Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 92 (2004) 477–484
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Cholinesterase inhibitory and spasmolytic potential of steroidal alkaloids

Asaad Khalid[a], Zaheer-ul-Haq[a], M. Nabeel Ghayur[b], Fareeda Feroz[a], Atta-ur-Rahman[a], A.H. Gilani[b], M. Iqbal Choudhary[a,*]

[a] *Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical Sciences, University of Karachi, Karachi 75270, Pakistan*
[b] *Department of Biological & Biomedical Sciences, The Aga Khan University, Karachi 74800, Pakistan*

Received 8 January 2004; accepted 6 August 2004

Abstract

A new steroidal alkaloid, isosarcodine (**1**) along with four known bases, sarcorine (**2**), sarcodine (**3**), sarcocine (**4**) and alkaloid-C (**5**) were isolated from the MeOH extract of *Sarcococca saligna*. The structures of these alkaloids were identified by spectral data interpretation. These compounds were subjected to acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition studies, and were found to be noncompetitive inhibitors of AChE (K_i = 21.8, 90.3, 32.2, 16.0 and 50.0 μM, respectively) and uncompetitive or noncompetitive inhibitors of BChE (K_i = 8.3, 7.5, 15.6, 5.0 and 12.0 μM, respectively).

The compounds (**2**–**5**) also showed dose-dependent spasmolytic activity in the rabbit jejunum intestinal preparations and also relaxed the high K^+ (80 mM)-induced contraction, indicative of a calcium channel-blocking mechanism.

Structure–activity relationship suggested that the nitrogen substituents at C-3 and/or C-20 of steroidal skeleton and the hydrophobic properties of the pregnane skeleton are the key structural features contributed to the inhibitory potency of these steroidal alkaloids against AChE and BChE.

Keywords: Steroidal alkaloids; Acetylcholinesterase; Butyrylcholinesterase; Inhibition studies; Spasmolytic activity; *Sarcococca saligna*

1. Introduction

The function of AChE (EC 3.1.1.7) in the cholinergic synapses is to catalyse the hydrolysis of acetylcholine (ACh) [1]. This enzyme has long been an attractive target for rational drug design and discovery of mechanism-based inhibitors for the treatment of many neurodegenerative diseases such as Alzheimer's (AD), Parkinson's diseases and myasthenia gravis [2]. AChE inhibitors are known to boost the cholinergic neurotransmission in vivo by inhibiting the AChE enzyme.

BChE (EC 3.1.1.8), on the other hand, is still an unexplored enzyme and there are several theories about its physiological functions, but none is universally acceptable. Cymserine is a derivative of the alkaloid physostigmine, which represents the first available potent, reversible, and selective inhibitor of BChE. Several cymserine derivatives are currently studied for the treatment of AD. This indicates that BChE inhibition may be a tool for the treatment of AD and related dementias [3].

The pregnane-type steroidal alkaloids have been found to possess a wide range of pharmacological properties, including antiulcer, antitumor and can cause a non-recoverable fall in blood pressures [4]. The genus *Sarcococca* belongs to the family Buxaceae, which is predominantly distributed in South Asia extending from Afghanistan through the Himalayas [5]. Some alkaloids of genus *Sarcococca* are known to potentiate the action of naturally secreted ACh in the isolated rat diaphragm and in the rabbit serum. Some of these alkaloids also exhibited gangloin-blocking activity and decrease the effects of nicotine on blood pressure [4]. In the course of our ongoing investigations on medicinal plants, we have studied the chemical constituents of genus *Sarco-*

* Corresponding author. Tel.: +92 21 9243211/24; fax: +92 21 9243190/91.
E-mail address: hej@cyber.net.pk (M.I. Choudhary).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.08.003

cocca. Our previous studies on this genus have resulted in the isolation of a number of new steroidal alkaloids [6–8]. This paper describes the isolation of a new steroidal alkaloid isosarcodine (**1**) along with four known alkaloids, sarcorine (**2**), sarcodine (**3**), sarcocine (**4**) and alkaloid-C (**5**) along with their cholinesterase (AChE) and (BChE) inhibitory and antispasmodic activities. All reported compounds were assayed for their cholinesterase inhibiting activities while compounds **2**–**5** were also tested for their mechanism of action on isolated rabbit jejunum tissues.

2. Materials and methods

2.1. Isolation and structure elucidation

2.1.1. Experimental

The optical rotations were measured on a Jasco digital polarimeter (model DIP-360) in methanol or chloroform. Ultraviolet spectra were recorded in methanol on a Hitachi spectrophotometer (model U-3200). Infrared spectra were recorded on a Jasco A-302 IR spectrophotometer as discs in KBr. The mass spectra were recorded on a double focussing mass spectrometer (Varian MAT 311 A). Peak matching, and field desorption (FD) measurements were performed on the Varian MAT 312 mass spectrometer. Accurate mass measurements were carried out with FAB source using glycerol as matrix while HREI-MS were recorded on Jeol HX 110 mass spectrometer. The NMR spectra were recorded on Bruker Avance 500 and AC 400 MHz spectrometers. Chemical shifts were recorded in δ (ppm) relative to $SiMe_4$, which was used as internal standard, while the coupling constants are reported in Hz. TLC separation were carried out on precoated plates (DC-Alufolien 60 F_{254} of E. Merck) and detected by Dragendorff spraying reagent. All reagents used were of analytical grades.

2.1.2. Collection and identification of plant material

The whole plants of *Sarcococca saligna* (D. Don.) Muel. (50 kg) were collected in October 1999 from the District Bagh of Azad Kashmir, Pakistan. It was identified by Mr. Tahir Ali, taxonomist, Department of Botany, University of Karachi, Pakistan, and a voucher specimen was deposited in the Herbarium of University of Karachi (KU # 19290).

2.1.3. Plant material and extraction

The air-dried plant material (14 kg) was crushed and soaked in MeOH (50 L) for 15 days. The methanolic extract was filtered and evaporated to a gum (1.25 kg), triturated in distilled water (2 L) and subsequently extracted with petroleum ether (251 g), chloroform (220 g), ethyl acetate (45 g) and finally with butanol (145 g).

Chloroform extract of *S. saligna* (220 g) was chromatographed over silica gel column (230–240 mesh, 1.5 kg) and eluted with increasing polarities of petroleum ether/acetone/diethylamine to obtain eight subfractions (ss-1 to ss-8). Fractions ss-2 (148.9 mg), ss-3 (124.8 mg), ss-4 (222.7 mg), ss-5 (128.8 mg) and ss-7 (109.8 mg) have exhibited 100% cholinesterase inhibition at 20 mg/mL. These subfractions were individually subjected to column chromatography on flash silica gel and eluted with increasing polarities of petroleum ether/acetone/diethylamine mixtures to obtain several semipure subfractions, which were finally purified over precoated TLC plates (silica gel) using petroleum ether/acetone/diethylamine (1.5:8.3:0.2) as solvent system, to obtain five steroidal alkaloids **1**–**5**.

Table 1
^{1}H and ^{13}C NMR data of isosarcodine (**1**) (in $CDCl_3$)

Carbon number	^{1}H NMR (δ) (J in Hz)	^{13}C NMR (δ)	Multiplicity
1	1.61, 1.52 m	32.6	CH_2
2	1.25, 1.35 m	28.7	CH_2
3	3.54 m	52.2	CH
4	1.98, 1.10 m	39.8	CH_2
5	1.22 m	46.0	CH
6	1.21, 1.25 m	28.8	CH_2
7	1.53, 1.62 m	31.9	CH_2
8	1.35 m	35.5	CH
9	1.24 m	54.9	CH
10	–	35.5	C
11	1.31, 1.52 m	21.1	CH_2
12	1.42, 1.91 m	31.5	CH_2
13	–	41.8	C
14	1.05 m	56.5	CH
15	1.61, 1.03 m	24.8	CH_2
16	2.85, 1.82 m	27.6	CH_2
17	1.37 m	54.2	CH
18	0.78, 0.63 s	12.0	CH_3
19	0.64, 0.83 s	12.3	CH_3
20	2.45 m	61.2	CH
21	0.88 d (J = 6.5)	9.9	CH_3
$N_a(CH_3)$	2.80 s	27.6	CH_3
$N_b(CH_3)_2$	2.16 s	39.8	C
1′	–	169.9	C
2′	2.05 s	22.5	CH_3

All assignments were confirmed by ^{1}H–^{1}H COSY, HMQC and DEPT spectra.

Isosarcodine (**1**), white amorphous. Percentage yield = 20.21 mg, 1.44×10^{-4}%, $[\alpha]_D^{20} = 48^\circ$ (c = 0.3, $CHCl_3$). UV (MeOH) λ_{max} in nm ($\log \varepsilon$): 198, 204. IR ($CHCl_3$) υ_{max} in cm^{-1}: 3640 (NH), 2810 (=CH stretching), 1648 (C=O), 1594 (C=C stretching), 1511 (NH bending). EI-MS m/z (relative intensity, %): 402 (29) [M^+], 387 (2) [$M^+ - 15$], 359 (1), 302 (3), 121 (2), 110 (62), 100 (15), 84 (22), 72 (100), 55 (5). FD-MS m/z 402 ($C_{26}H_{46}N_2O$). HREI-MS m/z (formula, calculated): 402.3579 ($C_{26}H_{46}N_2O$, 402.3601), 387.3233 ($C_{25}H_{43}N_2O$, 387.3273), 84.0822 ($C_5H_{10}N$, 84.0813), 72.0811 ($C_4H_{10}N$, 72.0813). ^{1}H and ^{13}C NMR values: δ, see Table 1.

2.2. Enzymology

2.2.1. Acetyl- and butyrylcholinesterase inhibition assays

AChE and BChE activities were measured in vitro by a modified spectrophotometric method developed by Ellman

et al. [9]. All the inhibition studies were performed using SpectraMax microplate spectrophotometer (Molecular Devices, CA, USA).

Electric eel AChE (type VI-S, Sigma), and horse serum BChE (Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma) were used as substrates in the reactions. Ellman reagent, i.e. 5,5-dithiobis(2-nitro)benzoic acid (DTNB, Sigma) was used as chromogenic marker for the measurement of the cholinesterases activity. Sodium phosphate buffer (1 mM) at pH 7.0 was used to prepare the enzyme working solution and at pH 8.0 for the assay mixture.

The assay procedure was previously described [7]. All the reactions were performed in triplicate and the initial rates were measured as the rate of change in optical density per minute (OD/min) and used in subsequent calculations. According to Ellman et al., since the extinction coefficient of the yellow anion is known, the rate of the enzymatic reaction can be calculated based upon the following equation [9]:

$$\text{rate (mol/L/min)} = \frac{\text{change in absorbace/min}}{13{,}600}.$$

The percent enzyme inhibition by test sample was calculated using the following formula:

$$\text{inhibition (\%)} = 100 - \left(\frac{\text{change of absorbance (test)}}{\text{change of absorbance (control)}} \times 100\right).$$

2.2.2. Estimation of inhibition and kinetic parameters

The IC_{50} values (the concentration of test compounds that inhibit the hydrolysis of substrates by 50%) were determined by spectrophotometric measurement of the effect of increasing concentrations of test compounds on enzyme activity. The IC_{50} values were calculated using EZ-Fit Enzyme Kinetics Program (Perrella Scientific Inc., Amherst, USA).

Two different methods were applied to estimate the effect of the inhibitors (test samples) on both K_m and V_{max} values. This was done firstly by plotting the reciprocal of the rate of the reactions against the reciprocal of the substrate concentration as Lineweaver–Burk plot [10], and secondly by the Dixon plot in which the reciprocal of the rate of the reactions was plotted against the inhibitor concentrations [11]. The secondary replots of the Lineweaver–Burk were also constructed in two ways; firstly, $1/V_{maxi}$ were determined at each intersection point of every inhibitor concentration line on the *y*-axis of Lineweaver–Burk plot and then replotted against different concentrations of the respective inhibitor. Secondly, in case of the noncompetitive and linear mixed-type inhibitions, the slope of each line of inhibitor concentration on Lineweaver–Burk plot was plotted against inhibitor concentrations. For the uncompetitive type of inhibition, K_{mi} was determined from the intersection of the inhibitor concentrations lines on the *x*-axis of Lineweaver–Burk plot and plotted against inhibitor concentrations. The secondary replot of Dixon plot was constructed as the slope of each line of substrate concentration in original Dixon plot against the reciprocals of the substrate concentrations.

K_i values (the rate constant of the dissociation of the enzyme–inhibitor complex into free enzyme and inhibitor) were determined by the interpretation of Dixon plots, Lineweaver–Burk plots, and their secondary replots by using initial velocities. These velocities were obtained over a range of substrate concentrations between 0.1 and 0.4 mM for ATCh and 0.05 and 0.2 mM for BTCh. The assay conditions for measurement of the residual activities of the enzymes in the presence of all inhibitors were identical to the aforementioned spectrophotometric assay procedure except that fixed concentrations of inhibiting compounds were used in the assay medium.

Types of inhibition were determined by the graphical analysis of Dixon plots, Lineweaver–Burk plots and their secondary plots.

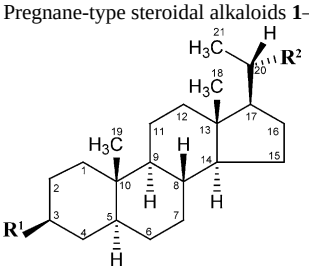
Assays were conducted in triplicate at each concentration of the inhibitor. Graphs were plotted using GraFit program [12]. Values of the correlation coefficient, slope, intercept and their standard errors were obtained by the linear regression analysis using the same software.

2.3. Antispasmodic pharmacological assays

Antispasmodic activities of the test compounds **2**–**5** were studied in isolated spontaneously contracting rabbit jejunum. Rabbits from a local breed of both sexes (1.5–2.0 kg), obtained from the Animal House of the Aga Khan Medical University, Karachi, were used in this study. Animals had free access to water but food was withdrawn 24 h prior to the experiment. Animals were sacrificed by a blow on the back of the head, the abdomen was cut open and a piece of jejunum was taken out. Segments 2 cm long were suspended in Tyrode's solution aerated with a mixture of 95% oxygen and 5% carbon dioxide, and maintained at 37 °C. Tyrode's solution composition was: 80 mM KCl, 91.04 mM NaCl, 1.05 mM $MgCl_2$, 11.87 mM $NaHCO_3$, 0.41 mM NaH_2PO_4, 1.8 mM $CaCl_2$, and 5.55 mM glucose. The spontaneous intestinal movements were recorded isotonically using Harvard transducers and Harvard Student Oscillograph. Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug. Under these experimental conditions, the rabbit jejunum exhibits spontaneous rhythmic contractions and therefore allows study of the relaxant (spasmolytic) activity directly without the use of an agonist.

Calcium channel-blocking activity of compounds **2**–**5** was determined as described by Farre et al. [13]. The data was analyzed and plotted using GraphPad Prism software [14]. The results were expressed as mean ± S.E.M (standard mean error). All statistical comparisons were made by using Student's *t*-test, and a *P*-values smaller than 0.05 were regarded as significant.

Table 2
Pregnane-type steroidal alkaloids **1**–**5**



Compound name	R^1	R^2	Unsaturation
Isosarcodine (**1**)	*N*CH_3Ac	*N*$(CH_3)_2$	
Sarcorine (**2**)	*N*HAc	*N*$(CH_3)_2$	
Sarcodine (**3**)	*N*$(CH_3)_2$	*N*CH_3Ac	
Sarcocine (**4**)	*N*$(CH_3)_2$	*N*CH_3Ac	$\Delta^{5,6}$
Alkaloid-C (**5**)	OCH_3	*N*$(CH_3)_2$	$\Delta^{5,6}$

3. Results

A new steroidal alkaloid, isosarcodine (**1**) was isolated along with four known bases, sarcorine (**2**), sarcodine (**3**), sarcocine (**4**) and alkaloid-C (**5**) from the MeOH extract of *S. saligna*. The structure of isosarcodine (**1**) was identified by spectral data interpretation. Where as the known compounds **2**–**5**, were identified by comparing their spectral data with the literature values [15–18] (Table 2).

All compounds **1**–**5** were found to inhibit both enzymes (AChE and BChE) in a concentration-dependent manner. Many of these compounds, i.e. **3**–**5**, as shown in Table 3, exhibited a pure noncompetitive type of inhibition against both enzymes as they decrease the V_{max} value without affecting the affinity of the enzyme for the substrate (K_m values). Compounds **1** and **2** were found to be uncompetitive inhibitors of BChE as they decrease K_m and V_{max} values.

As shown in Table 3, all compounds of this class were found to be selective towards the BChE, whereas the standard inhibitors were more selective towards the AChE.

All compounds reported in this study were found to be specific inhibitors of AChE and BChE and were inactive against some other enzymes such as phosholipase A_2, urease, acid phosphatase, β-glucuronidase, and α-glucosidase.

Structure–activity relationship, predicted from the results presented in Table 3, suggests that the nitrogen substituents at C-3 and C-20 of steroidal skeleton are the most important structural features that determine the inhibitory potency of these steroidal alkaloids. Moreover, steroidal alkaloids **1**–**5** possess a large hydrocarbon skeleton, which gives them interesting non-polar and hydrophobic character.

Interestingly, compounds **1** and **2** have also been found to be uncompetitive inhibitors of BChE, whereas compounds **3** and **4**, which contain acetylated nitrogen at C-20 were found to be noncompetitive inhibitors of BChE.

These cholinesterases inhibitors were also studied for their possible spasmogenic or spasmolytic effects in isolated rabbit jejunum. Table 4 shows the concentration ranges on which the inhibitors produced a dose-dependent inhibition of spontaneous contractions of the jejunum, thus showing spasmolytic (antispasmodic) activity. Similarly, Fig. 6(A) illustrates graphically the spasmolytic effect of compounds **2**–**5**. Compound 1 was not tested due to insufficient quantity.

The spasmolytic effects of these cholinesterases inhibiting compounds were found to be reversible, and the spontaneous activity returned to the normal level after washing the tissue a couple of times. The effect of verapamil which was used as a standard drug in this assay was also reversible but it takes slightly longer time than the tested compounds to restore the spontaneous activity.

4. Discussion

The new steroidal alkaloid, isosarcodine (**1**) (HREI-MS *m/z* 402.3610, $C_{26}H_{46}N_2O$), was isolated as white amorphous powder. The ion at *m/z* 387 was due to the loss of a methyl group from the M^+. The base peak at *m/z* 72 was due to *N*,*N*-dimethylaminium ion [19]. The other important fragments were at *m/z* 43, 74, 112 and 138 indicated the presence of a N_a-methyl-N_a-acetyl group at C-3. The IR spectrum of **1** showed strong absorption at 1648 cm^{-1} (amidic carbonyl). The UV spectrum displayed only terminal absorptions at 204 and 198 nm.

The ^{1}H NMR spectrum of compound **1** showed three tertiary methyl singlets at δ 0.78 (CH_3-18), 0.83 (CH_3-19) and

Table 3
Summary of the in vitro anticholinesterase activities of steroidal alkaloids **1**–**5**

Compound	Acetylcholinesterase			Butyrylcholinesterase		
	IC_{50} (μM) (mean ± S.E.M.)	K_i[a] (μM) (mean ± S.E.M.)	Type of inhibition	IC_{50} (μM) (mean ± S.E.M.)	K_i[a] (μM) (mean ± S.E.M.)	Type of inhibition
1	10.31 ± 0.13	21.8 ± 0.73	NC	1.893 ± 0.06	8.25 ± 3.15	UC
2	69.99 ± 2.6	90.3 ± 2.03	NC	10.33 ± 0.21	7.5 ± 1	UC
3	49.77 ± 1.26	32.2 ± 0.92	NC	18.31 ± 0.74	15.57 ± 0.8	NC
4	20 ± 1.30	16 ± 1.48	NC	3.86 ± 0.01	5 ± 0.15	NC
5	42.2 ± 0.26	50 ± 0.72	NC	22.13 ± 0.14	12 ± 0.8	NC
Eserine	0.041 ± 0.001	0.014 ± 0.001	MT	0.857 ± 0.01	0.9 ± 0.05	NC
Tacrine	0.021 ± 0.002	0.23 ± 0.02	MT	0.051 ± 0.005	0.025 ± 0.003	MT
Galanthamine	0.45 ± 0.02	0.19 ± 0.01	MT	39.1 ± 0.032	32 ± 0.33	NC

NC = noncompetitive; UC = uncompetitive; MT = mixed type; S.E.M. = standard mean error of three to five experiments.
[a] K_i is the mean of four values calculated from Lineweaver–Burk plot, its secondary replots, and Dixon plot.

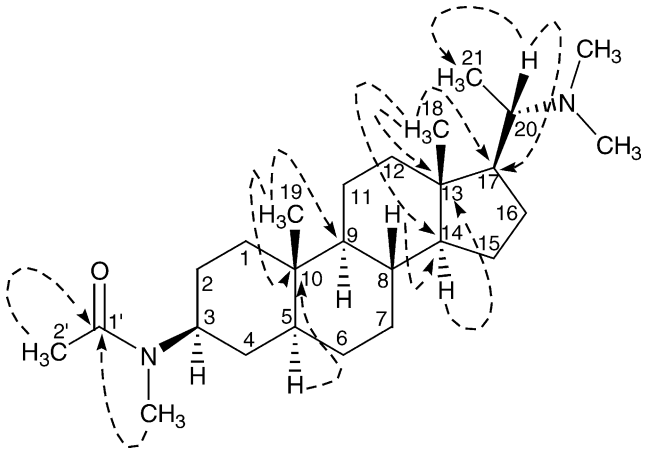


Fig. 1. Important HMBC correlations for isosarcodine (**1**).

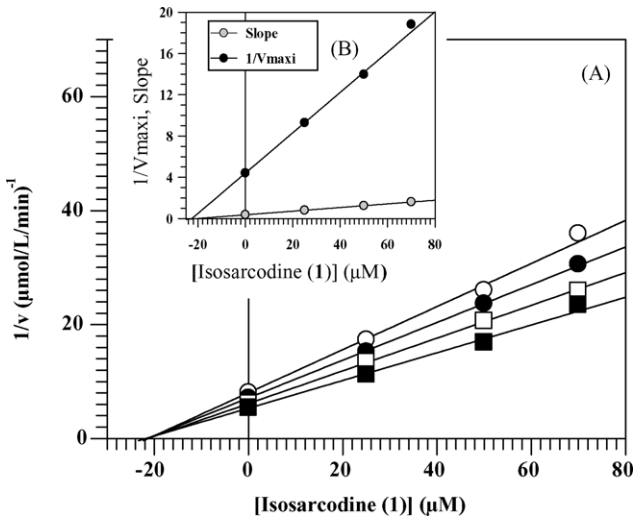


Fig. 3. Steady-state inhibition of AChE by isosarcodine (**1**). (A) Dixon plot of reciprocal of the initial velocities vs. various concentrations of inhibitor (at four fixed ATCh concentrations: (◯) 0.1 mM, (●) 0.13 mM, (□) 0.2 mM and (■) 0.4 mM). (B) Secondary replots of the Lineweaver–Burk plot: $1/V_{max,i}$ or slope vs. various concentrations of compound **1**. The correlation coefficient for all the lines of the graphs was >0.99, each point in the graphs represents the mean of three experiments.

2.05 (CH_3CO), along with a doublet at δ 0.88 ($J_{21,20}$ = 6.4 Hz) (CH_3-21). A six-proton singlet at δ 2.16, and a three-proton singlet at δ 2.80 were assigned to the $N_b(CH_3)_2$ and N_aCH_3 protons, respectively. Two downfield multiplets resonating at δ 2.45 and 3.54 were ascribed to the C-20 and C-3 methine protons, respectively. The ^{13}C NMR spectrum (broad-band decoupled) displayed 26 resonances for seven methyl, nine methylene, seven methine and three quaternary carbons. The C-19 methyl protons (δ_H 0.83) and C-1 methylene protons (δ_H 1.61) showed HMBC interactions with the C-10 (δ_C 35.5) (Fig. 1). The HMBC spectrum shows interactions of the C-18 methyl protons (δ_H 0.78) with the methine C-17 (δ_C 54.2) and quaternary C-13 (δ_C 41.8). The HMBC correlations also indicated the presence of a N_a-methyl-N_a-acetyl group at C-3. The N_a-CH_3 protons showed the long-range correlations with the C-3 methine carbon (δ_C 52.2). The C-3 methine proton (δ_H 3.54) also displayed HMBC couplings with C-1′ (δ_C 169.9) and C-4 (δ_C 39.8).

The stereochemistries at asymmetric centers in compound **1** were assigned on the basis of biogenetic considerations and cross-peaks in ROESY spectrum. The β-orientations of H-8 (axial), CH_3-18 (axial) and CH_3-19 (axial) were assigned on the basis of ROESY spectrum, since the H-8 (δ 1.35) and CH_3-18 (δ 0.78) signals showed cross-peaks with CH_3-19 (δ 0.83). Similarly C-18 methyl protons (δ 0.78) also showed correlations with H-8 and H-20 (Fig. 2). These spectral observations led to the structure **1** for isosarcodine.

The purity of the noncompetitive type of inhibition of compounds **3**–**5** against both enzymes and compounds **1** and **2** against AChE was confirmed by the secondary replots of Lineweaver–Burk plots as they show linear rather than hyperbolic lines as expected in the case of partial noncompetitive inhibition. Fig. 3 shows the graphical analysis of steady-state inhibition data of compound **1** for AChE as an example of this type of inhibition.

Compounds **1** and **2** were found to decrease both the affinity (K_m) and V_{max} values suggesting uncompetitive mechanism of inhibition against BChE. The graphical analysis of compound **1** provides an example of compounds acting through uncompetitive mechanism (Fig. 4). Interestingly,

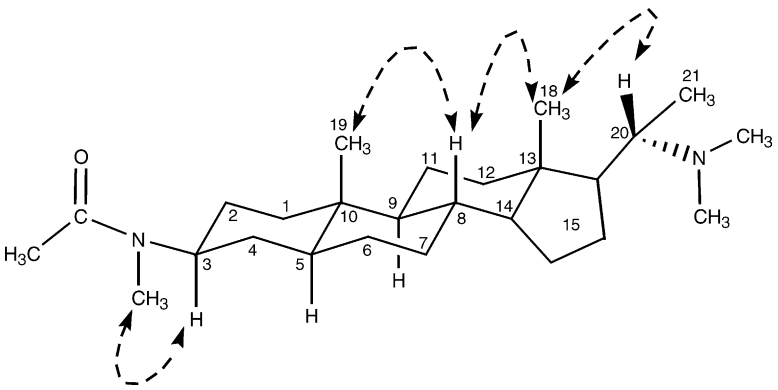


Fig. 2. Important ROESY correlations for isosarcodine (**1**).

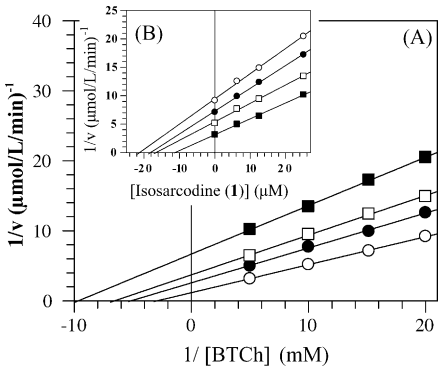


Fig. 4. Steady-state inhibition of BChE by isosarcodine (**1**). (A) Lineweaver–Burk plot of reciprocal of the initial velocities vs. reciprocal of BTCh in absence (◯) and presence of 6.25 μM (●), 12.5 μM (□) and 25.0 μM (■) of isosarcodine (**1**). (B) Dixon plot of reciprocal of the initial velocities vs. various concentrations of inhibitor (at four fixed BTCh concentrations: (◯) 0.05 mM, (●) 0.06 mM, (□) 0.1 mM and (■) 0.2 mM). The correlation coefficient for all the lines of all the graphs was >0.99; each point in all the graphs represents the mean of three experiments.

both the aforementioned uncompetitive inhibitors of BChE were found to be noncompetitive inhibitors of AChE.

In the structures of cholinesterases (AChE and BChE), aromatic gorge plays an extremely important role in ligand recognition [20]. This gorge has four loci, which are involved in the molecular recognition and catalysis. The acetyl specificity of AChE is based on the ligands interactions in the acyl binding locus [21,22]. The esteratic locus of AChE gorge consists of two subsites: (a) the oxyanion hole [23], and (b) the catalytic triad; on which Ser-200, Glu-327, and His-440 interact with the substrates such as ACh and hydrophobic ligands [24,25]. On the other hand, quaternary ammonium binding locus in aromatic gorge recognizes the quaternary ammonium function of the ligand via electrostatic interactions [23]. The peripheral anionic site is situated near the top of the aromatic gorge. Various classes of hydrophobic ligands, aromatic cations and bisquaternary ammonium ligands can interact with the peripheral anionic locus of enzyme [21,22] to influence its catalytic activity allosterically [26,27].

Since all these compounds were found to be noncompetitive or uncompetitive inhibitors of both cholinesterases, it was deduced that these compounds do not bind at the active site of the enzymes. They might, however, bind at the aromatic gorge of AChE/BChE enzymes both in the presence or absence of substrates. The alternative possibility of off-gorge binding, which requires major conformational changes in active site and gorge disruption, was not considered due to the same reason. Although, the precise dimensions of the aromatic gorges of AChE and BChE are still not known, they seem to be adequate in size as well as conformationally flexible enough to accommodate all inhibitors included in this study [28].

The selectivity of compounds of this class towards the BChE could be explained in part by the relatively large size of these compounds, which can diffuse easily inside the larger aromatic gorge of BChE. About halfway down the gorge of AChE, amino acids Phe-330 and Tyr-121, are pointing more or less inwards, forming a narrow bottleneck. Above and below this narrow point, the gorge is relatively wider [20]. Hydrophobic interactions, which are more prominent in BChE, might also play an important role in the selectivity of these compounds toward BChE.

Apparently, the nitrogen substituents at C-3 and C-20 of steroidal skeleton are the key structural features that determine the inhibitory potency of these steroidal alkaloids. They are protonated at physiological pH to afford quaternary centers, and thus mimic the quaternary nitrogen of the well-known quaternary nitrogen inhibitors of cholinesterases such as decamethonium. It is generally believed that due to the presence of the rings of the aromatic amino acids in AChE and BChE's gorges, the cationic ligands move toward the active sites by the diffusion on the surface of the enzyme [21]. The diffusion of the quaternary nitrogen containing compounds to the peripheral site of the aromatic gorge may be facilitated by the positive charge on these compounds.

Interestingly, the large hydrocarbon skeleton of alkaloids **1**–**5** gives them interesting non-polar and hydrophobic character. Hydrophobicity is thus another attribute common in these alkaloids and the residues of the aromatic gorge of cholinesterases. Apparently, this property plays an important role in the diffusion of these compounds inside the aromatic gorge of the enzyme.

Apparently, the presence of electron withdrawing substituents such as acetamide at C-3 (compound **1**) is comparatively more significant for the inhibition of AChE than the substitution of a similar group at C-20 (compound **3**). This highlights the impact of ligand interactions at the bottom of the gorge in contrast to the peripheral site on top of the gorge.

This prediction is supported by our ongoing molecular docking studies of these compounds against AChE, which highlighted the key role of acetamide group and hydrophobic skeleton. Since ring A of steroidal skeleton is buried at the bottom of the aromatic gorge, therefore the C-3 substituted acetamide group goes inside the aromatic gorge, while C-20 substituted acetamide group remains outside the gorge (Fig. 5). Detailed docking studies will be published elsewhere.

Compounds **1** and **2** have been found to be uncompetitive inhibitors of BChE, whereas compounds **3** and **4** which contain acetylated nitrogen at C-20 inhibited BChE noncompetitively. This shows that in case of BChE, substrate binding is essential in order to produce necessary conformational changes in the aromatic gorge of the enzyme to allow the binding of the inhibitor. When compound **2** was compared with the compound **1** (both contain an acetamide group at C-3), the latter appears to be more active. This could be due to the nature of substitution at the C-3 nitrogen. This suggests that the more substituted nitrogen favors the activity, probably due to the hydrophobic interactions it may have with the hydrophobic gorge of the enzyme.

Since none of the known inhibitors is structurally similar to the compounds under study, eserine (physostigmine),

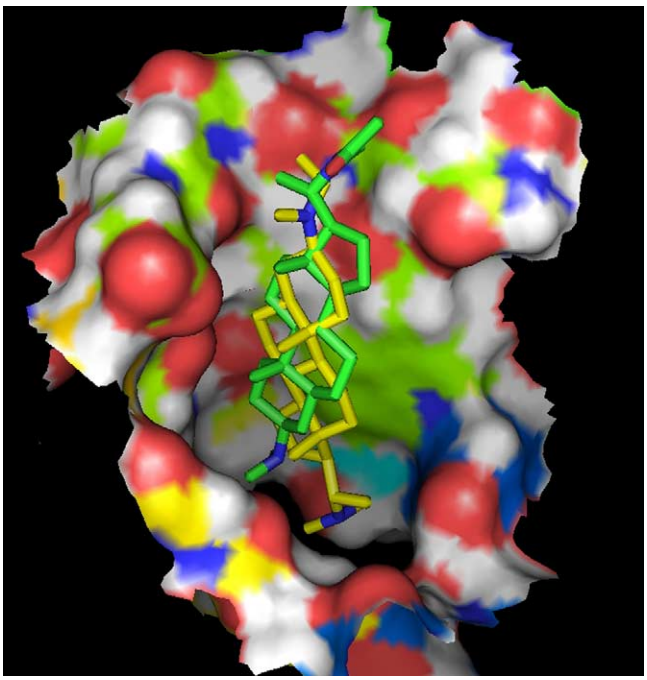


Fig. 5. Top view showing isosarcodine (**1**) and sarcodine (**3**) inside AChE aromatic gorge. Some amino acids were removed from the protein image for clarity.

tacrine and galanthamine were randomly selected as the standard inhibitors to compare the potency of the test compounds against both enzymes. Physostigmine and galanthamine are natural and reversible AChE and BChE inhibitors, which have a wide range of clinical applications. The K_i values and the type of inhibition of AChE and BChE by eserine, tacrine and galanthamine are shown in Table 3.

Compounds **2**–**5** were also studied for their possible spasmogenic or spasmolytic effects in isolated rabbit jejunum. Rabbit jejunum was selected due to its inherent spontaneous activity, which helps in screening both the muscle relaxing and muscle contracting agents [29]. In the absence of inhibitors, rabbit jejunum exhibited spontaneous contractions and the behavior of the tissue did not change over the time course of the experiments. Results in Table 4 shows that dose-dependent inhibition of spontaneous contractions of the jejunum produced by compounds **2**–**5** indicate a spasmolytic activity. Fig. 6(A) illustrates graphically the spasmolytic effect of compounds **2**–**5**.

Table 4
Summary of the antispasmodic activities of the cholinesterases inhibitors

Compound	EC_{50} (μg/ml)	
	Spontaneous	K^+ (80 mM)
2	16.3 ± 2.3	63.5 ± 6.5
3	7.1 ± 1.4	19.6 ± 6.1
4	5.8 ± 1.8	24.3 ± 2.6
5	213.3 ± 13.3	200.0 ± 0.0*
Verapamil	0.16 ± 0.04	0.14 ± 0.01

Values are mean ± S.E.M. of three to four determinations, except for the one denoted with an asterisk with $n = 1$.

Moreover, compounds **2**–**5** also inhibited the K^+-induced contractions (Fig. 6(B)) in similar dose ranges, thereby suggesting a calcium channel-blocking activity similar to that of verapamil, a standard calcium channel blocker (Table 4) [30].

The contractions of smooth muscle preparations including rabbit jejunum are dependent upon an increase in the cytoplasmic free Ca^{2+}, which activates the contractile elements [31]. The increase in intracellular Ca^{2+} is due to either influx via voltage-dependent Ca^{2+} channels (VDCs) or due to release from intracellular stores in the sarcoplasmic reticulum. Periodic depolarization and repolarization regulate the spontaneous movements of the intestine and at the height of depolarization, the action potential appears as a rapid influx of Ca^{2+} via VDCs [32]. Compounds **2**–**5** inhibited the spontaneous movements of rabbit jejunum (Fig. 6), and this may be due to interference either with the Ca^{2+} release or with the Ca^{2+} influx mechanisms through VDCs. However, in order to test whether the spasmolytic effect is mediated through the blockade of Ca^{2+} influx, a high dose of K^+ (80 mM) was introduced to depolarize the tissues. Addition of test compounds caused a dose-dependent inhibition of the K^+-precontracted jejunum, similar to the action of verapamil (Table 4). This effect is shown in Fig. 6(B) for the compounds **2**–**5**.

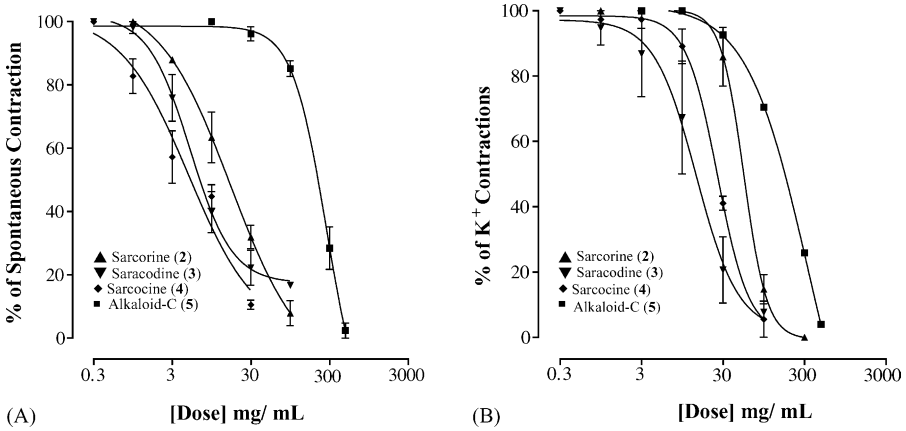


Fig. 6. Effect of the compounds **2**–**5** on the spontaneously beating (A) and high K^+ (80 mM)-induced contractions (B) on isolated rabbit jejunum.

The contractions induced by high K^+ are dependent on the entry of Ca^{2+} into the cells through VDCs [33], and a substance which can inhibit K^+-induced contractions is therefore considered to be a calcium channel blocker [34]. Thus, inhibition of high K^+ (80 mM)-induced contraction of rabbit jejunum by compounds **2–5** may reflect restricted Ca^{2+} entry via VDCs.

Acknowledgements

The authors wish to thank The Ministry of Science and Technology, Government of Pakistan for providing the financial support under the Pak–Kazakh Scientific Cooperation Program. We are extremely grateful to Dr. Rafat A. Siddiqui, Indiana University, Indianapolis, USA, for cooperation during this work. The authors are also grateful to Dr. M. Riaz Khan for providing the plant material.

References

[1] H.A. Lester, The response to acetylcholine, Sci. Am. 236 (1977) 106–118.
[2] P. Taylor, Anticholinesterase agents, in: A.G. Gilman, T.W. Rall, A.S. Nies, P. Taylor (Eds.), The Pharmacological Basis of Therapeutics, Pergamon Press, New York, 1990, pp. 131–149.
[3] Q. Yu, H.W. Holloway, T. Utsuki, A. Brossi, N.H. Greig, Synthesis of novel phenserine-based-selective inhibitors of butyrylcholinesterase for Alzheimer's disease, J. Med. Chem. 42 (1999) 1855–1861.
[4] M. Kiamuddin, H. Hye, Activity of an alkaloids form *Sarcococca saligna*, Pak. J. Sci. Ind. Res. 13 (1970) 59–62.
[5] E. Nasir, S.I. Ali, Buxaceae, vol. 457, Fakhri Printing Press, Karachi, 1972, 629 pp.
[6] Atta-ur-Rahman, Zaheer-ul-Haq, F. Feroz, A. Khalid, S.A. Nawaz, M.R. Khan, M.I. Choudhary, New cholinesterase-inhibiting steroidal alkaloids from *Sarcococca saligna*, Helv. Chim. Acta 87 (2004) 439–448.
[7] Atta-ur-Rahman, Zaheer-ul-Haq, A. Khalid, S. Anjum, M.R. Khan, M.I. Choudhary, Pregnane-type steroidal alkaloids of *Sarcococca saligna*: a new class of cholinesterases inhibitors, Helv. Chim. Acta 85 (2002) 678–688.
[8] S.K. Kalauni, M.I. Choudhary, F. Shaheen, M.D. Manandhar, Atta-ur-Rahman, M.B. Gewali, A. Khalid, Steroidal alkaloids from the leaves of *Sarcococca coriacea* of Nepalese origin, J. Nat. Prod. 64 (2001) 842–844.
[9] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[10] I.H. Segel, Enzyme Kinetics Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, Wiley, New York, 1975, pp. 101–112.
[11] M. Dixon, The determination of enzyme inhibitor constants, Biochem. J. 55 (1953) 170–171.
[12] R.J. Leatherbarrow, GraFit, Erithacus Software Ltd., Stains, UK, 1999.
[13] A.J. Farre, M. Colombo, M. Fort, B. Gutierrez, Differential effects of various Ca^{2+} antagonists, Gen. Pharmacol. 22 (1991) 177–181.
[14] T. Cass, Easy data analysis, Science 289 (2000) 1158.
[15] Atta-ur-Rahman, M.I. Choudhary, M.R. Khan, M.Z. Iqbal, Steroidal alkaloids from *Sarcococca saligna*, Phytochemistry 45 (1997) 861.
[16] A. Chaterjee, E.S. Mukherjee, Alkaloids of *Sarcococca pruniformis*, Chem. Ind. (1966) 769–770.
[17] R. Goutarel, C. Conreur, L. Diakoure, M. Leboeuf, A. Cave, Synthesis de mono- et di-amines steroidiques. alcaloides A (sarcodine), et B (sarcocine) du *Sarcococca pruniformis*, Tetrahedron 24 (1968) 7013–7026.
[18] J.M. Kholi, A. Zaman, A.R. Kidwai, Alkaloid C of *Sarcococca pruniformis*, Phytochemistry 10 (1971) 442–445.
[19] H. Budzkiewicz, C. Djerassi, H.D. Williams, Structure Elucidation of Natural Products by Mass Spectrometry, vol. 2, Holden-Day, Amsterdam, 1964, 5 pp.
[20] J.L. Sussman, M. Harel, F. Frolow, C. Oefner, A. Goldman, L. Toker, I. Silman, Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, Science 253 (1991) 872–879.
[21] D.M. Quinn, Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states, Chem. Rev. 87 (1987) 955–979.
[22] J. Massoulie, L. Pezzementi, S. Bon, E. Krejci, F.M. Vallette, Molecular and cellular biology of cholinesterases, Prog. Neurobiol. 41 (1993) 31–91.
[23] M. Harel, D.M. Quinn, H.K. Nair, I. Silman, J.L. Sussman, The X-ray structure of a transition state analog complex reveals the molecular origins of the catalytic power and substrate specificity of acetylcholinesterase, J. Am. Chem. Soc. 118 (1996) 2340–2346.
[24] T.L. Rosenberry, S.A. Bernhard, Studies of catalysis by acetylcholinesterase. Synergistic effects of inhibitors during the hydrolysis of acetic acid esters, Biochemistry 11 (1972) 4308–4321.
[25] T.L. Rosenberry, Acetylcholinesterase, Adv. Enzymol. Relat. Areas Mol. Biol. 43 (1975) 103–218.
[26] A. Shafferman, B. Velan, A. Ordentlich, C. Kronman, H. Grosfeld, M. Leitner, Y. Flashner, S. Cohen, D. Barak, N. Ariel, Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center, EMBO J. 11 (1992) 3561–3568.
[27] Z. Radic, D.M. Quinn, D.C. Vellom, S. Camp, P. Taylor, Allosteric control of acetylcholinesterase catalysis by fasciculin, J. Biol. Chem. 270 (1995) 20391–20399.
[28] Zaheer-ul-Haq, B. Wellenzohn, K.R. Liedl, B.M. Rode, Molecular docking studies of natural cholinesterase-inhibiting steroidal alkaloids from *Sarcococca saligna*, J. Med. Chem. 46 (2003) 5087–5090.
[29] E.M. Williamson, D.T. Okpako, F.J. Evans (Eds.), Selection, Preparation and Pharmacological Evaluation of Plant Material, vol. 1, Wiley, Chichester, 1996, 26 pp.
[30] D.J. Triggle, Drugs affecting calcium regulation and actions, in: G.M. Smith, A.M. Reynard (Eds.), Textbook of Pharmacology, Saunders, Philadelphia, 1992, pp. 453–479.
[31] H. Karaki, G. Weise, Mini reviews: calcium release in smooth muscles, Life Sci. 42 (1983) 111–122.
[32] A.F. Brading, How do drugs initiate contraction in smooth muscles, Trends Pharmacol. Sci. 2 (1981) 261–265.
[33] T.B. Bolton, Mechanism of action of transmitters and other substances on smooth muscles, Physiol. Rev. 59 (1979) 606–718.
[34] T. Godfraind, R. Miller, M. Wibo, Calcium antagonism and calcium entry blockade, Pharmacol. Rev. 38 (1986) 321–416.