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New diterpenoid alkaloids from *Aconitum heterophyllum Wall*: Selective butyrylcholinestrase inhibitors

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Abstract

Two new diterpenoid alkaloids, heterophyllinine-A (**1**) and heterophyllinine-B (**2**), along with two known alkaloids dihydroatisine (**3**) and lycoctonine (**4**) were isolated from the roots of *Aconitum heterophyllum* Wall. The structure of (**1**) and (**2**), were deduced on the basis of spectral data. Compounds **1**–**2** inhibited acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) enzymes in a concentration-dependent manner with percent inhibition ranging between 4.24% and 6.94 % and 79.1 % and 82.75 % for AChE and BChE, respectively indicating that compounds **1** and **2** are about thirteen times more specific to BChE than AChE.

Keywords: *Aconitum heterophyllum* wall, norditerpenoid alkaloids, heterophyllinine-A, heterophyllinine-B, acetylcholinesterase, butyrylcholinesterase

Introduction

Genus *Aconitum* is a rich source of diterpenoid alkaloids, many of which exhibit a broad spectrum of biological activities. Lappaconitine hydrobromide has been used as an antiarrhythmic drug [1]. Methyllycaconitine perchlorate is used in a curaremimetic preparation [2]. Some aconitine and mesaconitine derivatives possess potent analgesic and anti-inflammatory activities [3]. The methyllycaconitine and lycaconitine exhibited neuronal nicotinic acetylcholine receptor affinity [4]. Lycaconitine, obtained from several *Aconitum* species, was found to be effective against multi-drug resistant cancers. *Aconitum* plants are widely used in Chinese and Indian traditional systems of medicine [5,6]. Turkish *Aconitum* species are used externally in the treatment of rheumatic pain and sciatica and also against body lice [7]. Previously, heterophyllisine, heterophylline, heterophyllidine, heteratisine, atisine, atidine, F-dihydroatisine, hetisine, benzoylheteratisine and atisenol were reported from *A. heterophyllum* [8–11].

AChE (EC 3.1.1.7) is a key component of cholinergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine [12]. According to the cholinergic hypothesis, memory impairments in patients with the senile dementia diseases are due to a selective and irreversible deficiency in the cholinergic functions in the brain [13]. The role of BChE (EC 3.1.1.8) in normal aging and brain diseases is still elusive. It has been found that BChE is present in significantly higher quantities in Alzheimer's plaques than in plaques of normal age related non-demented brains [14]. The discovery of natural cholinesterase inhibitors has been a very challenging area of drug development due to the involvement of cholinesterases

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in Alzheimer's disease and other related dementias. We have previously reported a number of new natural inhibitors of cholinesterases (AChE and BChE) isolated from indigenous medicinal plants [15,16].

Herein we report the isolation and structure elucidation of two new diterpenoid alkaloids from *A. heterophyllum* and their cholinesterase inhibition potential.

Experimental

General experimental

Optical rotations were measured on a JASCO DIP 360 polarimeter. IR spectra were recorded on a JASCO 302-A spectrophotometer. EI-MS and HREI-MS were recorded on JMS HX 110 with data system and on JMS-DA 500 mass spectrometers. The ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker NMR spectrometers operating at 400 MHz, (100 and 125 MHz for ^{13}C). The chemical shifts values are reported in ppm (δ) units and the coupling constants ($\mathcal{J}$) are given in Hz.

Chromatographic conditions

For TLC, precoated aluminum sheets (silica gel 60F-254, E. Merck) were used. Visualization of the TLC plates was achieved under UV at 254 and 366 nm and by spraying with Dragendorff's reagent. Solvent system; "*n*-hexane-acetone-diethylamine (8:2:10)", was used to monitor the separation profile.

Plant material

The roots (5 kg, dry wt) of *Aconitum heterophyllum* Wall. were collected from Swat, N.W.F.P., Pakistan, at an elevation of 2000 m in August 2005 and identified by Mr. Mehboob ur Rahman, Assistant Professor, Department of Botany, Jahanzeb Post Graduate College, Saidu Sharif, Swat, NWFP, Pakistan. The voucher specimen (HA-014) is deposited in the herbarium of the botany department.

Extraction and isolation

Dried and powdered roots (5 Kg) of the plant were extracted exhaustively with *n*-hexane (3 × 8 L) followed by 80% EtOH (3 × 10 L) extraction at room temperature for 7 days (3-times). The filtrate was concentrated *in vacuo* to yield 60 g of residue. The residue was acidified to pH 1.5 with 0.5 N H_2SO_4 and extracted with CH_2Cl_2 (3 × 2 L) to afford alkaloid mixture (18 g). The acidic aqueous solution was basified (pH 8–10) by using 10% KOH (aq) and extracted with CH_2Cl_2 (5 × 2 L) to yield 13.8 g of alkaloid mixture. The crude basic fraction was fractionated on silica gel column (260 g) and five combined fractions were obtained. On repeated flash column chromatography using solvent system *n*-hexane-acetone (9:1) containing 10 drops of diethylamine per 100 ml. Heterophyllinine-A (**1**), Heterophyllinine-B (**2**), along with two known alkaloids dihydroatisine (**3**) and lycoctonine (**4**) were obtained.

*Heterophyllinine-A (**1**)*. Amorphous powder (15 mg). mp 110–112°C; $[\alpha]_D^{30}$ −81.1 (*c* 0.8, $CHCl_3$); IR $\upsilon_{max}CHCl_3$, 3492 (OH groups), 3086, 1658, 900 (C═CH_2, terminal methylene), 1376 (CCH_3), ^{1}H-NMR (400 MHz, $CDCl_3$): see Table I. ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table I, EIMS (M^+ m/z): $C_{22}H_{33}NO_2$, (343.518).

*Heterophyllinine-B (**2**)*. Amorphous powder (20 mg). mp., 68–70°C; $[\alpha]_D^{30}$ −68.0 (*c* 1.0, $CHCl_3$); IR $\upsilon_{max}CHCl_3$, 3492 (OH groups), 3086, 1658, 900 (C═CH_2, terminal methylene), 1376 (CCH_3), 1735 (C═O). ^{1}H-NMR (400 MHz, $CDCl_3$): see Table I. ^{13}C-NMR ($CDCl_3$, 100 MHz): see Table I, EIMS (M^+ m/z): $C_{24}H_{35}NO_4$, (401.398).

Enzyme inhibition assay

The standard operational assay protocol was employed to determine the AChE and BChE inhibition activities of natural products, by modifying the spectrophotometric method of Ellman *et al.* (1961) [17]. Enzymes and reagents such as electric-eel AChE (EC 3.1.1.7), horse-serum BChE (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). All other chemicals were of molecular biology grade. Acetylthiocholine iodide and butyrylthiocholine chloride were used as the substrates to assay AChE and BChE activities, respectively. The reaction mixture contained 150 μL of sodium phosphate buffer (100 mM) (pH 8.0), 10 μL of DTNB, 10 μL (0.2 mM) of test-compound solution and 20 μL of AChE or BChE solution, which were mixed and incubated for 15 min. (25°C). The reaction was then initiated with the addition of 10 μL acetylthiocholine or butyrylthiocholine, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion resulting from the reaction of DTNB with thiocholine, catalyzed by acetylthiocholine and butyrylthiocholine, respectively at a wavelength of 412 nm (15 min.). Test-compounds and the positive control (galanthamine) were dissolved in EtOH. As the extinction coefficient of the yellow anion is known, the rate of enzymatic reaction was

Table I. ^{1}H- and ^{13}C-NMR data of compound **1** and **2** (400 & 100 MHz, $CDCl_3$).

	Heterophyllinine-A (**1**)			Heterophyllinine-B (**2**)		
C. No	δ_C	Multiplicity	δ_H, $\mathcal{J}$ HZ	δ_C	Multiplicity	δ_H, $\mathcal{J}$ HZ
1	41.2	CH_2	1.89, 1.52, *m*	41.2	CH_2	1.05, 1.41, *m*
2	23.1	CH_2	1.74, 1.56, *m*	20.5	CH_2	1.61, 1.50, *m*
3	33.2	CH_2	1.34, 0.94, *m*	28.0	CH_2	1.58, 1.32, *m*
4	39.3	C	–	41.0	C	–
5	38.0	CH	2.26, br *s*	50.1	CH	0.88, br *s*
6	28.8	CH_2	1.71, *m*	77.9	CH	3.29, *m*
7	40.9	CH	2.40, *m*	41.8	CH_2	1.67, 1.44, *m*
8	38.7	C	–	41.0	C	–
9	50.1	CH	2.02, *t*, 11.6	41.3	CH	0.59, *m*
10	50.5	C	–	38.0	C	–
11	27.5	CH_2	0.93, 0.87, *m*	29.2	CH_2	0.98, 0.86, *m*
12	38.0	CH	1.46, *m*	33.2	CH	1.90, *m*
13	27.5	CH_2	1.41, 1.39, *m*	27.5	CH_2	1.74, 1.52, *m*
14	29.1	CH_2	1.70, 1.59, *m*	27.5	CH_2	1.45, 0.98, *m*
15	77.8	CH	3.50, br *s*	77.9	CH	3.49, br *s*
16	156.7	C	–	156.8	C	–
17	110.3	CH_2	5.03, 4.98, br *s*	110.2	CH_2	5.03, 4.98, br *s*
18	20.5	CH_3	0.82, br *s*	23.1	CH_3	0.98, br *s*
19	51.0	CH_2	2.91, 2.83, *d*, 10.27	100.0	CH	4.01, *s*
20	60.6	CH	3.62, *d*, 5.92	51.0	CH_2	2.91, 2.82, *d*,
N-CH_2 / −O-CH_2	59.6	CH_2	3.07, 2.45, *m*	55.8	CH_2	10.26
O-C=O / CH_3	55.8	CH_2	3.15, *t*, 10.88	59.6	CH_2	3.28, 2.54, *m*
	–	–	–	169.5	C	3.91, 3.79, *m*
	–	–	–	24.3	CH_3	– 2.02, br *s*

finally determined by applying the Ellman equation:

$$\text{Rate (mols/L/min.)} = \frac{\text{Change in absorbance/min.}}{13,600}$$

All the inhibition studies were conducted in 96-well micro-titer plates using Spectra Max-340 (Molecular Devices, CA, USA). Concentration of compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) was determined by monitoring effect of various concentrations of the compound on inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program [18].

Results and discussion

Heterophyllinine-A (**1**) was obtained as a white amorphous powder, and was assigned the molecular formula $C_{22}H_{33}NO_2$ on the basis of EI-MS (M^+ $m/z = 343$) and NMR spectral data. The ^{1}H- and ^{13}C-NMR spectra of heterophyllinine-A (**1**) exhibited a close resemblance to that of the known compound atidine [19] except for the presence of a methane group instead of carbonyl and methylene at both C-7 and C-20. Its IR spectrum also showed characteristic signals at 3492 (OH groups), 3086, 1658, 900 (C=CH_2, terminal methylene), 1376 (CCH_3). In the down field region of the ^{1}H-NMR spectrum of heterophyllinine-A (**1**) two broad singlets each of one proton at δ 5.03 and 4.98, were assigned to the methylene group. A broad singlet of one proton at δ 3.50 was assigned to H-15. A triplet of one proton integration at δ 2.02 ($\mathcal{J} = 11.6$ Hz), was due to the C-9 methine proton. Similarly, two doublets each of one proton integration at δ 2.91 and 2.83 ($\mathcal{J} = 10.27$), were assigned to the methylene protons attached at C-19. A broad singlet of one proton at δ 2.26 was assigned to the H-5, while, a multiplet of one proton at δ 2.40 was assigned to H-7. The ^{13}C-NMR spectrum (BB, DEPT) (Table I), showed 22 signals, including one methyl, eleven methylene, six methine, and four quaternary carbons. The ^{1}H- ^{13}C correlation was determined by the HMQC spectrum, while the long range ^{1}H- ^{13}C connectivities were obtained through HMBC technique (see HMBC of compound 1). The H-5 (δ 2.26) showed $^{2}\mathcal{J}$ and $^{3}\mathcal{J}$ correlation with C-4 (δ 39.3), C-10 (δ 50.5), C-6 (δ 28.8), and C-7 (δ 40.9), whereas H-15 (δ 3. 30), exhibited $^{1}\mathcal{J}$ and $^{2}\mathcal{J}$ correlation with C-15 (δ 77.8), C-16 (δ 156.7), and C-14 (δ 29.1), while, H-12 (δ 1.46), showed correlation with C-12 (δ 38.0), C-16 (δ 156.7), C-17 (δ 110.3), C-13 (δ 27.5), and C-11 (δ 27.5).

Thus on the basis of above spectral data, the structure of compound **1** was deduced as heterophyllinine-A.

Heterophyllinine-B (**2**), was obtained as a white amorphous powder, and was assigned the molecular formula $C_{24}H_{35}NO_4$, on the basis of EI-MS (M^+ $m/z = 401$), and NMR spectral data. The ^{1}H- and ^{13}C-NMR spectra of heterophyllinine-B (**2**) exhibited a close resemblance to that of the known compound isoatisine [20,21] except the presence of acetyl group instead of two hydroxyl groups at C-15 and at C-6. Its IR spectrum also showed characteristic signals at 3356 (OH groups), 3012, 1656, 893 (C═CH_2 terminal methylene), 1385 (CCH_3), 1685 (C═O). In the down field region of the ^{1}H-NMR spectrum of heterophyllinine-B (**2**) two broad singlets each of one proton at δ 5.03 and 4.98, were assigned to the methylene group. A broad singlet of one proton at δ 3.49 was assigned to H-15. Similarly, a singlet of one proton integration at δ 4.01 was assigned to the methine proton attached at C-19. A broad singlet of one proton at δ 0.88 was assigned to the H-5. The ^{13}C-NMR spectrum (BB, DEPT) (Table I), showed 24 signals, including two methyl, eleven methylene, six methine, and five quaternary carbons. The ^{1}H- ^{13}C correlation was determined by the HMQC spectrum, while the long-range ^{1}H- ^{13}C connectivities were obtained through HMBC technique (see HMBC of compound **2**). The H-5 (δ 0.88) showed ^2J and ^3J correlation with C-4 (δ 41.0), C-10 (δ 38.0), C-6 (δ 77.9), and C-7 (δ 41.8), whereas H-15 (δ 3.49), exhibited ^1J and ^2J correlation with C-15 (δ 77.9), C-16 (δ 156.8), and C-14 (δ 27.5).

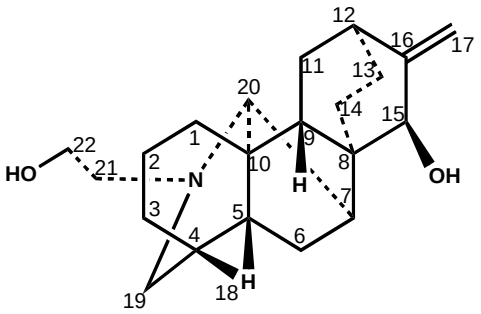
Table II. Inhibition of acetylcholinestrase (AChE) and butyrylcholinestrase (BChE) by compounds **1**, **2** and galanthamine.

Enzymes	Compounds	IC_{50} (μM) ± SEM	% Inhibition
AChE	Compound (**1**)	–	4.24
AChE	Compound (**2**)	–	6.94
BChE	Compound (**1**)	32.97 ± 0.533	79.1
BChE	Compound (**2**)	40.63 ± 1.11	82.75
AChE	Galanthamine[a]	0.5 ± 0.001	85.21
BChE	Galanthamine[b]	8.5 ± 0.02	81.36

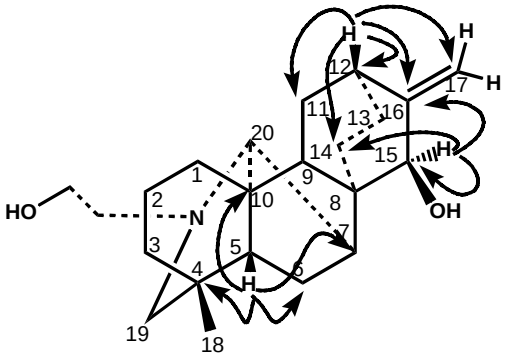
[a,b] Standard inhibitors of AChE and BChE

Thus on the basis of above spectral data, the structure of compound **2** was deduced as heterophyllinine-B.

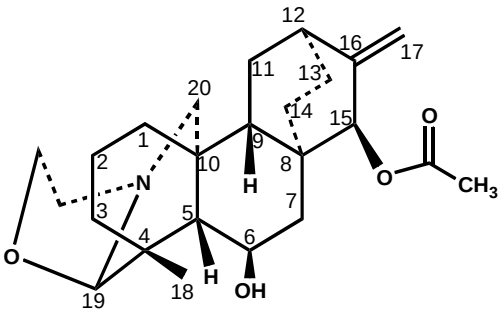
From the activity of both tested compounds (Table II) it can be concluded that compound **1** can be better accommodated than compound **2** for AChE and as well for BChE. Compound **1** has two hydroxyl groups at position C-15 and C-22 which facilitate penetration inside the aromatic pocket of both enzymes, while in case of compound **2** both hydroxyl groups are unavailable due to natural transformation in biological system. Therefore compound **2** is unable to penetrate into the active site of the enzymes. The size and shape of compound **1** supports its strong binding due its hydrophilic hydroxyl moieties which are readily available for hydrogen bonding with receptor, while compound **2** due to its hydrophobic nature is unable to form such strong hydrogen bonding with the target protein in both of the cases.



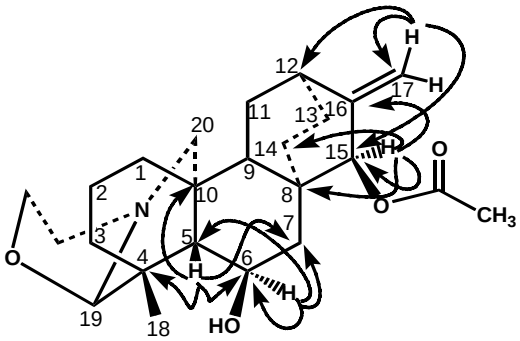
Structure of compound 1.



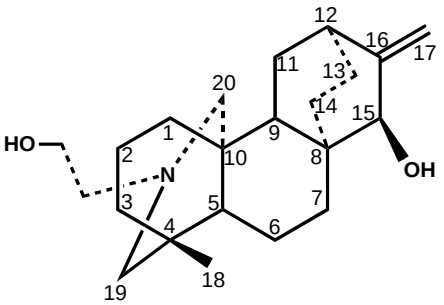
Key HMBC interaction in compound 1.



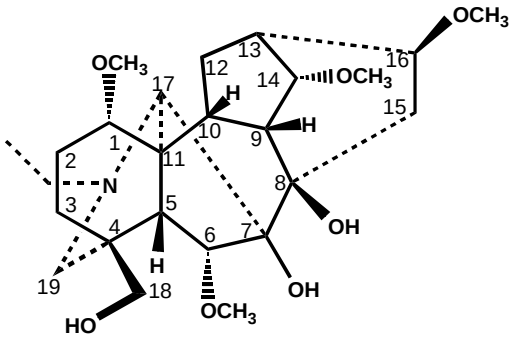
Structure of compound 2.



Key HMBC interaction in compound 2.



Dihydroatisine (3)



Lycoctonine (4)

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