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New cholinesterase inhibitors: synthesis and structure–activity relationship studies of 1,2-benzisoxazole series and novel imidazolyl-d^2-isoxazolines†

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ABSTRACT: The syntheses of a series of 3-(4-substituted-1-piperidinyl)-6-halo-1,2-benzisoxazole hydrochlorides (**5a–b**, **6a–b** and **7a–b**) and 3-(2-butyl-4-chloro-1*H*-imidazolyl)-substituted-d^2-isoxazolines (**10c–i**) by novel methods are described. The inhibitory activity of acetylcholinesterase (AChE) for the newly synthesized compounds against targets from different species, such as pure electric eel AChE, human serum AChE and rat brain AChE, was studied using Ellman *et al*.'s method. The benzisoxazole heterocycle was found to be an appropriate bioisosteric replacement for the benzyl functionality present in the *N*-benzylpiperidine class of inhibitors. Structure–activity relationships were studied by comparing the basicities of the different substituted heterocyclic ring systems at the C-3 position of the 1,2-benzisoxazoles derivatives. Maximum cholinesterase enzyme inhibition was revealed when there was a 6-fluoro substituent on the 1,2-benzisoxazole ring. The 1-morpholine hydrochloride substituent appeared less significant, although in most cases **5a**, **6a** and **10c** evoked maximum potency compared with the existing drug neostigmine. The most potent cholinesterase compound was found to be 1-[2-{6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole}ethyl]piperidine monohydrochloride (**5a**) by *in vitro* studies. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: 1,2-benzisoxazoles; isoxazolines; cholinesterases; neostigmine; Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that is the most common cause of dementia among the elderly. Neuropathological evidence has demonstrated that cholinergic functions declined in the basal forebrain and cortex in senile dementia of the Alzheimer type.[1] Accordingly, enhancement of cholinergic neurotransmission has been considered as one potential therapeutic approach against AD and also inhibition of acetylcholinesterase (AChE) enzyme is routinely employed for an increasing variety of clinical applications such as muscle relaxants and prophylactics in protection against nerve agent attack. One treatment strategy to enhance cholinergic functions is the use of AChE (EC 3.1.1.7) inhibitors to increase the amount of acetylcholine present in the synapses between cholinergic neurons.[2,3]

The first available treatment for AD was THA (1,2,3,4-tetrahydro-9-aminoacridine), which demonstrated moderate but significant efficacy in AD[4] but suffers from dose-limiting hepatotoxic effects.[5] Physostigmine, a carbamate-type inhibitor, is currently in phase III trials by Forest Laboratories. The third class of novel AChE inhibitors is *N*-benzyl piperidines and 1,2-benzisoxazoles, which have been demonstrated to be efficient both *in vitro* and *in vivo* and exhibited minimal side-effects, which has been confirmed by clinical studies.[6]

We report here the synthesis and the AChE enzyme inhibitory activity against various targets of a series of 3-(4-substituted-1-piperidinyl)-6-halo-1,2-benzisoxazole hydrochlorides[7] and 3-(-2-butyl-4-chloro-1*H*-imidazolyl)-substituted-d^2-isoxazolines.[8]

RESULTS AND DISCUSSION

Compounds

The synthesis of the 1,2-benzisoxazole series began with the synthesis of *N*-formylated piperidine-substituted benzoyls (**2a**,**b**) via Friedel–Crafts acylation by using isonipecotic acid **1**. 1,2-Benzisoxazoles such as 6-fluoro(or chloro)-3-(4-piperidinyl)-1,2-benzisoxazole hydrochlorides (**4a**,**b**) was obtained in one step from the key intermediate (2,4-difluoro(or chloro)phenyl)(1-formyl-4-piperidinyl)methanone (**3a**,**b**) (Table 1). This reaction

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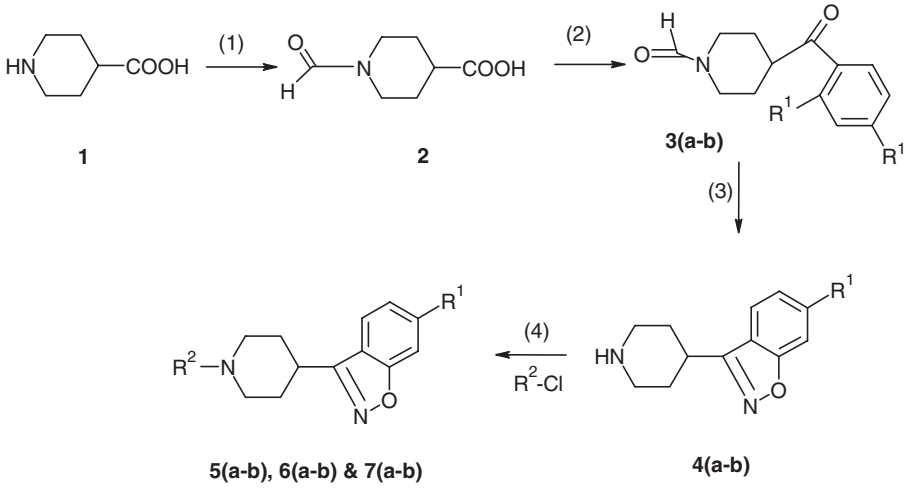
Table 1. Physical data for compounds **3a**,**b** and **4a**,**b**

Compound	R^{1} [a]	M.p. (°C)	Compound	R^{1} [a]	M.p. (°C)
3a: 4-(4-Fluorobenzoyl)-piperidine-1-carbaldehyde	2,4-Difluoro	64–66	**4a**: 6-Fluoro-3-piperidin-4yl-benzo[*d*]isoxazole	6-Fluoro	302–306
3b: 4-(4-Choro-benzoyl)-piperidine-1-carbaldehyde	2,4-Dichloro	43–45	**4b**: 6-Chloro-3-piperidin-4yl-benzo[*d*]isoxazole	6-Chloro	180–184

[a] See Scheme 1.



Where [a]R^{1} = F, **3a**–**7a**; [b]R^{1} = Cl, **3b**–**7b**; R^{2} = ethyl-1-piperidine, **5**(**a**–**b**); R^{2} = ethyl-1-pyrrolidine, **6**(**a**–**b**) and R^{2} = ethyl-1-morpholine **7**(**a**–**b**).

Reaction conditions: (1) AC_2O, HCOOH, IPA; (2) $SOCl_2$, methylene dichloride, DMF, $AlCl_3$, 1,3-difluorobenzene; (3) $(NH_2OH)_2.H_2SO_4$, KOH, MeOH/H_2O (4) K_2CO_3/DMF

Scheme 1

involves hydroxylamine sulfate/powdered potassium hydroxide-mediated oxime formation and subsequent internal cyclization followed by alkaline hydrolysis of the protected piperidinyl group simultaneously, which is reported for the first time. The target compounds **6a**,**b**, **7a**,**b**, and **8a**,**b** were synthesized via nucleophilic substitution reactions with different alkyl halides (Scheme 1 and Table 2) by using potassium carbonate as a base and *N*,*N*-dimethylformamide as a solvent.

3-(-2-Butyl-4-chloro-1*H*-imidazolyl)substituted-d^{2}-isoxazolines (**10c**–**i**) were obtained from the 2-butyl-5-chloro-3*H*-imidazolyl-4-carbaldehyde[10] through 1,3-dipolar cycloaddition reactions[9,11] of aldoxime[12] with the monosubstituted alkenes as shown in Scheme 2. The target molecules were synthesized by trapping the respective nitryl oxide in presence of a monosubstituted dipolarophile by using sodium hypochlorite as an oxidant, which is a high-yield solution-phase synthetic process.

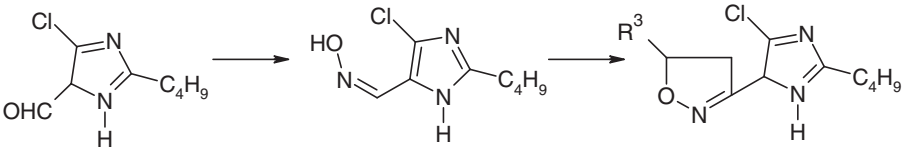
Biology and structure–activity relationship (SAR)

The inhibitory activities of the newly synthesized compounds against AChE was studied using the method of Ellman *et al.*[13] to determine the rate of hydrolysis of acetylthiocholine (ATCh) in the presence of the inhibitor, against different sources such as electric eel AChE, human serum AChE and rat brain homogenate AChE, as shown in Figs 1, 2 and 3, respectively.

Table 2. Physical data for compounds **5a**,**b**, **6a**,**b** and **7a**,**b**

Compound	R^{1} [a]	R^{2} [a]	M.p. (°C)
5a: 6-Fluoro-3-[1-(2-piperidin-1-ylethyl)piperidin-4-yl]benzo[*d*]isoxazole	6-F	1-Piperidinylethane	242–244
6a: 6-Fluoro-3-[1-(2-pyrrolidin-1-ylethyl)piperidin-4-yl]benzo[*d*]isoxazole	6-F	1-Pyrrolidinylethane	238–240
7a: 6-Fluoro-3-[1-(2-morpholin-4-ylethyl)piperidin-4-yl]benzo[*d*]isoxazole	6-F	4-Morpholinylethane	204–206
5b: 6-Chloro-3-[1-(2-piperidin-1-ylethyl)piperidin-4-yl]benzo[*d*]isoxazole	6-Cl	1-piperidinylethane	162–164
6b: 6-Choro-3-[1-(2-morpholin-4-ylethyl)piperidin-4-yl]benzo[*d*]isoxazole	6-Cl	1-Pyrrolidinylethane	191–193
7b: 6-Chloro-3-[1-(2-morpholin-4-ylethyl)piperidin-4-yl]benzo[*d*]isoxazole	6-Cl	4-Morpholinylethane	204–205

[a] See Scheme 1.



8 **9** **10 c-i**

Where **10c**, $R^3 = —CN$; **10d**, $R^3 = —C_6H_5$; **10e**, $R^3 = —COOC_6H_5$; **10f**, $R^3 = —COOC_2H_5$; **10g**, $R^3 = —CH_2COOCH_3$; **10h**, $R^3 = —CH_2OH$; **10i**, $R^3 = —COOCH_3$

Scheme 2

Activities of the synthesized compounds were compared with the inhibitory activity shown by the known standard inhibitor neostigmine. According to Boudan *et al*.'s calculations,[14] the 6-fluoro-3-substituted-1,2-benzisoxazoles are active compounds ($IC_{50} = 6.7$ μM) and Tong *et al*.[15] found that the presence of bulky groups and/or lipophilic groups at the benzisoxazole moiety would enhance the inhibitory effect on AChE. Also, changing the length of the chain that connects the piperidine to the benzisoxazole substituent has been shown to have a profound effect on the potency. The optimal length is an ethyl spacer (**5**, **6** and **7** series) of the 4-ethylpiperidine fragment, which is ideally situated, and the nature of the spacer will control the positioning of the benzisoxazole within the gorge and hence the extent of contact with the protein. The experimental results reveal that the possible structure–activity relationship of 1,2-benzisoxazoles exhibits profound inhibition on AChE when there is a strongly basic nitrogen-containing heterocyclic cation (ethylpyperidine) present on the 6-fluoro-3-(4-pyperidinyl)-1,2-benzisoxazole ring. It is noted that a fluorine atom present at the 6-position on the benzisoxazole ring resulted in better activity than a chlorine atom. The order of basicity and the potency for the different substituted heterocyclic rings are piperidine > pyrrolidine > morpholine, which imparts inhibition accordingly when the respective ring is present at the C-3 position of the 3-pyperidinyl-1,2-benzisoxazoles. The compounds **5a** ($IC_{50} = 1.5$, 2.22, 1.29 μM), **6a** ($IC_{50} = 2.51$, 3.29, 5.3 μM) and **10c** ($IC_{50} = 2$, 1.9, 4.85 μM) possess strong inhibition against AChE from different sources: electric eel, human serum and rat brain homogenate, respectively. Compounds **5b** ($IC_{50} = 9.4$ μM) and **10h** ($IC_{50} = 21$ μM) showed species specificity by moderately inhibiting electric eel AChE, but showed inhibition only at high concentrations against human serum and rat brain homogenate AChE (Table 3). Compounds **6b**, **7a** and **7b** did not show significant inhibitory activity or a species-specific mode of action against any

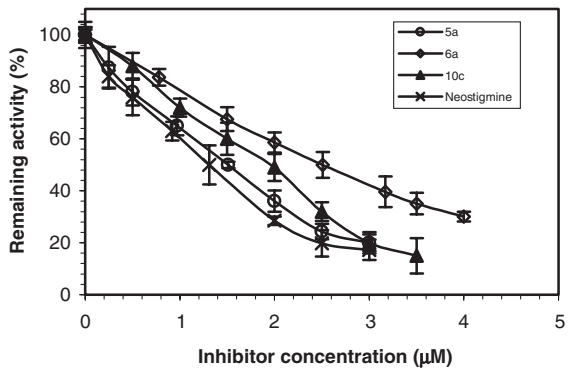


Figure 1. Concentration-dependent inhibition of electrical eel AChE by **5a**, **6a** and **10c** and neostigmine

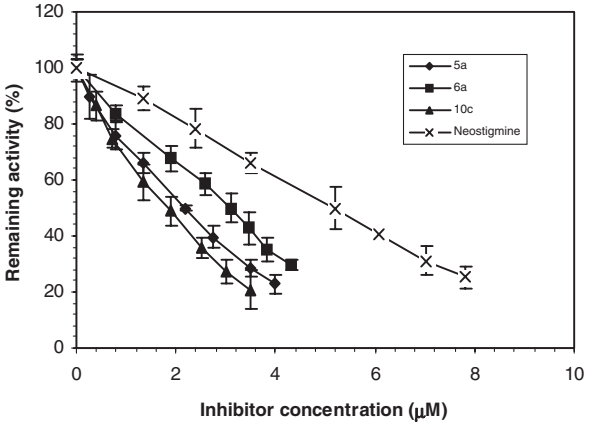


Figure 2. Concentration-dependent inhibition of human serum AChE by **5a**, **6a** and **10c** and neostigmine

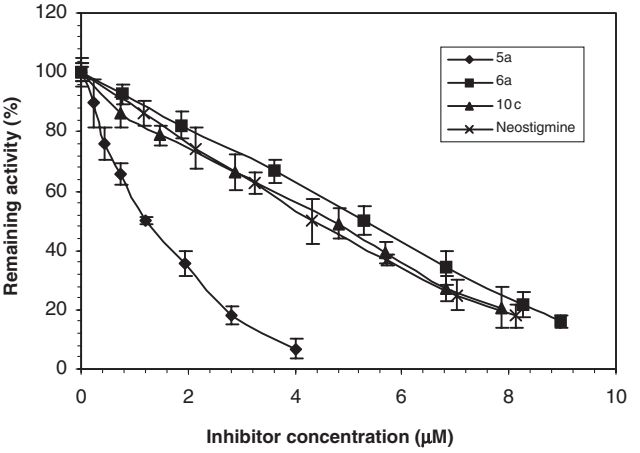


Figure 3. Concentration-dependent inhibition of rat brain homogenate AChE by **5a**, **6a** and **10c** and neostigmine

Table 3. Comparative inhibitory activities shown by the synthesized inhibitors against AChE from different sources

Compound	IC_{50} (μM)[a]		
	Electric eel	Human serum	Rat brain homogenate
5a	1.57 ± 0.06	2.22 ± 0.11	1.29 ± 0.03
6a	2.51 ± 0.09	3.29 ± 0.11	5.3 ± 0.21
7a	68.47 ± 2.9	293.1 ± 10	75.4 ± 3.0
5b	9.44 ± 0.37	1259 ± 52	1634 ± 75
6b	397.62 ± 17	1132 ± 46	675 ± 29
7b	603.18 ± 26	981.1 ± 39	>1000 ± 40
10c	2 ± 0.08	1.9 ± 0.06	4.85 ± 0.14
10d	131 ± 5.5	165 ± 7.2	114 ± 4.7
10e	72.1 ± 2.6	81 ± 2.5	156 ± 6.2
10f	69.73 ± 2.4	54.6 ± 2.0	771 ± 28.5
10g	160.4 ± 6	182.3 ± 6	543 ± 17.1
10h	21 ± 0.8	34.9 ± 1.2	62 ± 1.1
10i	241 ± 9	217 ± 8	341 ± 11
Neostigmine	1.39 ± 0.03	5.27 ± 0.15	4.3 ± 0.15

[a] Values are means of three determinations, the ranges of which were less than 5% of the mean in all cases.

of the cholinesterases from different sources (Table 3). Hence these results indicate that the benzisoxazole heterocycle is an appropriate bioisosteric replacement for the benzyl functionality present in the *N*-benzylpiperidine class of inhibitors (Fig. 4).

Among the imidazolyl-substituted isoxazolines, 3-(-2-butyl-4-chloro-1*H*-imidazolyl)-substituted-d^2-isoxazolines **10c**–**i**, the compound **10c** was found to be a better inhibitor against all the three cholinesterases tested compared with standard neostigmine (Table 3), possibly owing to the presence of nitrile at the isoxazoline position, and **10h** exhibited moderate inhibition (Table 3), possibly owing to presence of a hydroxymethyl group on the isoxazoline ring.

The other compounds (**10d**–**g** and **i**) had an insignificant effect, showing higher IC_{50} values, possibly owing to the presence of hydrolysable groups.

CONCLUSION

The benzisoxazole **5a** was found to be a potent inhibitor and **6a** and **10c** were found to be selective and significant inhibitors of AChE *in vitro* (Fig. 1). This study reveals that the synthesized compounds **5a**, **6a** and **10c** showed significant inhibition against cholinesterases from different sources. Therefore, they could be used as biochemical tools for the development of new compounds for treating AD.

EXPERIMENTAL

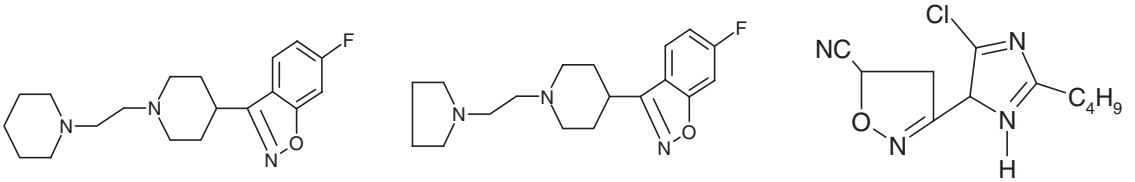
Compounds

Melting-points were determined on a SELACO-650 hot-stage apparatus and are uncorrected. IR (Nujol) spectra were measured on a Shimadzu 8300 IR spectrophotometer. ^{1}H NMR spectra were recorded on a Shimadzu AMX 400 MHz spectrophotometer by using $CDCl_3$ as solvent and TMS as an internal standard (chemical shift δ in ppm). Elemental analyses were obtained on a Vario-EL instrument. TLC was conducted on 0.25 mm silica gel $60F_{254}$ plates (Merck) and column chromatography was carried out on 60–120 mesh silica gel. All extracted solvents were dried over Na_2SO_4, followed by evaporation *in vacuo*.

Compounds **3a**, **4a**, **5a**, **6a** and **7a**. Syntheses of these compounds have been reported previously.[7]

4-(2,4-Dichlorobenzoyl)piperidine-1-carbaldehyde (**3b**). Compound **3b** was obtained by reaction of *N*-formylisonipecotic acid (50 g, 0.318 mol) (**2**), 1,3-dichlorobenzene (46.74 g, 0.382 mol), thionyl chloride (50 ml), aluminium chloride (74.20 g) and 2–3 drops of DMF following our reported procedure;[7] yield, 32.5 g (65%). IR: 1715, 1621, cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.15 (s, 1H, NCHO), 1.72 (q, 4H, CH_2), 3.1 (m, 1H, CH CO), 2.6–2.8 (t, 4H, CH_2N), 7.6 (d, 1H, ArH), 7.3 (s, 1H, ArH), 7.45 (dd, 1H, ArH). Anal. Calculated for $C_{13}H_{13}C_{l2}N_2O_2$: C, 54.56; H, 4.58; N, 4.89. Found: C, 54.29; H, 4.69; N, 4.74%.

4-(6-Chloro-benzo[d]isoxazol-3-yl)piperidiniuim chloride (**4b**). Compound **4b** was obtained by reaction of **3b** (25 g, 0.0876 mol), hydroxylamine hydrochloride (9.13 g) and sodium acetate (21.55 g) in 100 ml of methanol by following our reported method;[7] yield, 18 g (72%). IR: 1484 cm^{-1}. ^{1}H NMR (D_2O): δ (ppm): 1.84 (q, 4H), 2.65 (t, 4H) 2.79 (m, 1H), 3.45 (s, NH), 7.3 (t, 1H, ArH), 7.19 (d, 1H, ArH), 7.58 (dd, 1H, ArH). Anal. Calculated for $C_{12}H_{14}C_{l2}N_2O_4$: C, 52.76; H, 5.17; N, 10.26. Found: C, 52.48; H, 7.3; N, 10.39%.



5a **6b** **10 c**

Figure 4. The most significant molecules screened from both series

1-{2-[4-(6-Chlorobenzo[d]isoxazol-3-yl)-piperidin-1-yl]ethyl}-piperidinium chloride (**5b**). A finely powdered compound (6.4 g) was obtained from **4b** (5 g, 0.0183 mol), 1-(2-chloroethyl)piperidinium chloride (3.36 g) and powdered K_2CO_3 (7.58 g) by following our reported procedure.[7] IR: 2995, 1632, 3320 cm^{-1}. ^1H NMR (D_2O): δ (ppm) 1.5–1.65 (q, 2H), 1.8–2.1 (m, 4H), 2.2–2.5 (t, 4H), 3.1 (s, 1H), 3.4–3.7 (d, 2H), 4.4–4.8 [s, $N(CH_2)_3$], 7.15 (t, 1H, ArH), 7.29 (d, 1H, ArH), 7.43 (q, 1H, ArH) ^{13}C NMR (D_2O, 100 MHz): δ (ppm) 27.1, 27.6, 32.14, 34.2, 34.8, 36.2, 50.4 (2), 51.8, 55.3, 57.45, 96.4, 121.8, 120.4, 123.9, 125.24, 142, 149, 165.2. Anal. Calculated for $C_{19}H_{27}Cl_2N_3O$: C, 59.37; H, 7.08; N, 10.93. Found: C, 59.20; H, 6.95; N, 10.76%.

1-{2-[4-(6-Chlorobenzo[d]isoxazol-3-yl)piperidin-1-yl]ethyl}-pyrrolidinium chloride (**6b**). Compound **6b** was obtained from **4b** (5 g, 0.0183 mol), 1-(2-chloroethyl)pyrrolidine hydrochloride (3.11 g) and K_2CO_3 (7.58 g) by following our reported method;[7] yield 4.1 g yield (82%). IR: 2155, 1624, 1438 cm^{-1}. ^1H NMR (D_2O): δ (ppm) 1.7–1.82 (m, 8H), 2.0–2.2 [d, 4H, $N(CH_2)$], 2.8–3.1 (dd, 4H, CH_2), 2.75 (q, 1H, CH) 04.3 [s, 4H, $N(CH_2)_2$], 7.23 (t, 1H, ArH), 7.15 (d, 1H, ArH), 7.46 (q, 1H, ArH). ^{13}C NMR (D_2O, 100 MHz): δ (ppm) 26, 30.39, 33.9, 51.5, 54.54, 56.19, 58.37, 100.4, 100.77, 116.40, 116.6, 126.3, 126.51. Anal. Calculated for $C_{18}H_{25}Cl_2N_3O$: C, 58.38; H, 6.80; N, 11.35. Found: C, 58.43; H, 6.72; N, 11.28%.

4-{2-[4-(6-Chlorobenzo[d]isoxazol-3-yl)piperidin-1-yl]ethyl}-morpholinium chloride (**7b**). Compound **7b** was obtained from **4b** (5 g, 0.0183 mol), 4-(2-chloroethyl)morpholine hydrochloride (3.4 g) and K_2CO_3 (7.58 g); yield: 3.5 g (70%). IR: 2148, 1626, 1442 cm^{-1}. ^1H NMR (D_2O): δ (ppm): 2.2 (q, 4H, CH_2), 2.46 (d, 4H, CH_2), 3.2 [t, 4H, $N(CH_2)_2$], 3.7 (quintet, 1H, CH), 4.0 (m, 4H, OCH_2), 4.6 [s, 2H, $(NCH_2)_2$], 7.34 (t, 1H, ArH), 7.29 (d, 1H, ArH), 7.56 (q, 2H, ArH). ^{13}C NMR (D_2O, 100 MHz): δ (ppm) 30.3, 33.67, 53.2, 53.6, 55.9, 67.1 (2), 100.5, 116.5, 116.7, 119.8, 126.37, 126.62, 163.3, 166.59, 169.12. Anal. Calculated for $C_{18}H_{25}Cl_2N_3O_2$: C, 55.96; H, 6.52; N, 10.88; Found: C, 55.87; H, 6.62; N, 10.91%.

Compounds **10c–i**. Syntheses of these compounds have been reported previously.[8]

Biology

Materials. Electric eel AChE (Type-VI-S), acetylthiocholine iodide, tetraisopropylpyrophosphoramide (*iso*-OMPA) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma. Crude human AChE was prepared by mixing 9 ml of fresh blood (collected from healthy volunteers by vein puncture) with 1 ml of 3.8% (w/v) trisodium citrate and centrifuging at 3000 rpm at 0 °C for 20 min. The supernatant was used as a source of AChE. Rat brain homogenate was obtained as follows: a male rat was killed by decapitation and the whole brain removed and placed on ice. The cerebellum was excised and, if relevant, the brain could be stored for at most 2 months at −70 °C following thawing, if relevant the brain being weighed and homogenized in 10 volumes of ice-cold sucrose solution using a Potter–Elvehsen homogenizer (10 strokes up and down at 1000 rpm). The centrifugate was centrifuged at 10 000 N kg^{-1} for 10 min at 4 °C using Centrium centrifuge. The supernatant was separated and the residual pellet discarded. The supernatant was homogenized for 5 min in a Polytron homogenizer (setting: half of the maximum speed) and 1 ml samples were collected. These samples are stable for 2 months at −70 °C. Just before use, each sample was allowed to thaw and made up to 4 ml with ice-cold sucrose solution, resulting in a homogenate containing 25 mg ml^{-1} of wet tissue.

Method. Cholinesterase activity was determined by the method of Ellman *et al*.[13] The activity of AChE was expressed as micromoles of acetylthiocholine metabolized per minute per milligram of protein. Protein content was estimated by the method of Lowry *et al*. A typical enzyme assay comprised 25 µl of enzyme, 100 µM *iso*-OMPA and 10 µl of inhibitor sample dissolved in a suitable solvent and 0.1 M sodium phosphate buffer (pH 8.0), to make a total volume of 3 ml. To this enzyme–inhibitor mixture, after incubation at 27 °C for 1 min, 0.5 mM DTNB reagent was added and the enzyme reaction was initiated by addition of 0.5 mM acetylthiocholine iodide (to give an increase in absorbance of 0.6 units at the end of 3 min) and the increase in absorbance at 412 nm was recorded. All assays were carried out in duplicate and mean values are reported here. The relative activity was expressed as the percentage ratio of enzyme activity in the presence of inhibitor to enzyme activity in the absence of inhibitor at the end of 3 min of enzyme reaction.

Inhibitor concentration causing 50% inhibition of enzyme activity (IC_{50}) values. AChE was studied in both the presence and absence of different concentrations of compounds and the percentage inhibition of enzyme activity was calculated. The IC_{50} was determined from a graph of percentage inhibition versus concentration of inhibitor. For comparison, the IC_{50} value was obtained with a non-specific inhibitor of cholinesterases, neostigmine. Inhibitor solutions were prepared using methanol or DMSO as solvent, and the concentration of any of the solvents used in the present study had no influence on AChE activity.

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