New Chemical Derivatives of the Natural Compound Dictyophlebine Inhibiting Acetyl- and Butyrylcholinesterase

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The acetyl- and butyrylcholinesterase inhibiting natural product dictyophlebine **1** was subjected to different *N*-alkylation and hydrochlorination reactions by which five new and bioactive chemical derivatives (**2** – **6**) with pentyl, pent-4-en-1-yl, hex-5-en-1-yl, 4-chloropentyl and 5-chlorohexyl substituents at the 3-*N* position were obtained with high yield. The alkylated and chlorinated products **3** – **6** were found to have significantly higher inhibitory potential towards cholinesterase than the parent compound **1**.

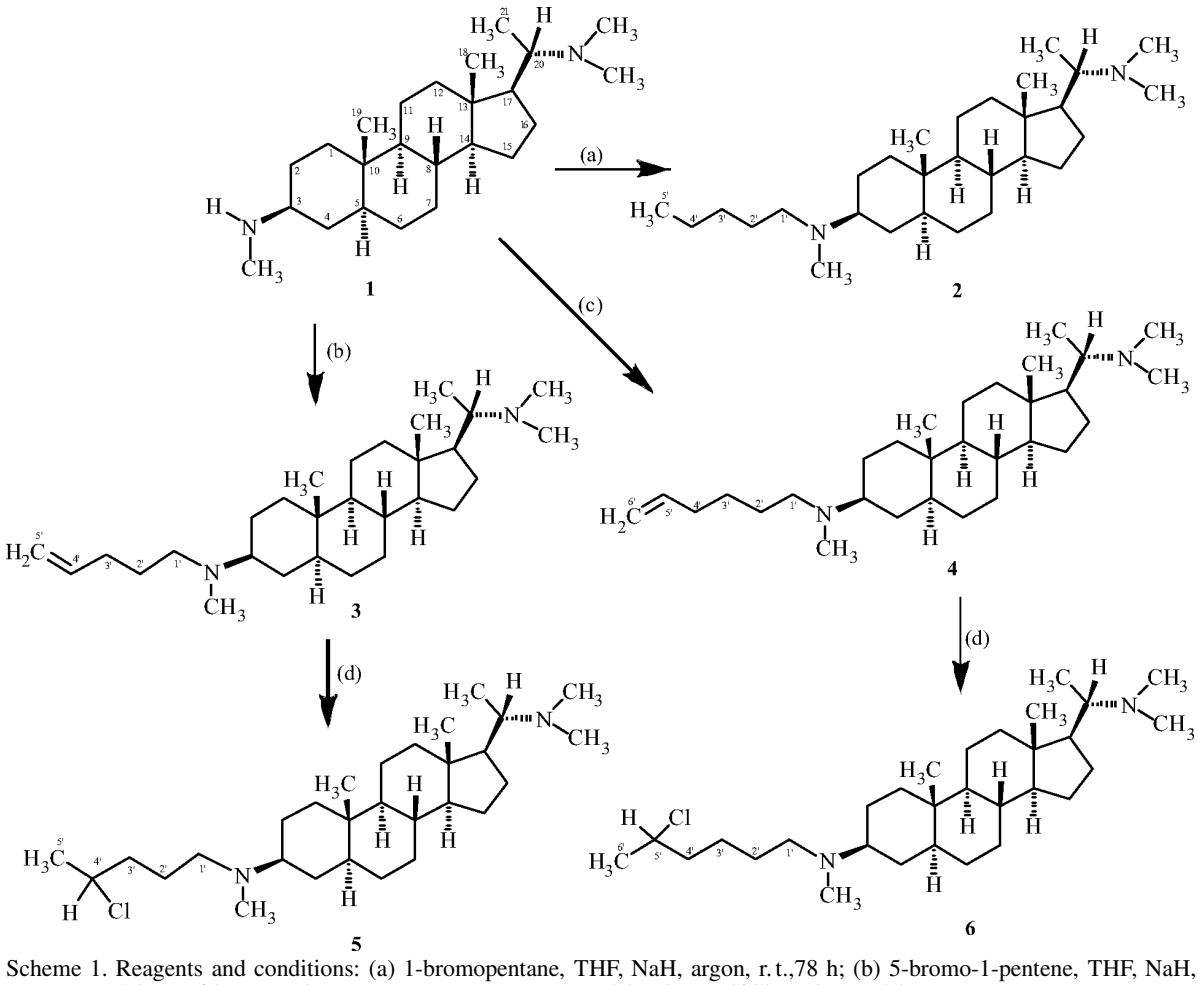
Key words: Dictyophlebine, Acetyl- and Butyrylcholinesterase, Chemical Derivatives, *N*-Alkylation, Hydrochlorination

Introduction

Cholinesterases (ChE) form an important class of enzymes intimately connected with the nervous system. Acetylcholine (ACh) was first discovered in 1867 as a synthetic compound and later on detected in the adrenal gland of human tissue in 1906 as a neurotransmitter, which transmits signals from one nerve cell to another [1]. ACh is hydrolyzed by the enzymes ChE into choline and acetic acid and thus inactivated. Any compound that inhibits cholinesterase activity increases the availability of ACh for nerve cell communications [2]. Alzheimer's disease (AD) is characterized by a progressive memory loss that leads to a profound emotional disturbance in later stages. The disease is accompanied by dysfunctions in cholinergic neurotransmission of the central nervous system [3]. Hence, cholinesterase inhibitors may act as potential leads in the discovery of therapeutics for such nervous-system disorders.

Acetylcholinesterase (AChE) is a key component of cholinergic brain synapses and neuromuscular junctions. This enzyme is produced in the liver and enriched in the circulation. The main role of this enzyme is the termination of signal transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine [4, 5]. According to the cholinergic hypothesis, the memory impairment in the patients with senile dementia of Alzheimer's type results from a deficiency in cholinergic function in the brain [6]. Hence the most promising therapeutic strategy for activating central cholinergic functions has been the use of cholinomimetic agents. The aim of acetylcholinesterase inhibitors is to boost the endogenous levels of acetylcholine in the brains of AD patients and thereby to boost cholinergic neurotransmission. The exact physiological role of butyrylcholinesterase (BChE) is still elusive, but it is generally viewed as a back-up for the homologous AChE [7].

It has been estimated that AD affects more than 18 million people, mostly in the developed world [8]. This disease is the most common form of dementia in elderly people, affecting 25 % of the population over 80 years of age [9]. It is now well known that inhibitors of AChE and BChE play a role in the treatment of AD. As no safe and effective drug is yet available for the treatment of AD, secondary metabolites from plants may be instrumental in meeting this challenge. Alkaloids such as physostigmine, galanthamine, huperzine A *etc.* are some examples of natural products which exhibit potent anticholinesterase and competitive antagonistic activity at central and peripheral



Scheme 1. Reagents and conditions: (a) 1-bromopentane, THF, NaH, argon, r. t.,78 h; (b) 5-bromo-1-pentene, THF, NaH, argon, r. t.,78 h; (c) 6-bromo-1-hexene, THF, NaH, argon, r. t.,78 h; (d) Me_3SiCl, H_2O, r. t., 8 h.

cholinergic synapsis. The drugs currently used against AD are expensive, relatively toxic and cause severe side effects. So, the development of drugs against AD and related dementia from natural products and their chemical derivatives needs to be pursued with high priority.

In our ongoing study to find potent cholinesterase inhibitors from the plant *Sarcococca hookeriana* Baill., we reported a series of steroidal alkaloids displaying this activity [10, 11]. Among the compounds isolated from this plant, dictyophlebine (**1**) was obtained in a relatively large quantity in crystalline form. We reported its X-ray structure, which was the first report of this skeleton from the genus *Sarcococca* [12]. This compound was employed in microbial transformations with the fungus *Rhizopus stolonifer* to produce new AChE and BChE inhibitors [13]. Natural product **1** was further subjected to a number of other chemical reactions, to give five new derivatives (**2**–**6**) which were studied with respect to their cholinesterase inhibitory activities.

Results and Discussion

Dictyophlebine (**1**) isolated from the alkaline fraction of the dichloromethane extract of *S. hookeriana* [12] was subjected to chemical derivatization and five new products (**2**–**6**) were obtained (see Scheme 1). The structures of compounds **2**–**4** have been determined by comparison of their spectroscopic data with those of the parent compound **1**, while compounds **5** and **6** were characterized by comparing their spectroscopic data with those of compounds **3** and **4**.

N-alkylation of compound **1** by 1-bromopentane in the presence of sodium hydride in THF under argon

Table 1. *In vitro* anticholinesterase activities of compounds **1** – **6**.

Compounds	$IC_{50}(\mu M) \pm SEM$[a]	
	AChE	BChE
1	6.21 ± 0.23	3.65 ± 0.023
2	8.24 ± 0.04	4.15 ± 0.1
3	1.83 ± 0.1	0.29 ± 0.001
4	1.97 ± 0.02	0.33 ± 0.01
5	1.12 ± 0.01	0.54 ± 0.001
6	1.07 ± 0.03	0.13 ± 0.01
Galanthamine[b]	0.5 ± 0.01	8.2 ± 0.02
Eserine[b]	0.04 ± 0.0001	0.82 ± 0.001

[a] Standard error of the mean of five assays; [b] positive control used in the assays.

afforded the alkylated derivative **2** ($C_{29}H_{54}N_2$, *m/z* = 430 $[M]^+$). The ^1H NMR spectrum of **2** showed new signals as compared to the parent compound **1** comprising a triplet at δ = 0.84 ($J_{5',4'}$ = 6.6 Hz) assigned to 5′-H methyl protons, while a multiplet at δ = 2.32 – 2.40 is due to the diastereotopic 1′-H methylene protons. The 2′-H to 4′-H methylene protons resonated as multiplets in the range of δ = 1.20 – 1.86.

N-Alkylation of compound **1** by 5-bromo-1-pentene in the presence of sodium hydride in THF under argon atmosphere afforded the alkylated derivative **3** ($C_{29}H_{52}N_2$, *m/z* = 428 $[M]^+$). The ^1H NMR spectrum of **3** showed new signals comprising two overlapping multiplets integrating for two protons resonating at δ = 2.32 – 2.43 which correspond to the diastereotopic 1′-H methylene protons. The 2′-H and 3′-H methylene protons resonate as multiplets in the range of δ = 1.33 – 1.89. One doublet at δ = 4.91 (J = 10.2 Hz), which is further split because of gem-alkene and allyl couplings, is assigned to 5′-H *cis* to 4′-H, while the double multiplet at δ = 4.97 (J = 17.1 Hz) is assigned to the 5′-H *trans* to 4′-H. A multiplet appearing at δ = 5.81 is due to the 4′-H olefinic methine proton.

N-Alkylation of compound **1** with 6-bromo-1-hexene in the presence of sodium hydride in THF under argon atmosphere afforded the alkylated derivative **4** ($C_{30}H_{54}N_2$ *m/z* = 442 $[M]^+$). The ^1H NMR spectrum of **4** showed new signals including two overlapping multiplets integrating for two protons resonating at δ = 2.35 – 2.41 which correspond to the diastereotopic 1′-H methylene protons. The 2′-H to 4′-H methylene protons resonate as multiplets in the range of δ = 1.22 – 1.82. One doublet at δ = 4.94 (J = 10.4 Hz), which is further split because of gem-alkene and allyl couplings, is assigned to 6′-H *cis* to 5′-H, while the double multiplet at δ = 4.99 (J = 17.1 Hz) is assigned to the 6′-H *trans* to 5′-H, and a multiplet at δ = 5.83 assigned to the 5′-H olefinic methine proton.

Hydrochlorination of compound **3** by trimethylsilylchloride in the presence of water afforded the chlorinated derivative **5** as a diastereomeric mixture ($C_{29}H_{53}N_2Cl$, *m/z* = 464 $[M]^+$). The ^1H NMR spectrum of **5** showed one prominent signal compared to compound **3**, integrating for three protons and resonating as a doublet at δ = 1.49 ($J_{5',4'}$ = 6.0 Hz) which has been assigned to 5′-H methyl protons of both diastereomers. Similarly, a downfield multiplet appearing at δ = 4.22 was due to the 4′-H geminal to chlorine. This proves the Markovnikov-type HCl addition.

Hydrochlorination of compound **4** with trimethylsilylchloride in the presence of water afforded the alkylated derivative **6** as a diastereomeric mixture ($C_{30}H_{55}N_2Cl$, *m/z* = 478 $[M]^+$). The ^1H NMR spectrum of **6** showed one prominent signal as compared to compound **4**, integrating for three protons and resonating as a doublet at δ = 1.49 ($J_{5',4'}$ = 6.0 Hz), which has been assigned to the 6′-H methyl protons of both diastereomers. Similarly, a downfield multiplet appearing at δ = 4.00 is due to 5′-H geminal to chlorine, proving the regioselectivity.

The AChE and BChE inhibitory activities of compounds **1** – **6** are presented in Table 1. In a test for the inhibitory potential of the parent compound **1** with the *N*-alkylated products **2** – **4**, the *N*-pentyl product **2** was found to have comparatively less activity than **1**. But interestingly, the *N*-alkylated products **3** and **4** with a terminal double bond showed higher inhibitory potential compared to compound **1**. This activity was even higher than that of the standard drugs galanthamine and eserine in the case of BChE, and comparable for AChE (see Table 1). Furthermore, the chlorinated products **5** and **6** also display anti-cholinesterase activities comparable to that of compounds **3** and **4**.

The drugs that are used in the treatment of AD cause severe adverse effects such as high toxicity, low bioavailability, short duration of action and narrow therapeutic windows. To reduce these problems, a great deal of emphasis has been placed on the use of natural alkaloids and their chemical derivatives as cholinesterase-inhibiting drugs. As some of the compounds in the present study display anticholinesterase activity comparable to that of the standards galanthamine and eserine, our findings of this activity in 5α-pregnane-type steroidal alkaloid class of compounds may act as leads in the discovery of clinically useful inhibitors for nervous system disorders, particularly by reducing memory deficiency in AD patients by poten-

tiating and effecting the cholinergic transmission process.

Experimental Section

Optical rotations were measured on a Jasco digital polarimeter (model DIP-3600) in methanol. IR spectra were recorded in $CHCl_3$ on a Jasco A-302 IR spectrophotometer. The EI-MS spectra were recorded on a double focusing mass spectrometer (Varian MAT 311A). The ^{1}H NMR spectra were recorded on Bruker AM 400 and Bruker AMX 500 instruments at 400 and 500 MHz, respectively. Proton chemical shifts are reported in δ (ppm) relative to the residual $CDCl_3$ signal at δ = 7.26. Coupling constants J are given in Hz. Column chromatography was carried out on silica gel 60 (70 – 230 mesh sizes, E. Merck). Pre-coated silica gel TLC plates (E. Merck, F_{254}) were used to check the purity of compounds, and Dragendorff's spray reagent was used for the staining of compounds on TLC.

All chemicals used in the experiments were of analytical grade.

*Dictyophlebine (**1**)*

Compound **1** was isolated from different sources, such as *Dictyophlebia lucidia*, *Funtumia latifolia*, *Sarcococca saligna* and *S. hookeriana*, and characterized by using spectroscopic and X-ray analysis techniques [12, 14].

*N^3-Pentyldictyophlebine (**2**)*

Compound **1** (15.0 mg, 0.041 mmol) was dissolved in THF (2.0 mL) and 1.6 mg (0.984 mmol) of NaH was added. The mixture was then treated under argon with 0.5 mL (61.9 mmol) of 1-bromopentane with stirring. The reaction mixture was continuously stirred for 78 h at r. t. The resulting mixture was extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The residues were subjected to column chromatography and eluted with pet. ether/acetone/Et_2NH (70 : 27 : 3) to give 14.6 mg (85.8 %) of *N*-alkylated derivative **2** as a colorless gummy solid. – R_f = 0.47 (pet. ether/acetone/Et_2NH, 40 : 56 : 4). – $[\alpha]_D^{25}$ = 26 (c = 0.05, MeOH). – ^{1}H NMR (500 MHz, $CDCl_3$, only characteristic signals are given): δ = 0.61 (s, 3H, 18-Me), 0.73 (s, 3H, 19-Me), 0.84 (t, 3H, ^3J = 6.6 Hz, 5′-Me), 0.89 (d, 3H, ^3J = 6.8 Hz, 21-Me), 2.13 (s, 6H, NMe_2), 2.22 (s, 3H, NMe), 1.20 – 1.86 (m, 6H, 2′ to 4′-H), 2.32 – 2.40 (m, 2H, 1′-H). – MS (EI, 70 eV): *m/z* (%) = 430 (8) $[M]^+$, 415 (28), 100 (8), 72 (100), 71 (6).

*N^3-Penten-4′-yl-dictophlebine (**3**)*

Compound **1** (50.0 mg, 0.138 mmol) was dissolved in THF (5.0 mL) and 4.16 mg (3.31 mmol) of NaH was added. The mixture was then treated with 0.16 mL (20.5 mmol) of 5-bromo-1-pentene under argon with stirring. The reaction mixture was continuously stirred for 78 h at r. t. The resulting mixture was extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The residues were subjected to column chromatography and eluted with pet. ether/acetone/Et_2NH (75 : 22 : 3) to give 45.7 mg (76.9 %) of *N*-alkylated derivative **3** as a colorless gummy solid. – R_f = 0.44 (pet. ether/acetone/Et_2NH, 44 : 52 : 4). – $[\alpha]_D^{25}$ = 46 (c = 0.02, MeOH). – IR ($CHCl_3$): ν = 2930 (CH), 1598 cm^{-1} (C=C). – ^{1}H NMR (500 MHz, $CDCl_3$, only characteristic signals are given): δ = 0.61 (s, 3H, 18-Me), 0.74 (s, 3H, 19-Me), 0.84 (d, 3H, ^3J = 6.3 Hz, 21-Me), 2.13 (s, 6H, NMe_2), 2.23 (s, 3H, NMe), 2.32 – 2.42 (m, 2H, 1′-H), 1.33 – 1.89 (m, 4H, 2′ and 4′-H), 4.91 (d, 1H, ^3J = 10.2 Hz, 5′-H *cis* to 4′-H), 4.97 (d, 1H, ^3J = 17.1 Hz, 5′-H *trans* to 4′-H), 5.81 (m, 1H, 4′-H). – MS (EI, 70 eV): *m/z* (%) = 428 (5) $[M]^+$, 413 (35), 98 (5), 72 (100), 69 (26).

*N^3-Hexen-5′-yl-dictophlebine (**4**)*

Compound **1** (50.0 mg, 0.138 mmol) was dissolved in THF (5.0 mL) and 4.16 mg (3.31 mmol) of NaH was added. The mixture was then treated with 0.18 mL (22.4 mmol) of 6-bromo-1-hexene under argon with stirring. The reaction mixture was continuously stirred for 78 h at r. t. The resulting mixture was extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The residues were subjected to column chromatography and eluted with pet. ether/acetone/Et_2NH (80 : 17 : 3) to give 43.2 mg (70.3 %) of an *N*-alkylated derivative **4** as a colorless gummy solid. – R_f = 0.52 (pet. ether/acetone/Et_2NH, 40 : 58 : 2). – $[\alpha]_D^{25}$ = 16 (c = 0.01, MeOH). – IR ($CHCl_3$): ν = 2928 (CH), 1595 cm^{-1} (C=C). – ^{1}H NMR (400 MHz, $CDCl_3$, only characteristic signals are given): δ = 0.61 (s, 3H, 18-Me), 0.74 (s, 3H, 19-Me), 0.84 (d, 3H, ^3J= 6.6 Hz, 21-Me), 2.13 (s, 6H, NMe_2), 2.22 (s, 3H, NMe), 2.35 – 2.43 (m, 2H, 1′-H), 1.22 – 1.82 (m, 2H, 2′ to 4′-H), 4.94 (dd, 2H, ^3J(*cis*) = 10.4 Hz, ^3J(*trans*) = 17.1 Hz, 6′-H), 5.83 (m, 1H, 5′-H). – MS (EI, 70 eV): *m/z* (%) = 442 (2) $[M]^+$, 427 (29), 83 (8), 72 (100).

*N^3-4′-Chloropentyldictophlebine (**5**)*

Compound **3** (5.0 mg, 1.168 mmol) was dissolved in distilled water (5.0 mL) and 0.014 mL (1.166 mmol) of Me_3SiCl was added. The reaction mixture was continuously stirred for 8 h at r. t. The resulting mixture was extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The residues were subjected to column chromatography and eluted with pet. ether/acetone/Et_2NH (70 : 27 : 3) to yield 4.9 mg (92.0 %) of chlorinated derivative **5** as a white powder (mixture of diastereomers). – R_f = 0.41 (pet. ether/acetone/Et_2NH, 40 : 55 : 5). – $[\alpha]_D^{25}$ = 106 (c = 0.01, MeOH). – ^{1}H NMR (400 MHz, $CDCl_3$, only characteristic signals are given): δ = 0.69 (s, 3H, 18-Me), 0.79 (s, 3H,

19-Me), 1.22 (d, 3H, ^3J = 6.3 Hz, 21-Me), 2.60 (s, 6H, NMe_2), 2.84 (s, 3H, NMe), 1.51 (d, 3H, ^3J = 6.0 Hz, 5′-Me), 1.40 – 1.61 (m, 2H, 3′-H), 2.21 – 2.14 (m, 2H, 1′-H), 4.22 (1H, m, 4′-H). – MS (EI, 70 eV): *m/z* (%) = 464 (12) [M^+], 443 (28), 214 (5), 72 (100).

*N^3-5′-Chlorohexyldictophlebine (**6**)*

Compound **4** (5.0 mg, 1.168 mmol) was dissolved in distilled water (5.0 mL) and 0.014 mL (1.166 mmol) of Me_3SiCl was added. The reaction mixture was continuously stirred for 8 h at r. t. The resulting mixture was extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4. The residues were subjected to column chromatography and eluted with pet. ether/acetone/Et_2NH (70 : 27 : 3) to yield 5.1 mg (94.6 %) of chlorinated derivative **6** as a white powder (mixture of diastereomers). – R_f = 0.44 (pet. ether/acetone/Et_2NH, 40 : 55 : 5). – $[\alpha]_D^{25}$ = 118 (*c* = 0.01, MeOH). – ^{1}H NMR (400 MHz, $CDCl_3$, only characteristic signals are given): δ = 0.70 (s, 3H, 18-Me), 0.80 (s, 3H, 19-Me), 1.23 (d, 3H, ^3J = 6.4 Hz, 21-Me), 2.63 (s, 6H, NMe_2), 2.85 (s, 3H, NMe), 1.49 (d, 3H, ^3J = 6.0 Hz, 6′-Me), 1.54 – 1.77 (m, 2H, 4′-H), 2.01 – 2.20 (m, 2H, 1′-H), 4.00 (m, 1H, 5′-H). – MS (EI, 70 eV): *m/z* (%) = 478 (18) [$M]^+$, 443 (30), 214 (12), 72 (100).

In vitro cholinesterase inhibition assay and determination of IC_{50}

Acetylcholinesterase (electric eel EC 3.1.1.7), butyrylcholinesterase (horse serum E.C. 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic-acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO,USA). Buffer and other chemicals were of analytical grade. Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured by a slightly modified spectrophotometric method developed by Ellman *et al*. [15]. Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. 5,5′-Dithiobis[2-nitrobenzoic-acid] (DTNB) was used for the measurement of cholinesterase activity. 140 μL of (100 mM) sodium phosphate buffer (pH = 8.0), 10 μL of DTNB, 20 μL of test compound solution and 20 μL of acetylcholinesterase or butyrylcholinesterase solution were mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μL acetylthiocholine or butyrylthiocholine, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). Test compounds and control were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro plates and monitored in a SpectraMax 340 (Molecular Devices, USA) spectrometer.

Estimation of IC_{50} values: The concentrations of test compounds which inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50 % (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA).

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