However, **3** suffers from a short half-life, variable bioavailability, and a narrow therapeutic index, which may account for the inconsistent clinical efficacy and for the continuing effort to design more selective and more stable analogues.[4,5,7−15]

We report, herein, the synthesis and initial pharmacological evaluation of compounds **4**−**8** and enantiomers (+)-**4** and (−)-**4** against human AChE and butyrylcholinesterase (BChE), compared to those of caproctamine (**1**), its constrained analogue (**2**), and physostigmine (**3**).

An analysis of the results shown in Table 1 reveals that the position of the carbamate moiety in the phenyl ring is of paramount importance for the anticholinesterase activity. The 6-substituted derivative **4** is 60−550-fold more potent than **5** and **6**, which bear the carbamate group at position 7 and 8, respectively, at both AChE and BChE. Substituted hexahydrochromeno[4,3-*b*]pyrroles **4**−**6** did not discriminate, like physostigmine (**3**), between AChE and BChE. As expected,[4] the quaternary derivative **7** was as active as the parent tertiary amino

Table 2. Values of the Rate Constant k_3 and Stability Constant K_c for **4** and Physostigmine (**3**)

compd	k_3 (min^{-1})	K_c (M)
3	9.0 ± 0.7	$2.5 \pm 0.4 \times 10^{-7}$
4	26.5 ± 1.8	$7.3 \pm 0.5 \times 10^{-7}$

derivative **5**. Similarly, the finding that the phenolic compound **8**, lacking the carbamate function, was more than 3 orders of magnitude less potent than **4** at both AChE and BChE parallels the result observed following removal of the carbamate group from the physostigmine structure.[5]

It is known that inhibition of both AChE and BChE by **3** is highly enantioselective, resting almost exclusively on the 3a*S* enantiomer.[5] However, enantiomers (−)-**4** and (+)-**4** did not follow the same trend as revealed by a comparison of their pIC_{50} values at AChE and BChE. It turned out that (−)-**4** was 3- and 14-fold more potent than enantiomer (+)-**4** at AChE and BChE, respectively. An analysis of Dreiding stereomodels of reference compound **3** (3a*S* enantiomer), enantiomers (−)-**4** and (+)-**4**, and compounds **5** and **6** revealed that only (−)-**4** can be superimposed rather easily to **3**, which might explain why **5** and **6** were significantly less potent than both **3** and (−)-**4**. However, this reasoning does not apply to (+)-**4**, which was only slightly less potent than the other enantiomer. Although the reason for this discrepancy is not clear yet, the low enantioselectivity observed for the enantiomers of **4** is not surprising as other physostigmine derivatives also showed a similar pattern.[5] Interestingly, the NH group of the carbamate function and the nuclear oxygen of the dehydropyran ring of **4** are in a suitable position for the formation of a hydrogen bond, which may have relevance because it could stabilize a conformation close to that required for the interaction with the enzyme. A study is in progress to verify this hypothesis.

As previously reported,[16−18] the inhibition of AChE by **3** involves a reversible complex formation followed by carbamylation of the enzyme, yielding a covalent adduct. The carbamylated enzyme is then hydrolyzed to regenerate the free enzyme according to the following mechanism:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_3} F \xrightarrow{k_5} E + Q$$
$$EI \xrightarrow{} P$$

where E and I are the enzyme and the inhibitor, respectively. The carbamylation phase of the reaction is considerably more rapid than the decarbamylation phase, that is $k_3 > k_5$, and therefore the successive phases can be characterized separately.[18]

To compare the mode of action of **4** to **3**, their equilibrium ($K_c = k_2/k_1$) and rate (k_3) constants were calculated by performing a traditional stopped assay.[17] Inhibitor−AChE complex constant K_c and the rate constant k_3 calculated for **4** were comparable with those found for **3** (Table 2). This would suggest a similar 'time-dependent' pattern of inhibition for the two examined inhibitors. The physostigmine rate constant k_3 value was similar to that obtained with bovine AChE (10.8 ± 0.34 min^{-1}).[16] Similarly, the K_c value calculated for **3** was in agreement with the value reported in the literature.[18]

In conclusion, the most interesting finding of the present investigation is that the hexahydropyrrolo[2,3-*b*]indole moiety of physostigmine (**3**) can be replaced by a hexahydrochromeno[4,3-*b*]pyrrole, as in **4**, without affecting the affinity for AChE. Clearly, compound **4** could form a lead for the development of new AChE inhibitors. However, it remains to determine whether this new scaffold ring system improves the stability of the compound relative to the prototype.

Experimental Section

Chemistry. Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian VXR 200 or 300 instruments, respectively. Although the IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. The mass spectra were obtained on a VG707EH-F spectrometer. The elemental compositions of the compounds agreed to within ±0.4% of the calculated value. When the elemental analysis is not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040−0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063−0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F_{254}) that were visualized in an iodine chamber. The term "dried" refers to the use of anhydrous sodium sulfate.

***cis*-6-Methoxy-1-methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]pyrrole (11).** A mixture of *N*-methylglycine (2.13 g, 23.9 mmol) and chlorotrimethylsilane (30.3 mL, 2.39 mmol) was gently refluxed under nitrogen for 3 h. After cooling, the formed solid was collected by filtration and washed with dry ether. To a suspension of this solid in dry toluene (70 mL) were added diisopropylethylamine (6.56 mL, 37.7 mmol) and 2-allyloxy-3-methoxybenzaldehyde[19] (1.25 g, 6.5 mmol) and the reaction mixture was heated under reflux and the water formed continuously removed for 18 h. Removal of the solvent gave an oil that was partitioned between aqueous $NaHCO_3$−CH_2Cl_2. The organic phase was dried and evaporated to give a residue that was purified by flash chromatography. Eluting with CH_2Cl_2−EtOH (9.7:0.3) afforded **11** as the free base: 84% yield; mp 44−47 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.75−6.85 (m, 3), 4.09−4.15 (m, 1), 3.90 (t, 1), 3.83 (s, 3), 3.03 (dt, 1), 2.90 (d, 1), 2.37−2.42 (s + m, 3 + 2), 1.91−2.09 (m, 1), 1.31−1.52 (m, 1).

***cis*-7-Methoxy-1-methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]pyrrole (12).** It was synthesized from 2-allyloxy-4-methoxybenzaldehyde[19] following the procedure described for **11**: 60% yield; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.10 (d, 1), 6.41−6.53 (m, 2), 3.96−4.06 (m, 1), 3.88 (t, 1), 3.76 (s, 3), 3.08 (dt, 1), 2.87 (d, 1), 2.20−2.46 (s + m, 3 + 2), 1.94−2.13 (m, 1), 1.32−1.51 (m, 1).

***cis*-8-Methoxy-1-methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]pyrrole (13).** It was synthesized from 2-allyloxy-5-methoxybenzaldehyde[19] following the procedure described for **11**: 67% yield; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.67−6.86 (m, 3), 3.91−4.01 (m, 1), 3.85 (t, 1), 3.74 (s, 3), 3.05 (dt, 1), 2.90 (d, 1), 2.38−2.49 (s + m, 3 + 2), 1.95−2.10 (m, 1), 1.40−1.53 (m, 1).

***cis*-1-Methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]-pyrrol-6-ol Hydrochloride (8).** 1 M BBr_3 (4 mL) was added to a stirred and cooled (0 °C) solution of **11** (0.6 g, 2.7 mmol) in anhydrous $CHCl_3$ (17 mL) under a stream of dry nitrogen. When the addition was completed, the reaction mixture was stirred at room temperature for 3 h. After cooling at 0 °C, excess BBr_3 was destroyed by cautious dropwise addition of anhydrous MeOH (16 mL). The resulting mixture was heated at 100 °C for 2 h. Removal of the solvent gave a viscous solid that was taken up in water. The resulting solution was washed twice with ether, made basic with $NaHCO_3$ and then extracted

with CH_2Cl_2 (3 × 25 mL). Removal of dried solvents gave **8** as the free base, which was transformed into the hydrochloride salt: 60% yield; mp 254−260 °C (from 2-PrOH/MeOH); ^{1}H NMR (D_2O, 200 MHz) δ 6.94−7.04 (m, 3), 4.59 (d, 1), 4.12−4.21 (m, 1), 3.92−4.02 (m, 1), 3.56−3.71 (m, 1), 3.25 (q, 1), 2.97−3.14 (s + m, 3 + 1), 2.38−2.58 (m, 1), 1.90−2.11 (m, 1). Anal. ($C_{12}H_{16}ClNO_2$) C, H, N.

***cis*-1-Methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]-pyrrol-7-ol Hydrochloride (9).** It was synthesized from **12** following the procedure described for **8**: yield 60%; mp 249−254 °C (from 2-PrOH/MeOH); ^{1}H NMR (D_2O, 300 MHz) δ 7.31 (d, 1), 6.61 (dd, 1), 6.45−6.47 (m, 1), 4.49 (d, 1), 4.09−4.15 (m, 1), 3.83−3.90 (m, 1), 3.58−3.70 (m, 1), 3.25 (q, 1), 2.92−3.06 (s + m, 3 + 1), 2.39−2.52 (m, 1), 1.88−2.01 (m, 1).

***cis*-1-Methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]-pyrrol-8-ol Hydrochloride (10).** It was synthesized from **13** following the procedure described for **8**: 50% yield; mp 228−233 °C (2-PrOH/MeOH); ^{1}H NMR (D_2O, 200 MHz) δ 6.86−6.93 (m, 3), 4.53 (d, 1), 3.81−4.09 (m, 2), 3.54−3.69 (m, 1), 3.23 (q, 1), 2.92−3.10 (s + m, 3 + 1), 2.33−2.54 (m, 1), 1.87−2.10 (m, 1).

***cis*-1-Methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]-pyrrol-6-yl *N*-Methylcarbamate Hydrochloride (4).** MeNCO (0.02 mL) was added to a stirred mixture of **8** (0.09 g, 0.44 mmol) and NaH (5 mg, 0.22 mmol) in anhydrous THF (5 mL). Stirring was continued for 20 h at room temperature, and then removal of the solvent gave a residue that was transformed into the hydrochloride salt: 80% yield; mp 237−240 °C (from EtOH/ether); EI-MS m/z = 262 (M^+); ^{1}H NMR (free base, $CDCl_3$, 200 MHz) δ 7.04 (t, 2), 6.85−6.90 (m, 1), 5.20 (br s, exchangeable with D_2O, 1), 4.07 (dd, 1), 3.89 (t, 1), 3.07 (dt, 1), 2.95 (d, 1), 2.85 (d, 3), 2.34−2.42 (m + s, 1 + 3), 2.04 (q, 1) 1.93−2.13 (m, 1), 1.36−1.52 (m, 1). Anal. ($C_{14}H_{19}ClN_2O_3$) C, H, N.

***cis*-1-Methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]-pyrrol-7-yl *N*-Methylcarbamate Oxalate (5).** It was synthesized from **9** following the procedure described for **4**. The free base was transformed into the oxalate salt: 85% yield; mp 128−133 °C (from EtOH/ether); EI-MS m/z = 262 (M^+); ^{1}H NMR (free base, $CDCl_3$, 200 MHz) δ 7.15 (d, 1), 6.62−6.75 (m, 2), 5.06 (br s, exchangeable with D_2O, 1), 4.02 (dd, 1), 3.88 (t, 1), 3.08 (dt, 1), 2.81−2.92 (s + d, 3 + 1), 2.48−2.35 (s + m, 3 + 1), 2.26 (q, 1), 1.85−2.12 (m, 1), 1.32−1.5 2 (m, 1). Anal. ($C_{16}H_{20}N_2O_7$) C, H, N.

***cis*-1-Methyl-1,2,3,3a,4,9b-hexahydrochromeno[4,3-*b*]-pyrrol-8-yl *N*-Methylcarbamate Oxalate (6).** It was synthesized from **10** following the procedure described for **4**. The free base was transformed into the oxalate salt: 90% yield; mp 186−191 °C (from EtOH/ether); EI-MS m/z = 262 (M^+); ^{1}H NMR (free base,$CDCl_3$, 200 MHz) δ 6.83−6.97 (m, 3), 4.98 (br s, exchangeable with D_2O, 1), 4.0 (dd, 1), 3.84 (t, 1), 3.08 (dt, 1), 2.93 (d, 1), 2.87 (d, 3), 2.32−2.49 (s + m, 3 + 1), 2.30 (q, 1), 1.96−2.12 (m, 1), 1.35−1.54 (m, 1). Anal. ($C_{16}H_{20}N_2O_7$) C, H, N.

***cis*-1,1-Dimethyl-7-{{(methylamino)carbonyl}oxy}-1,2,3,-3a,4,9b-hexahydrochromeno[4,3-*b*]pyrrol-1-ium Iodide (7).** A solution of **5** (0.04 g, 0.15 mmol) and methyl iodide (0.093 mL) in acetone (7 mL) was left in the dark at 5 °C for 10 days, and then the solid was filtered and crystallized: 94% yield; mp 182−187 °C (from EtOH); ^{1}H NMR (D_2O, 200 MHz) δ 7.49 (d, 1), 6.81−6.96 (m, 2), 4.98 (d, 1), 4.05 (dq, 2) 3.58−3.69 (m, 2), 3.17−3.32 (s + m, 3 + 1), 2.75 (s, 3), 2.51−2.62 (s + m, 3 + 1), 2.20−2.38 (m, 1). Anal. ($C_{15}H_{21}IN_2O_3$) C, H, N.

HPLC Separation of (+)-4 and (−)-4. The enantioselective separation and the determination of the enantiomeric excess (ee) of enantiomers (+)-**4** and (−)-**4** were performed on a Jasco HPLC system consisting of a Jasco PU-980 solvent delivery system equipped with a Reodyne model 7125 injector with a 20-μL sample loop. The eluents were monitored by a Jasco MD 910 diode array detector set at 260 nm and connected to a computer station. The chromatographic column was a Chiracel OD (250 × 4.6 mm i.d.) (Daicel Chemical Industries, Ltd.). The mobile phase composition was 2-propanol−hexane 20:80 (v/v) and the flow rate was 1 mL min^{-1}.

The single enantiomers (+)-**4** and (−)-**4** were obtained by chromatographic resolution of racemic **4** using the same condition as for the analytical separation. Low-loading repetitive injections (60 μg each injection, one injection every 25 min) allowed the collection of about 1 mg of each enantiomeric fraction in about 15 h. All solvents used for the preparation of the mobile phases were HPLC grade from Merck.

Inhibition of AChE and BChE. The method of Ellmann et al. was followed.[20] Five different concentrations of each compound were used in order to obtain inhibition of AChE or BChE activity comprised between 20% and 80%. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5′-dithiobis(2-nitrobenzoic acid), 0.035 unit/mL AChE or BChE derived from human erythrocytes (0.5 and 3.4 UI/mg, respectively; Sigma Chemical), and 550 μM acetylthiocoline iodide. Test compounds were added to the assay solution and preincubated with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE or BChE in order to account for nonenzymatic reaction. The reaction rates were compared, and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration−inhibition curves.

Kinetic Characterization of AChE Inhibition. The stopped time assay was performed,[17] in which AChE and inhibitors physostigmine and **4** at five concentrations comprised between 10 and 200 nM were mixed in the assay buffer pH 8.0, and at specific incubation time intervals at 37 °C, the determination of residual activity of the AChE-catalyzed hydrolysis of acetylthiocholine was followed spectrophotometrically at 412 nm. Each concentration was analyzed in triplicate. A parallel control, that is with no inhibitor in the mixture, allowed to adjust activities measured at various times. k_3 and K_c values were calculated as the mean of two independent measurements.

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