Journal of Enzyme Inhibition and Medicinal Chemistry, December 2006; 21(6): 703–710

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healthcare

New natural cholinesterase inhibiting and calcium channel blocking quinoline alkaloids

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(Received 25 August 2005; accepted 25 March 2006)

Abstract

During this study, one new coumarin; 7-*O*-β-D-glucopyranoside-2*H*-1-benzopyran-2-one (**1**) and three quinoline alkaloids; 3-hydroxy, 2, 2, 6-trimethyl–3, 4, 5, 6-tetrahydro-2*H*-pyrano[3,2-c] quinoline 5-one (**2**), ribalinine (**3**) and methyl isoplatydesmine (**4**) were isolated from the aerial parts of *Skimmia laureola* and their structures established by spectroscopic studies. Compounds **2**-**4** were found to be linear mixed type inhibitors of acetylcholinesterase (K_i = 110.0, 30.0 and 30.0 μM, respectively). Compounds **2** and **3** were also found to be linear mixed type inhibitors of butyrylcholinesterase, while compound **4** was a noncompetitive inhibitor of the enzyme (K_i = 90.0, 70.0 and 19.0 μM, respectively). The inhibition of acetyl- and butyryl-cholinesterase enzymes persists as the most promising therapeutic strategy for activating the impaired cholinergic functions in Alzheimer's disease and related dementias.

Compound **4** also showed dose-dependent spasmolytic activity in the isolated rabbit jejunum intestinal preparation by relaxing the spontaneous (EC_{50} = 0.1 mg/mL) and K^+-induced contractions (EC_{50} = 0.4 mg/mL), suggesting that the spasmolytic effect of compound **4** is mediated through the blockade of voltage-dependent Ca^{2+} channels.

Keywords: *Quinoline alkaloids*, Skimmia laureola, *acetylcholinesterase*, *butyrylcholinesterase*, *enzyme inhibition*, *calcium channel blocker*

Abbreviations: *ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; BChE, butyrylcholinesterase; HMBC, Heteronuclear Multiple Bond Connectivity; PDB, Protein Data Bank; VDCs, voltage dependent Ca^{2+} channels.*

Introduction

Skimmia laureola Hook. is found in Kashmir and in the mountains of Northern Pakistan [1]. The pharmacological properties of quinoline alkaloids of *Skimmia laureola* have been previously investigated to a limited degree. Cytotoxic, phyototoxic, mutagenic, antibacterial, antifungal and anti-viral (HIV) activities have been reported for some quinoline alkaloids [2].

Acetylcholinesterase (acetylcholine acetyl hydrolase, AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) are cholinesterases; a family of related enzymes with divergent properties dependent on the species, tissue and source. AChE catalyzes the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) in the cholinergic synapses [3]. This enzyme is an attractive target for rational drug design and discovery of mechanism-based inhibitors for the treatment of many neurodegenerative disorders such as Alzheimer's disease (AD) and myasthenia gravis [4]. On the other hand, the physiological function of BChE is still unknown. However, it has been found

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ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360600889708

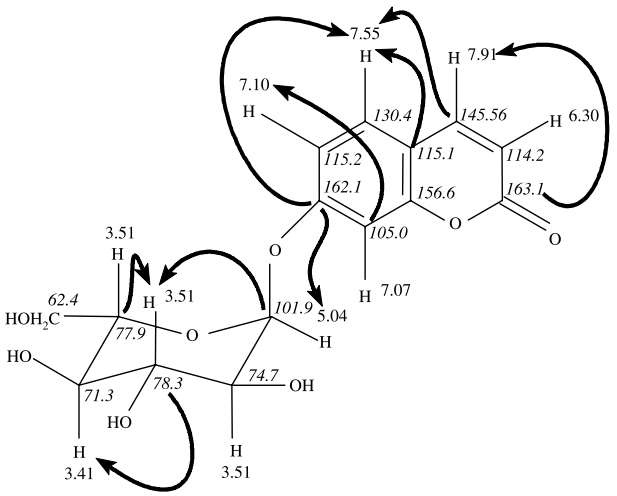


Figure 1. Key heteronuclear multiple bond connectivity (HMBC) interactions in compound **1**.

that BChE is present in significantly higher quantities in Alzheimer's plaques than in plaques of normal age-related non-demented brains [5]. Selective inhibitors of BChE such as cymserine analogues and the dual inhibitor of AChE and BChE, rivastigmine, showed potential therapeutic benefits in AD and related dementias [6,7]. The list of medications approved by the U.S. Food and Drug Administration (FDA) to delay the worsening of certain symptoms of AD contains only cholinesterase inhibitors such as donepezil (Aricept), rivastigmine (Exelon) and galantamine (Reminyl).

The active site of *Torpedo californica* AChE (which shares more than 70% sequence homology with the human enzyme) is buried near the bottom of a 20Å deep narrow gorge that penetrates halfway into the enzyme and widens out close to its base. This gorge is lined by 14 conserved aromatic amino acids and hence frequently called the 'aromatic gorge' [8]. The recently solved crystal structure of the BChE enzyme[9] was shown to be very similar to that of *Torpedo californica* AChE. However, several aromatic amino acids, lining the gorge of the AChE, have been replaced in BChE by hydrophobic analogues. Due to this, the BChE gorge becomes relatively larger and capable of accommodating bulkier and relatively non-polar ligands.

Since the discovery of physostigmine [10] as the first natural AChE inhibitor, only few other natural inhibitors have attracted the attention of neuropharmacologists [11]. We have previously reported a number of novel natural inhibitors of cholinesterases isolated from various medicinal plants [12–18]. Inhibition kinetics, SAR and CoMFA studies have also been conducted for an appreciable number of these inhibitors [17,19,20]. We report in this paper the isolation and AChE-and BChE-inhibitory properties of the natural quinoline alkaloids **2-4**, isolated from *Skimmia laureola*, and their mechanism of AChE and BChE inhibition. Spasmolytic and calcium channel blocking potential of compound **4** on isolated rabbit jejunum tissues is also described (Figure 1,2).

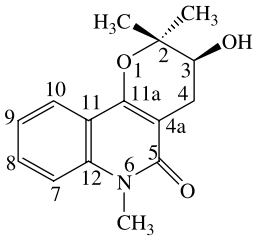
Materials and methods

Instrumentation

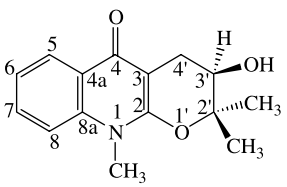
The aerial parts of *Skimmia laureola* Hook. (20 kg) were collected from Azad Kashmir, Pakistan. A voucher specimen (KUH # 58106) was deposited in the Herbarium of Department of Botany, University of Karachi. The mass spectra were recorded on a JEOL HX-110 instrument. The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ at 500, 400 and 125, 75 MHz, on a Bruker AM-500 NMR spectrometer. The UV and IR spectra were recorded on Shimadzu UV-240 and JASCO A-320 spectrophotometers, respectively. Optical rotations were measured on a polatronic D polarimeter. The purity of the compounds was checked on TLC (silica gel, Merck PF_{254}, 0.25 mm thickness). Melting points (uncorrected) were determined in glass capillary tubes using Buchi 535 and Gallenkamp 30/MF-370 melting point apparatus.

Isolation and structure elucidation

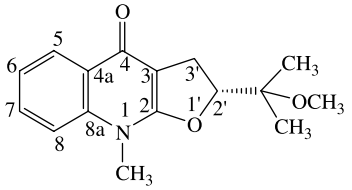
Air-dried aerial parts of *S. laureola* (20 kg dry weight) were extracted with EtOH (100 L). The EtOH extract was concentrated to a gum (822 g), dissolved in



2,2,6-Trimethyl-3,4,5,6-tetrahydro-2*H*-pyrano [3, 2-c] quinoline-5-one (**2**)



Ribalinine (**3**)



Methyl isoplatydesmine (**4**)

Figure 2. Chemical structures of compounds **2-4**.

distilled water and extracted with petroleum ether (45 L). The concentrated extract (66.92 g) was evaporated under reduced pressure and chromatographed on a silica gel column (Merck, 70–230 mesh, 2025 g). The combined sub-fractions 99–145 (1.91 g), obtained by elution of the column with petroleum ether-acetone (20:80, 500 mL each), were collected and again subjected to Column Chromatography (70–230 mesh, 60.20 g). Sub-fractions 7–18 (0.92 g, 500 mL each), obtained with the solvent system 20:80 hexane-acetone, showed similar behaviour and were combined and finally purified by preparative TLC (Merck PF_{254},0.2 mm) using $CHCl_3$ as eluent to obtain pure compound **3** (28 mg, $1.4 \times 10^{-4\%}$ yield, $R_f = 0.1$). After this, the original aqueous layer was acidified with acetic acid to pH 3.0, and then extracted with $CHCl_3$. The remaining aqueous acidic layer was made alkaline with NH_4OH to pH 12.0 and extracted with $CHCl_3$ (40 L). The $CHCl_3$ soluble portion was dried to give a crude alkaloidal mixture (74.96 g) which was chromatographed on a silica gel column (Merck, 70–230 mesh, 2015 g). Elution of this column with the solvent system 96% $CHCl_3$-MeOH, afforded fractions 20–26 (11.02 g, each fraction 500 mL each), which contained an impure mixture of compounds **2** and **4**. This mixture was repeatedly chromatographed on a SiO_2 gel column (2.5 cm × 70 cm) (Merck, 70–230 mesh, 322.11 g) and first eluted with $CHCl_3$ and then with MeOH: $CHCl_3$ (5:95). Fractions 55–90 (500 mL each, 3.96 g), obtained with 10% MeOH-90% $CHCl_3$ were again subjected to column chromatography over silica gel (70–230 mesh size, 99.24 g). The column (1.5 cm × 50 cm) was initially eluted with $CHCl_3$-MeOH (92:8) to afford eighteen fractions. All of these fractions were combined and finally purified by repeated TLC plates (Merck, PF 254, 0.5 mm) using $CHCl_3$-MeOH (70:30) to afford compound **4** (18.13 mg, 9.0x 10^{-5}% yield, $R_f = 0.32$). Fractions 90–96 (500 ml each) were obtained on elution with $CHCl_3$-MeOH (80:20) from the same column. These fractions were purified by preparative TLC (silica gel) using $CHCl_3$-MeOH (70:30) to afford compound **2** (20 mg, 1.0×10^{-4}% yield, $R_f = 0.47$). The remaining aqueous layer was freeze-dried (89.31 g) and chromatographed on a silica gel column (70–230 mesh size, 2930 g). Fractions 30–35 (500 mL each) obtained on elution with solvent system MeOH-$CHCl_3$ (25:75) showed similar behavior on TLC using ceric sulphate reagent and were combined (1.98 g) and subjected to chromatography (70–230 mesh, 32.89 g) and the column was eluted with the solvent system 73:27 $CHCl_3$-MeOH. This afforded semi-pure fractions (29–45, 1.20 g), which were subjected to preparative TLC (Merck, PF_{254}, 0.2 mm) by using $CHCl_3$-MeOH (72: 28) to afford pure compound **1** (12.52 mg, 6.2×10^{-5}% yield, $R_f = 0.6$).

***7-O-β-D-Glucopyranoside-2H-1-benzopyran-2-one (1)*:-**. White crystalline substance, M.P. 195–198° C; $[\alpha]_D^{29} = 92$ ($c = 0.010$, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε), 317 (3.9), 256 (3.1), 239 (3.5), 234 (3.5), 203 (4.2), 192 (4.7), 187 (4.7); IR (KBr) ν_{max} cm^{-1}, 3445 (OH), 1720 (six-membered lactone carbonyl carbon), 1622 (C = C); FAB MS (-ve) *m/z* 323; HREI MS; *m/z* 324.1616, Calcd. for $C_{15}H_{16}O_8$ 324.1628; ^{1}H-NMR (CD_3OD, 400 MHz): and ^{13}C-NMR; (CD_3OD, 125 MHz); δ (see Table I).

***3-Hydroxy-2, 2, 6-trimethyl-3, 4, 5, 6-tetrahydro-2H-pyrano [3,2-c] quinoline-5-one (2)*:-**. White amorphous substance; $[\alpha]_D^{29} = -57$ ($c = 0.138$, MeOH); UV λ_{max} (MeOH), 265 (2.67), 304 (3.84), 318 (3.86), 325 (3.74), 330 (3.76) nm; IR ($CHCl_3$) ν_{max}, 3340 (OH), 1120 (O-C) 1640, (C = O) cm^{-1}; 259.2212 (Calcd. 259.2220); HREI MS: *m/z* 188.0744 ($C_{11}H_{10}NO_2$), 200.0711 ($C_{12}H_{10}NO_2$); ^{1}H-NMR (D_2O, 400 MHz): ^{13}C-NMR (D_2O, 75 MHz) δ: (see Table I).

***Ribalinine (3)*:-**. The compound gave a red color test with Dragendorff's reagent; pale yellow gummy substance; $[\alpha]_D^{29} = 10$ ($CHCl_3$, $c = 1$); UV**:** (MeOH) λ_{max} nm, 238, 361, 328; IR ($CHCl_3$) ν_{max} cm^{-1}, 3295–3315 (OH), 1719 (six-membered lactone carbonyl carbon); HREI MS: *m/z* 188.0744 (calcd. 188.071149, $C_{11}H_{10}NO_2$), 218.0133 (calcd. 218.0817, $C_{12}H_{12}NO_3$); FD MS**:** *m/z* 259; 1H-NMR: ($CDCl_3$, 500 MHz); 13C-NMR ($CDCl_3$, 125 MH_z): δ.reported in the literature [21].

***Methyl isoplatydesmine (4)*:-**. White crystalline compound, M.P. = 73–75° C; $[\alpha]_D^{29} = 40$ ($c = 0.10$, $CHCl_3$); UV: (MeOH) λ_{max} 327 nm (log ε = 3.0), 314 nm (log ε = 3.1), 308 nm (log ϵ = 2.7), 237 nm (log ε = 3.2), 222 nm (log ε = 2.8), 211 nm (log ε = 3.1); IR ($CHCl_3$) ν_{max} cm^{-1}, 1620 (C = O), 1100 (OCH_3); EI MS: *m/z* (rel. int%); 156 (59%), 127 (18%), 200 (39%), 188 (100%), 202 (15%); HREI MS**:** *m/z* 200.0710 (calcd. 200.071149, $C_{12}H_{10}NO_2$), 202.0823 (calcd. 202.0867, $C_{12}H_{12}NO_2$), 188.0686 (calcd. 188.0711, $C_{11}H_{10}NO_2$), 172.0751 (calcd. 172.0762, $C_{11}H_{10}NO$), 127.0410 (calcd. 127.0421, C_9H_5N); FD MS: *m/z* 273, ^{1}H-NMR (D_2O, 400 MHz): δ; reported in the literature [22].

Enzyme Inhibition Assays

AChE and BChE inhibitory activities were measured *in vitro* by a modified spectrophotometric method developed by Ellman et al. [23]. The assay procedure has been previously described [18]. All the inhibition studies were performed using a SpectraMax microplate spectrophotometer (Molecular Devices, CA, USA).

Table I. ^{1}H- and ^{13}C-NMR chemical shift assignments for compounds **1** and **2**.

Carbon Nos	1			Carbon Nos	2		
	δ^{13}C	Multiplicity♣	δ^{1}H ($\mathcal{J}$ in Hz)#		δ^{13}C	Multiplicity♣	δ^{1}H ($\mathcal{J}$ in Hz)#
C-2	163.1	C	–	C-2	86.9	C	–
C-3	114.2	CH	6.30 (d, $\mathcal{J}_{3,4}$ = 9.4)	C-3	69.1	CH	3.92 (t, $\mathcal{J}_{3\alpha,4\alpha}$ = 9.4)
C-4	145.5	CH	7.91 (d, $\mathcal{J}_{4,3}$ = 9.4)	C-4	28.1	CH_2	2.71 (dd, $\mathcal{J}_{4\beta,4\alpha}$ = 16.4, $\mathcal{J}_{4\beta,3\alpha}$ = 4.9), 2.41 (dd, $\mathcal{J}_{4\alpha,4\beta}$ = 16.2, $\mathcal{J}_{4\alpha,3\alpha}$ = 6.6)
C-4a	115.1	C	–	C-4a	93.6	C	–
C-5	130.4	CH	7.55 (dd, $\mathcal{J}_{5,6}$ = 8.4, $\mathcal{J}_{5,8}$ = 1.2)	C-5	157.3	C	–
C-6	115.2	CH	7.10 (dd, $\mathcal{J}_{6,5}$ = 8.2, $\mathcal{J}_{6,8}$ = 3.9)	C-7	119.2	CH	7.42 (d, $\mathcal{J}_{7,8}$ = 8.0)
C-7	162.1	C	–	C-8	135.8	CH	7.21 (dd, $\mathcal{J}_{8,9}$ = 15.8, $\mathcal{J}_{8,7}$ = 8.1)
C-8	105.0	CH	7.07 (d, $\mathcal{J}_{8,6}$ = 3.9)	C-9	125.0	CH	7.55 (dd, $\mathcal{J}_{9,8}$ = 15.8, $\mathcal{J}_{9,10}$ = 8.3)
C-8a	156.6	C	–	C-10	127.4	CH	7.64 (d, $\mathcal{J}_{10,9}$ = 8.1)
C-1′	101.9	CH	5.04 (d, $\mathcal{J}_{1',2}$ = 7.6)	C-11	115.9	C	–
C-2′	74.7	CH	3.51 (m)	C-11a	157.2	C	–
C-3′	78.3	CH	3.51 (m)	C-12	139.0	C	–
C-4′	71.3	CH	3.41 (m)	C-2′	26.4	CH_3	1.59 (s)
C-5′	77.9	CH	3.51 (m)				
C-6′							

♣Multiplicity assignments based on DEPT experiment. #One-bond heteronuclear correlations determined by Heteronuclear Multiple Quantum Coherence (HMQC) experiment

Torpedo californica AChE (type VI-S, Sigma), and horse serum BChE (Sigma) were used as enzyme sources, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma) were used as substrates. Ellman reagent i.e. 5,5-dithiobis (2-nitro) benzoic acid (DTNB, Sigma) was used to develop the chromogenic marker for the measurement of the cholinesterase activity. Sodium phosphate buffers (1 mM) at pH 7.0 and 8.0 were used to prepare the enzyme working solutions and to assay enzyme activity at 30°C, respectively.

The initial rates of the enzymatic reactions were measured as the rate of change in OD/ min. (optical density/min.) and used in subsequent calculations. According to Ellman et al., the rate of the enzymatic reaction is calculated by the following equation[23];

$$\text{Rate} \quad (\text{mole/L/min}) = \frac{\text{change in absorbance/min.}}{13,600} \tag{1}$$

Compounds **2-4** were also studied for the inhibition of phosphodiesterase, urease, β-glucuronidase, and α- glucosidase enzymes using known methodology, and found to be inactive against them.

Estimation of kinetic parameters. The dissociation constant for the enzyme-inhibitor complex, K_i, and type of inhibition was determined from of Dixon plots, Lineweaver-Burk plots, and their secondary replots by using initial velocities. These velocities were measured over a range of substrate concentrations, 0.1–0.4 mM for ATCh and 0.05–0.2 mM for BTCh. The assay conditions for measurement of the residual activities of all inhibitors were identical to the aforementioned spectrophotometric assay procedure except that fixed concentrations of test compounds **2-4** were used in the assay medium.

Two graphical methods were applied to monitor the effect of the inhibitor on the V_{max} and K_m values (V_{maxapp} and K_{mapp}). Firstly, the reciprocal of the rate of the reactions were plotted against the reciprocal of the substrate concentration as a Lineweaver-Burk plot to determine V_{max}, K_m, V_{maxapp} and K_{mapp} values [24]. Secondly, Dixon plots were constructed by plotting the reciprocals of the rate of the reaction against the inhibitor concentration to estimate the K_i values [25].

The secondary replots of the Lineweaver-Burk plots were also constructed in two ways to estimate the K_i values; firstly, $1/V_{maxapp}$ values for every inhibitor concentration were replotted against the relevant concentration of the respective inhibitor. Secondly, in the case of the noncompetitive and linear mixed-type inhibitions, the slope of each line for each inhibitor concentration on the Lineweaver-Burk plot was plotted against inhibitor concentration. For the uncompetitive

type of inhibition, K_{mapp} was determined from the intersection of the lines of each inhibitor concentration on the x-axis of the Lineweaver-Burk plot and plotted against the inhibitor concentrations. The secondary replot of the Dixon plot was constructed as the slope of each line of substrate concentration obtained from original Dixon plot against the reciprocals of the substrate concentrations.

Antispasmodic assays

Antispasmodic activity of the test compound **4** was studied in isolated spontaneously contracting rabbit jejunum. Rabbits from a local breed of either sex (~1 kg), were obtained from the animal house of the Aga Khan University, Karachi. Animals were given free access to water but food was withdrawn 24 h prior to the experiment. Animals were sacrificed by a blow on the back of their heads, the abdomen was cut open and a piece of jejunum was taken out. Segments of 2 cm length 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: KCl 80 mM, NaCl 91.04 mM, $MgCl_2$ 1.05 mM, $NaHCO_3$ 11.87 mM, NaH_2PO_4 0.41 mM, $CaCl_2$ 1.8 mM, and glucose 5.55 mM. The spontaneous intestinal movements were recorded isotonically using Harvard transducers coupled with Harvard Student Oscillograph. Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug.

Calcium channel blocking activity of compound **4** was determined as described by Farre et al. [26].

Statistical analysis

All experiments described in this study were performed in triplicate. Enzyme inhibition graphs were plotted using GraFit program [27]. Values of the correlation coefficient, slope, intercept and their standard errors were obtained by linear regression analysis using the same software. The correlation coefficient for all the lines of the graphs was > 0.99.

The data from the pharmacological experiments were analyzed and plotted using GraphPad Prism software [28]. The results were expressed as mean ± standard mean of error (S.E.M). All statistical comparisons were made using Student's *t*-test, and a *P* value smaller than 0.05 was regarded as significant.

Results and discussion

Our phytochemical investigations on the arial part of *Skimmia laureola* resulted in isolation of a new coumarin; 7-*O*-β-D-glucopyranoside-2*H*-1-benzopyran-2-one (**1**) and three quinoline alkaloids, 3-hydroxy, 2, 2, 6-trimethyl−3, 4, 5, 6-tetrahydro-2*H*-pyrano[3,2-c] quinoline 5-one (**2**), ribalinine (**3**) and methyl isoplatydesmine (**4**). Compound **2** was isolated from this plant for the first time, although it has been previously reported as a synthetic compound [2]. Compounds **3** and **4** are known compounds, as identified by spectroscopic studies (Table I).

The new coumarin **1**, $C_{15}H_{16}O_8$, was isolated as a white crystalline substance. Its UV spectrum showed absorptions at 317, 256, 239, 234, 203, 192 and 187 nm indicating the presence of a coumarin skeleton [29]. The IR spectrum displayed strong absorptions at 3445 (OH), 1622 (C=C) and 1720 (C=O) cm^{-1}. FAB MS (−ve) showed a quasi-molecular ion at *m/z* 323 $[M-1]^-$. The HREI mass spectrum of **1** showed the M^+ at *m/z* 324.1616 (calcd. 324.1628) indicating the presence of eight double bond equivalents in the molecule. The peak at *m/z* 162.0321 (calcd. 162.0315, $C_9H_6O_3$) resulted from the cleavage of C-1′ and O-7.

The ^{1}H-NMR spectrum of **1** (Table I) showed two doublets at δ 7.91 and 6.30 which were assigned to the C-4 and C-3 vicinal methine protons of the α,β-unsaturated lactone ring [30]. Aromatic H-5 and H-6 appeared as double doublets at δ 7.55 and 7.10. The H-8 appeared as a doublet at δ 7.07. This pattern clearly indicated substitution at C-7 of ring 'A' of coumarin. The anomeric proton appeared at δ 5.04 ($\mathcal{J} = 7.6$ Hz), which was assigned to D-glucopyranoside [31]. The ^{13}C-NMR spectrum indicated the presence of 15 carbons, and closely resembled the ^{13}C-NMR data of other known C-7 glycosidic coumarins [32].

The anomeric (H-1′) signal at δ 5.04 showed coupling with C-7 in the HMBC experiment, indicating that the glucose moiety is linked to the C-7 oxygen of ring 'A' (Figure 1). On the basis of the above spectral data, structure **1** was assigned as 7-*O*-β-D-glucopyranoside-2*H*-1-benzopyran-2-one (**1**).

Compound **2** was obtained as a white amorphous substance. Compound **2** is a known synthetic compound[2] isolated from *Skimmia laureola* for the first time. The ^{1}H-NMR data of compound **2** has been partially reported but not the ^{13}C-NMR in the literature [2]. The IR spectrum showed absorptions at 3340 (OH), 1120 (O-C) and 1640 (C = O) cm^{-1} [33]. The HREI MS showed the M^+ at *m/z* 259.2212 (calcd. 259.2220). The peak at *m/z* 188.0744 ($C_{11}H_{10}NO_2$) may arise from the loss of C_4H_7O from M^+. The ^{1}H-NMR spectrum (Table I) showed three singlets at δ 3.52, 1.59 and 1.49 due to the NCH_3 and *gem*-dimethyl C-2′ and C-2″ protons, respectively. H-10 appeared as a doublet at δ 7.64 and its chemical shift indicated that the compound has a quinoline-5−one skeleton [34]. Two double doublets at δ 7.55, and 7.21 were assigned to H-9 and H-8. The downfield doublet at δ 7.42 was assigned to H-7. Two double doublets at δ 2.71 and 2.41 were assigned to H-4β and H-4α, respectively. The downfield chemical shift of the C-3 methine proton indicated the presence

Table II. Kinetic parameters of cholinesterases inhibited by quinoline alkaloids **2-4**.

Compound	Acetylcholinesterase		Butyrylcholinesterase	
	K_i * (μM) mean ± SEM	Type of Inhibition	K_i * (μM) mean ± SEM	Type of Inhibition
2	110.0 ± 1.5	LM	90.0 ± 1.5	LM
3	30.0 ± 1.05	LM	70.0 ± 1.05	LM
4	30.0 ± 1.9	LM	19.0 ± 1.05	NC
Tacrine	0.23 ± 0.02	MT	0.025 ± 0.003	MT
Galanthamine	0.19 ± 0.01	MT	32 ± 0.33	NC

* K_i is the mean of four values calculated from a Lineweaver-Burk plot, its secondary replot, and Dixon plot. SEM = standard mean error of 3-5 experiments. NC = noncompetitive; LM = linear mixed; MT = mixed type.

of an OH group at C-3 [35]. The ^{13}C-NMR spectrum indicated the presence of 15 carbons. On the basis of these spectroscopic studies, the structure of the compound was assigned as 3-hydroxy-2, 2, 6–trimethyl–3, 4, 5, 6–tetrahydro–2*H*–pyrano [3,2-c] quinoline–5–one (**2**).

On the basis of spectroscopic studies, compound **3**, was identified as ribalinine, previously isolated from *Balfourodendron riedelianum* [21]. Methyl isoplatydesmine (**4**) ($[\alpha]_D^{29}$=40) was earlier isolated from this plant by us [22].

The quinoline alkaloids, **2-4** were screened for their inhibition potential on AChE, BChE, phospholipase A_2, phosphodiesterase, urease, acid phosphatase, β-glucuronidase, and α- glucosidase enzymes. These compounds were found to be specific concentration-dependent inhibitors of AChE and BChE (Table II) and were inactive at concentrations upto 1 mM against all the other enzymes.

All these compounds were found to be linear mixed inhibitors of both cholinesterases except compound **3** which exhibited a pure noncompetitive inhibition against BChE. Linear mixed inhibitors decrease both the V_{max} values and the affinity of the substrate to the enzyme e.g. AChE inhibition by compound **3** (Figure 3). The mixed inhibition showed by quinoline alkaloids under study was found to be a combination of partially competitive and pure noncompetitive inhibitions. This suggest that these compounds are structurally capable of competing with the substrate for the active site, while they can also bind to other subsites of the AChE and BChE aromatic gorge such as the peripheral anionic site at the top of the gorge.

The pure noncompetitive inhibition of BChE by compound **3** was envisaged from the ability of the compound to decrease the V_{max} value without affecting the affinity of the enzyme for the substrate (K_m values). The purity of the noncompetitive inhibition was further predicted by the linear lines obtained in the secondary replots of Lineweaver-Burk plots.

As shown in Table II, compound **4** was found to be the most potent inhibitor of BChE, while compounds **3** and **4** were equally potent against AChE. On the other hand, compounds **2** and **4** were found to be slightly selective toward the BChE, while compound **3** was found to be a selective inhibitor of AChE.

It was mentioned earlier that several amino acids of the AChE gorge has been replaced in BChE by hydrophobic and/or smaller amino acids residues to enable BChE to accommodate bulkier ligands. The selectivity of compounds **2** and **4** toward BChE could be, therefore, explained in part by their ability to easily go through the relatively larger aromatic gorge of BChE [8]. Hydrophobic interactions, conversely, which are more prominent in the case of BChE, might play an important role in the selectivity of these compounds toward the enzyme.

Since none of the known AChE inhibitors is structurally similar to the compounds under study, tacrine and galanthamine were randomly selected as standards to compare the potency of the test compounds against both enzymes. Galanthamine is a

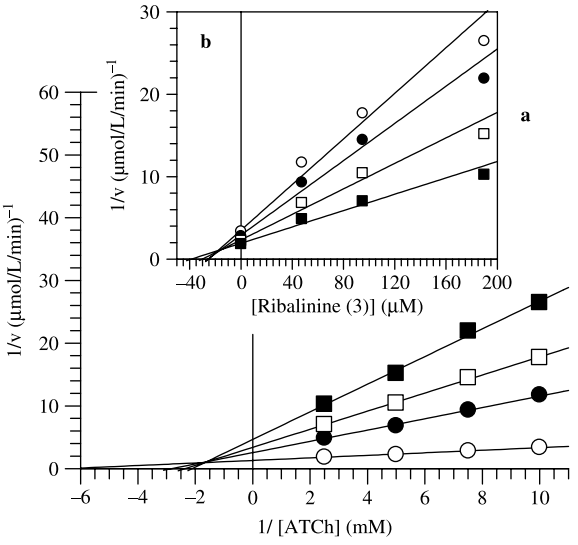


Figure 3. Steady state inhibition of AChE by ribalinine (**3**), (a) Lineweaver-Burk plot of reciprocal of the initial velocities versus reciprocal of ATCh in absence (○) and presence of 47.4 μM (•), 94.9 μM (□) and 189.8 μM (■) of ribalinine (**3**), (b) Dixon plot of reciprocal of the initial velocities versus various concentrations of inhibitor at four fixed ATCh concentrations: (○), 0.1 mM; (•), 0.13 mM; (□) 0.2 mM and (■), 0.4 mM.

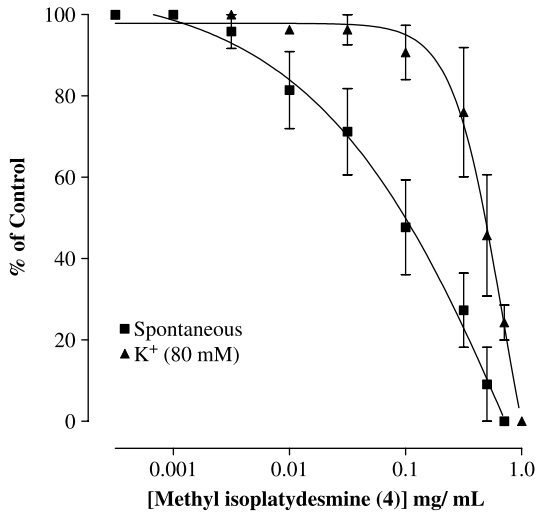


Figure 4. Inhibitory effect of the quinoline alkaloid **4** on the spontaneous and K^+-induced contractions in isolated rabbit jejunum.

natural and reversible AChE and BChE inhibitor, which has a wide range of clinical applications. The K_i values and the type of inhibition of AChE and BChE by tacrine and galanthamine were also investigated and are shown in Table II.

Compound **4** was further studied for its possible spasmolytic (antispasmodic) effect in isolated rabbit jejunum. Rabbit jejunum was selected due to its spontaneous activity, which helps in screening both muscle relaxing as well as muscle contracting agents [36]. In the absence of compound **4**, rabbit jejunum exhibited spontaneous contractions and the behavior of the tissue did not change over the time course of the experiments. It was found that compound **4** produced a dose-dependent inhibition of spontaneous contractions of the jejunum ($EC_{50} = 0.1 \pm 0.04$ mg/ml, $n = 3$), suggesting spasmolytic activity (Figure 4). The contractions of smooth muscle preparations are dependent upon the increase in the cytoplasmic free Ca^{2+}, which activates the contractile elements [37]. The increase in intracellular Ca^{2+} is due to either influx *via* voltage dependent Ca^{2+} channels (VDCs) or to the release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum. To test whether the spasmolytic effect of compound **4** is mediated through the interference with the Ca^{2+} release or through the blockade of Ca^{2+} influx mechanisms through VDCs, a high dose of K^+(80 mM) was applied to depolarize the tissue. Compound **4** showed a dose-dependent inhibition of K^+-induced contractions ($EC_{50} = 0.4 \pm 0.08$ mg/mL, $n = 3$), in a mode similar to that of verapamil, a standard calcium channel blocker [38], which exhibits calcium channel blocking activity (Figure 4).

It is important to mention that the calcium channel blockers, which are already used widely to treat high blood pressure and other problems, also have potential anti-Alzheimer's activity. In elderly patients with isolated systolic hypertension, active treatment starting with the calcium-channel blocker nitrendipine reduces the incidence of dementia by 50% [39,40]. Currently, several calcium channel blockers such as nimodipine and sabeluzole are undergoing phase III clinical trials for the treatment of AD [41,42]. All the results of this study indicated the quinoline alkaloids **2-4** as interesting leads for anti-Alzheimer's drug development being cholinesterase inhibitors, and potential Ca^{2+} channels blockers.

Acknowledgements

The authors wish to thank the Ministry of Science and Technology, Government of Pakistan for providing the financial support for the current study under the Pak-Kazakh Scientific Cooperation Program.

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