Four New Diterpenoids from *Ballota limbata*

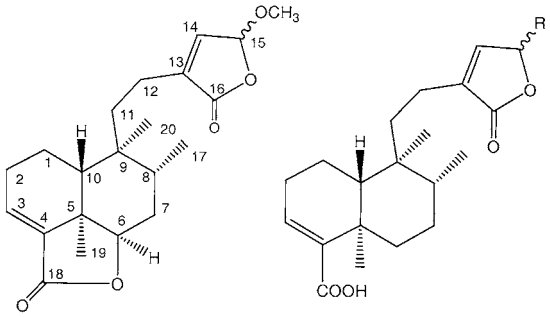
by **Viqar Uddin Ahmad***, **Umar Farooq**, **Ahmed Abbaskhan**, **Javid Hussain**, **Muhammad Athar Abbasi**, **Sarfraz Ahmad Nawaz**, and **M. Iqbal Choudhary**

International Center for Chemical Sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan
(phone: +92 21-9243223; fax: +92 21-9243190; e-mail: vuahmad@cyber.net.pk)

Six diterpenoids (**1**–**6**) belonging to the clerodane and tetracyclic diterpene types were isolated from the $CHCl_3$ extract of *Ballota limbata*. The structures of the new compounds **1** and **4**–**6** (named ballatenolide A and limbatenolides A–C, resp.) were established by their spectral data, and their relative configuration was determined by 2D NMR. Compounds **2** and **3** were identified as known clerodanes. Compound **1** had a unique feature, *i.e.*, an α,β-unsaturated γ-lactone moiety at C(4)/C(6), whereas limbatenolides A–C (**4**–**6**) belonged to the rare class of tetracyclic diterpenoids, which are known to occur only in the genus *Ballota*. All of the isolated compounds showed inhibitory potential in a cholinesterase-inhibition assay.

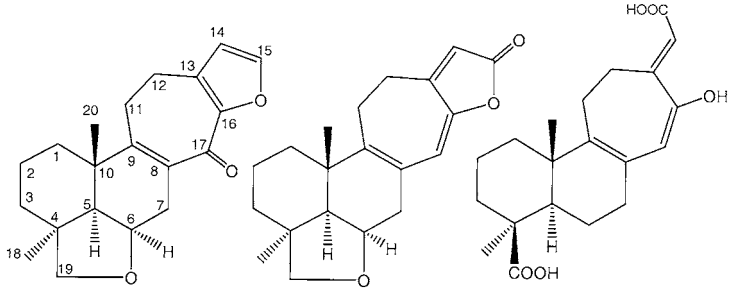
Introduction. – The genus *Ballota* (Lamiaceae) comprises *ca.* 33 species, mainly occurring in the Mediterranean region [1]. In Pakistan, only two species have been found, namely *Ballota aucheri* and *Ballota limbata* BOISS. (syn. *Otostigia limbata*; Lamiaceae). *Ballota limbata* is locally called 'Bui' or 'Phut kandu' [2]. *Ballota limbata* is widely distributed in the Northwest Frontier Province and lower hills of West Punjab in Pakistan, and traditionally, it has been used in the treatment of children gum diseases and for ophthalmia in man [3]. Moreover, the species of the genus *Ballota* are widely used by the traditional practitioners against various diseases, and its constituents have shown to possess antiulcer, antispasmodic, antidepressant, anxiolytic, and sedative activities [4]. Here, we report the isolation and structure elucidation of six diterpenoids among which four are new and two are known constituents. Among the new compounds, one is a clerodane-type diterpenoid, and the other three are of the rare tetracyclic-diterpenoid type.

All the compounds **1**–**6** showed inhibitory potential against butyrylcholinesterase. Against acetylcholinestrase, only **1**–**3** displayed inhibition, in a concentration-dependent fashion with promising IC_{50} values (see below). Acetylcholinesterase (AChE, EC 3.1.1.7) is known to be a key component of cholinergic brain synapses and neuromuscular junctions as it reduces memory deficiency in *Alzheimer*'s disease patients by potentiating and affecting the cholinergic transmission process [5]. Acetylcholinesterase terminates the impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine [6]. The inhibitors of acetylcholinesterase increase the endogenous level of this compound in the brains of *Alzheimer*-disease patients and, thereby, boost cholinergic neurotransmission. Recently, it has been found that butyrylcholinesterase (BChE, EC 3.1.1.8) inhibition may also be an effective tool for the treatment of *Alzheimer*'s disease and related dementias [7].



1

2 R=OMe
3 R=H



4 **5** **6**

Results and Discussion. – The $CHCl_3$ extract of the air-dried whole plant of *Ballota limbata* was subjected to chromatography (silica gel) to give one new clerodane-type diterpene, named ballatenolide A (**1**), and three new representatives of the rare class of tetracyclic diterpenoids, named limbatenolides A – C (**4** – **6**), along with the two known compounds **2** and **3**.

Compounds **1** – **3** were identified as clerodane-type diterpenoids. Their UV and IR data indicated the presence of an α,β-unsaturated γ-lactone, and their structures were established by a detailed analysis of their spectral data and comparison with those of closely related known compounds.

The α,β-unsaturated γ-lactone and the α,β-unsaturated carbonyl group of **1** – **3** gave rise to a λ_{max} at 210 nm and absorptions at 1752 – 1769 cm^{-1} and 1678 – 1681 cm^{-1}, respectively, similar to the absorption maxima of 'neocleroda-3,13-diene-15,16-olide-18-oic acid' [8]. An absorption at 2980 cm^{-1} suggested the presence of a COOH group in **2** and **3** [8][9]. The tricyclic clerodane skeleton of **1** – **3** was identified by their unique ^{1}H-NMR spectrum (*Table 1*) in which the two tertiary Me groups appeared as *s* at δ 1.01 – 1.22 and 0.86 – 0.74, respectively, and one secondary Me group as *d* at δ 0.8 – 0.96 ($J = 6.0$ Hz). Apart from these typical highfield Me signals, the downfield region showed two olefinic CH signals at δ 6.46 – 6.83 and 6.75 – 7.07 with very small *J* values, which is typical for most of the clerodanes. These compounds were distinguished as β-substituted analogues of clerodanes [9] as both of these individual downfield olefinic H-atoms showed interactions with a downfield quatenary olefinic C-atom at δ(C) 134 – 140.4 and a C=O at δ(C) 171 – 174.3 in the HMBC spectrum.

The high-resolution mass spectrum of **1** exhibited the highest peak at *m/z* 342 ($[M - H_2O]^+$, $C_{21}H_{28}O_5^+$). The ^{13}C-NMR DEPT experiment disclosed the presence of six quaternary C-atoms including the two olefinic C-atoms and the two C=O groups (*Table 2*). The presence of two γ-lactone moieties in **1** was confirmed by

Table 1. *^{1}H-NMR* ($CDCl_3$) *Data of Compounds* **1**–**6**. δ in ppm, J in Hz.

1	**2**	**3**	**4**	**5**	**6**	
CH_2(1)	1.50–1.67 (*m*)[a])	0.73[a])	0.74[a])	1.26–1.38 (*m*)[a]) 2.01–1.82 (*m*)[a])	1.90–1.97 (br. *m*)	2.15–2.4 (*m*)[a])
CH_2(2)	2.43–2.49 (*m*)	2.20–2.31 (*m*)	2.39–2.43 (*m*)	1.80–1.89 (*m*)[a])	1.81–1.90 (br. *m*)	1.53–1.55 (br. *m*)
H–C(3) or CH_2(3)	6.46 (br. *s*)	6.83 (br. *t*, *J* = 2.1)	6.83 (br. *t*, *J* = 3.1)	2.49–2.62 (*m*)[a])	1.20–1.29[a])	1.95–2.0 (*m*) 2.07 (*dd*, *J* = 5.17)
H–C(5)	–	–	–	1.33[a])	1.19[a])	1.36 (br. *d*, *J* = 12)
H–C(6) or CH_2(6)	3.69 (*dd*, *J* = 3.5, 7.5)	2.30 (*m*), 1.12 (*dd*, *J* = 5.5, 12)	2.32–1.13 (*m*)	3.46 (*dd*, *J* = 5.3, 11)	3.45 (*dd*, *J* = 5, 11)	1.69–1.79 (*m*) 1.95–2.0 (*m*)
CH_2(7)	1.99–2.03 (*m*)	1.40–1.51 (*m*)[a])	1.37–1.50 (*m*)[a])	1.90–1.81 (*m*)	2.230–2.27 (*m*)[a])	1.79–1.95 (*m*)[a])
H–C(8)	1.78 (*m*)	1.44[a])	1.59–1.69 (*m*)	–	–	–
H–C(10)	1.43 (br. *d*, *J* = 12)	1.30 (br. *d*, *J* = 11.5)	1.31 (br. *d*, *J* = 11.5)	–	–	–
CH_2(11)	1.44–1.60 (*m*)[a]), 2.10 (*ddd*, *J* = 4.8, 11.6)	1.40–1.5[a])	1.42–1.53 (*m*)[a])	2.56–2.47 (*m*)[a])	1.31–1.39 (*m*)	1.79–1.88 (br. *m*) 2.41–2.47 (*m*)
CH_2(12)	2.15–2.21 (*m*)	2.01 (br. *t*, *J* = 14), 2.18 (*m*)	2.01 (br. *t*, *J* = 12.5) 2.15 (br. *t*, *J* = 13)	2.80–2.62[a])	2.30–2.25 (*m*)[a])	2.32–2.41 (*m*)[a])
H–C(14)	6.75 (*t*, *J* = 1.4)	6.73 (br. *s*)	7.07 (br. *s*)	6.31 (*d*, *J* = 1.6)	5.78 (*s*)	5.63 (*s*)
H–C(15) or CH_2(15)	5.70 (br. *s*)	5.69 (br. *s*)	4.74 (*d*, *J* = 1.5)	7.45 (*d*, *J* = 1.6)		
Me(17) or H–C(17)	0.96 (*d*, *J* = 6.5)[b]), 0.98 (*d*, *J* = 6.5)[b])	0.79 (*d*, *J* = 6)[b]), 0.77 (*d*, *J* = 6)[b])	0.80 (*d*, *J* = 7)	–	5.87 (*s*)	5.65 (*s*)
Me(18)	–	–	–	1.24 (*s*)	1.25 (*s*)	1.24 (*s*)
Me(19) or CH_2(19)	1.01 (*s*)	1.21 (*s*)	1.22 (*s*)	4.20 (*d*, *J* = 11) 3.35 (*d*, *J* = 11)	4.19 (*d*, *J* = 11) 3.31 (*d*, *J* = 11)	–
Me(20)	0.86 (*s*)	0.73 (*s*)	0.74 (*s*)	1.01 (*s*)	0.96 (*s*)	0.83 (*s*)
MeO	3.56 (*s*), 3.55 (*s*)[b])	3.55 (*s*), 3.54 (*s*)[b])	–	–	–	–

[a]) Overlapped. [b]) Epimer mixture.

^{1}H,^{1}H-COSY and HMBC experiments (*Fig. 1*). Thus, the ^{1}H,^{1}H COSY indicated the presence of two partial structures, from C(3) to C(1) and C(6) to C(8) and from C(14) to C(15). In the HMBC spectrum, correlations between H–C(6) (δ(H) 3.69) and C(7) (δ(C) 31.4), C(8) (δ(C) 38.1), C(4) (δ(C) 139.7), C(10) (δ(C) 44.9), and C(18)=O (δ(C) 171) were observed. The HMBC correlations of the olefinic H–C(3) (δ(H) 6.46) with C(18)=O and C(6) established the presence of a lactone ring linking the rings A and B of clerodane through C(4) and C(6) [10][11], thus connecting the partial structures C(3) to C(1) and C(6) to C(8). The configuration of the lactone ring at C(4) and C(6) was deduced by a 1D-NOESY experiment. The second lactone moiety was inferred to be at C(12) along the side chain whose quaternary C(13)-atom appeared at δ(C) 138.8 in the broad-band experiment [9]. The presence of a MeO substituent at C(15) was inferred from the chemical shift of H–C(15) at δ(H) 5.70 coupled to the vicinal olefinic H–C(14) (δ(H) 6.75), which, in turn, showed long-range allylic coupling to CH_2(12) (δ(H) 2.15–2.21) as indicated by the ^{1}H,^{1}H-COSY and 2D-NOESY experiments. Furthermore, the substitution at the lactone ring was confirmed by the HMBC cross-peaks of H–C(15) with the MeO C-atom (δ(C) 57.1), C(13) (δ(C) 138.8), C(14) (δ(C) 141.6), and C(16) (δ(C) 172) (*Fig. 1*). The relative configuration of **1** was determined by a 1D-NOESY experiment. Since the irradiated signals of H–C(10) and Me(19) showed no mutual correlation, the A/B ring junction was deduced to be *trans*. The observed NOE interactions were Me(19)/H–C(6) and Me(20), and Me(17)/Me(20). Hence Me(17), Me(19), and Me(20) were disposed *cis* to each other, and the lactone ring was deduced to be oriented axially at C(6). The *cis* disposition of the three Me groups was also confirmed by their δ(H) and δ(C) values [8][9]. Furthermore, the Me(17) signal (δ(H) 0.96) of **1** was splitted suggesting the presence of an epimer mixture, which we could not separate.

Table 2. *^{13}C-NMR* ($CDCl_3$, 125 MHz) *Data of Compounds* **1**–**6**. δ in ppm.

	1	**2**	**3**	**4**	**5**	**6**		**1**	**2**	**3**	**4**	**5**	**6**
C(1)	17.5	17.4	17.3	34.2	34.5	37.2	C(12)	19.9	19.3	19.2	27.9	27.0	27.8
C(2)	27.3	27.2	27.4	18.1	18.6	19.5	C(13)	138.8	134	134.9	135.6	151	130.1
C(3)	130.1	141.3	141.2	27.5	29.6	36.0	C(14)	141.6	143	143.5	112.7	113.7	115.1
C(4)	139.7	140.4	140.4	42.8	42.7	43.5	C(15)	102.5	102.5	70.2	145.9	172	172.9
C(5)	39.1	37.5	37.5	51.1	51.1	53.0	C(16)	172	172.2	174.3	149.1	157.9	143.2
C(6)	86.1	35.7	35.7	80.1	80.1	20.8	C(17)	15.5	15.5	15.9	183	116.1	114.9
C(7)	31.4	27.4	27.2	28.0	33.8	35.6	C(18)	171	171.3	172.5	22.5	22.4	28.1
C(8)	38.1	36.3	36.2	134.2	125.7	127.5	C(19)	16.1	20.5	20.5	64.1	64.0	182.2
C(9)	42.3	38.8	38.7	154.1	147.9	142.4	C(20)	19.7	18.1	18.2	19.9	20.2	17.2
C(10)	44.9	46.7	46.6	40.1	39.0	40.5	MeO	57.1,	56.9,				
C(11)	36.4	36.31	36.2	25.0	24.7	25.2		57.1[a])	57.0[a])				

[a]) Epimer mixture.

The clerodane structure of **2** (FAB-MS: *m*/*z* 425 ([*M* + glycerol – 1]$^-$) was suggested by its UV, IR, and ^{1}H-NMR spectra, which were similar to those discussed above. Compound **2** lacked the C(4)/C(6) lactone moiety but contained an α,β-unsaturated acid function at the olefinic quaternary C(4). This COOH group was inferred from the fact that **2** contained six CH_2 as compared to five CH_2 in **1**, and also from the absence of any other HMBC cross-peak to *C*OOH except for that from of *H*–C(3). With the aid of 2D NMR experiments and literature data, **2** was found to be identical with the compound reported by *Krishna et al.* [9].

Compound **3** (FAB-MS: *m*/*z* 425 ([*M* + glycerol + 1]$^+$) was devoid of the MeO substituent at C(15) and had a CH_2 group instead (δ(H) 4.74, δ(C) 70.2). Comparison of its NMR data with literature data established that **3** was identical to the compound reported in [8].

Limbatenolide A (**4**) had the molecular formula $C_{20}H_{24}O_3$ corresponding to *m*/*z* 312 in the EI mass spectrum. Its structure was established by its spectral data and comparison with literature data.

The basic skeleton of **4** was identified on the basis of its ^{1}H- and ^{13}C-NMR and characteristic IR data. The IR spectrum showed an absorption band for a furan ring at 871 cm^{-1}, whereas a strong carbonyl absorption at 1616 cm^{-1} was attributed to an α,β-unsaturated ketone, which was also conjugated to the furan ring. The UV

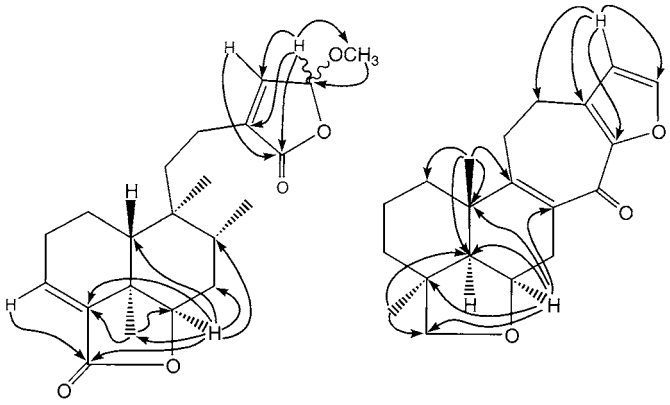


Fig. 1. *Important HMBC correlations of compounds* **1** *and* **4**

absorptions at 272, 285, 305, and 311 were typical for this furan and α,β-unsaturated-ketone moieties. In the ^{1}H-NMR spectrum of **4**, only two downfield Me *s* were observed, which were unusual for clerodanes, whereas the ^{13}C-NMR spectrum showed several low-field C-atoms, again unusual for clerodanes. The presence of the furan ring was also confirmed by the methine signals (CH(14) and CH(15)) at δ(H) 6.31 and 7.45 and δ(C) 112 and 145.9. HMBC Correlations suggested that the furan ring was fused with the hydrocarbon skeleton, as δ(H) 6.31 (H–C(14)) showed cross-peaks with the downfield olefinic quaternary C(13) (δ(C) 135.6) and C(16) (δ(C) 149.1), and with CH_2(12) (δ(C) 27.9) (*Fig. 1*). The empirical formula of **4** and DEPT measurement suggested the presence of six high-field CH_2 groups and of a seven-membered ring. A tetrasubstituted C=C bond and characteristic IR absorptions indicated an $\alpha,\beta:\alpha',\beta'$-diunsaturation with the carbonyl absorption at 1616 cm^{-1} and the δ(C) at 183.0. The identity of the fused furan and carbonyl system in the seven-membered ring was established upon comparison of these features with those of hispanonic acid [12]. The carbonyl group was placed at C(17) due to the γ-shielding effect for C(7) (δ(C) 28) [12]. The signals of the two tertiary Me(18) and Me(20) groups at δ(H) 1.24 (δ(C) 22.5) and 1.01 (δ(C) 19.9), respectively, were in good agreement with those of hispanonic acid and hispaninic acid [13]. However, Me(18) at δ(H) 1.24 showed HMBC cross-peaks with an OCH_2 appearing as an *AB* system ('*q*') at δ(H) 4.20 and 3.35 ($J = 11$ Hz each) (*Fig. 1*). Furthermore, the same OCH_2 showed cross-peaks with C(6) (δ(C) 80.1), and H–C(6) resonated at δ(H) 3.46 (*dd*, $J = 5.3$ and 11 Hz). In turn, H–C(6) showed the corresponding HMBC correlations with O*C*H$_2$(19) (δ(C) 64.1) and with C(4) (δ(C) 42.8), C(5) (δ(C) 51.5), C(7) (δ(C) 28.0), and C(8) (δ(C) 134.2). These data were in accordance with the presence of a C(4)/C(6) oxymethylene bridging resulting in a fused tetrahydrofuran ring. The configuration of the fused tetrahydrofuran ring was confirmed by a 1D-NOESY experiment showing mutual NOEs for CH_2(19) and Me(20) and suggesting an axially oriented oxymethylene moiety.

Limbatane B (**5**) and limbatane C (**6**) had the molecular ion at *m*/*z* 312 and 346 in the EI mass spectra corresponding to molecular formulae $C_{20}H_{24}O_3$ and $C_{20}H_{26}O_5$, respectively. Their structures were deduced from their spectral data and by comparison with those of **4**.

The IR spectra of **5** showed an absorption at 1743 cm^{-1} for an α,β-unsaturated γ-lactone but no $\alpha,\beta:\alpha',\beta'$-diunsaturated carbonyl group as in **4**. The UV spectra confirmed the absence of a furan ring as the absorption maxima were observed at 349, 304, 255, 230 nm (*cf.* data of **4**). The NMR spectrum suggested the presence of two tertiary Me, six high-field CH_2, and one downfield CH_2 for an oxymethylene bridging with chemical-shift values similar to those of **4**, indicating a tetracyclic parent skeleton for **5**. However, in the ^{1}H-NMR spectrum, the two olefinic signals were at δ(H) 5.78 and 5.87. The latter signal arising from H–C(17) showed cross-peaks in the HMBC spectrum with downfield C(13) (δ(C) 151.0), C(16) (δ(C) 157.9), C(9) (δ(C) 147.9), and C(8) (δ(C) 125.7) and with *C*H$_2$(7) (δ(C) 33.8), suggesting the absence of the γ-shielding effect observed for C(7) of

4 and explaining the different IR data. The olefinic H–C(14) at δ(H) 5.78 showed correlations with the lactone C(15)=O (δ(C) 172.0) and the high-field *C*H_2(12) of the seven-membered ring.

Compound **6** lacked the OCH_2 bridge present in **4** and **5** but contained a COOH group at C(4) as deduced from the IR absorption at 1710 cm^{-1}; also the H-atoms of Me(18) showed a ^3J HMBC correlation with *C*OOH (δ(C) 182.2). The two downfield olefinic signals of **6** at δ(H) 5.63 (H–C(14)) and 5.65 (H–C(17)) gave similar HMBC correlations for the seven-membered ring as discussed for **5**. The IR data (no absorption at 1730–1740 cm^{-1}) of **6** suggested the presence of a free α,β-unsaturated-acid function at C(13) rather than a lactone moiety such as **5**, which was confirmed by an absorption at 1656 cm^{-1} (free acid). The C(13)=C(14) bond apparently had (*E*)-configuration, because the COOH group at C(14) did not form a lactone ring with OH–C(16). The signal of the tetrasubstituted quaternary C(16) appeared at δ(C) 142 in the ^{13}C-NMR suggesting OH substitution. This was confirmed by the empirical formula of **6**, which had eight unsaturations compared to nine in **4** and **5**. The IR spectrum also confirmed the presence of a free OH group at C(16).

Galanthamine was used as a positive control in a cholinesterase inhibition assay, in which compounds **1**–**6** showed inhibitory potential against butyrylcholinesterase (*Table 3*). Compounds **1**–**3**, belonging to the clerodane class of diterpenoids, showed inhibition against both the enzymes butyrylcholinesterase and acetylcholinesterase. Among **1**–**3**, some degree of selectivity was observed for **3**, which was most active against butyrylcholinesterase while it was a weak inhibitor of acetylcholinesterase as compared to **1** and **2**, which displayed strong acetylcholinestrase inhibition. The tetracyclic diterpenoids **4**–**6** were moderately active against butyrylcholinesterase in a selective manner, but they were inactive against acetylcholinesterase (*Table 3*, *Fig. 2*).

Table 3. In vitro *Quantitative Inhibition of Acetylcholinesterase* (AChE) *and Butyrylcholinesterase* (BChE)

	$IC_{50} \pm$ s.e.m.[a]) [μM]	
	BChE	AChE
1	24.9 ± 0.2	50.0 ± 2.0
2	25.4 ± 0.4	52.0 ± 1.5
3	14.0 ± 1.2	102 ± 0.5
4	45.1 ± 2.0	–
5	51.0 ± 1.0	–
6	40.2 ± 0.05	–
Galanthamine[b])	8.5 ± 0.05	0.50 ± 0.002

[a]) Standard error of the mean of five assays. [b]) Standard inhibitor of acetylcholinesterase and butyrylcholinesterase.

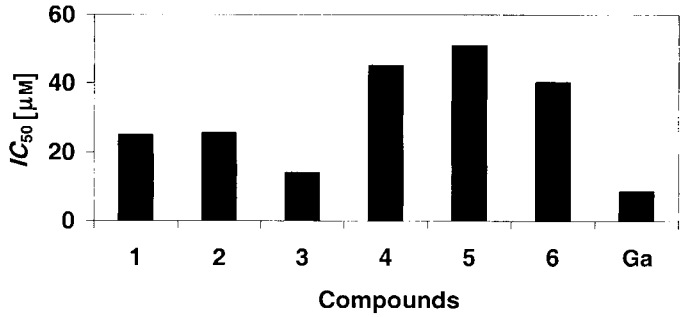


Fig. 2. IC_{50} *Values of compounds* **1**–**6** *compared with that of the galanthamine* (Ga) *standard inhibitor of butyrylcholinesterase*

Experimental Part

General. TLC: precoated silica gel *G-25-UV$_{254}$* plates; detection at 254 nm and by ceric sulfate in 10% H_2SO_4 soln. Column chromatography (CC): silica gel (*E. Merck*, 230–400 mesh). Optical rotations: *Jasco-DIP-360* digital polarimeter; 10-cm cell tube. UV and IR Spectra: *Hitachi UV-3200* and *Jasco 320-A* spectrophotometers, resp.; in nm (log ε) and cm^{-1}, resp. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AM-400* and *AMX-500* (for 2D) spectrometers; $SiMe_4$ as an external standard; δ in ppm, J in Hz. EI- and HR-EI-MS: *Finnigan MAT-12* or *MAT-312* spectrometers; in m/z (rel. %). Fast-atom-bombardment (FAB) MS: *Jeol HX-110* mass spectrometer.

Plant Material. The plant *Ballota limbata* (Lamiaceae) was collected from Abbottabad, Pakistan, in June 2001, and identified by Dr. *Manzoor Ahmad* (taxonomist) at the Department of Botany, Post-Graduate College, Abbottabad, Pakistan. A voucher specimen (No. 6872) has been deposited at the herbarium of the Botany Department of Post-Graduate College, Abbottabad, Pakistan.

Extraction and Purification. The air-dried whole plant (35 kg) was exhaustively extracted with MeOH (3 × 40 l) at r.t. After evaporation, the residue (315 g) was divided into hexane- (47 g), $CHCl_3$- (95 g), AcOEt- (69 g), BuOH- (33 g), and H_2O-soluble portions (59 g). The $CHCl_3$ extract was subjected to CC (silica gel; $CHCl_3$/hexane gradient, $CHCl_3$, and MeOH/$CHCl_3$ gradient): *Fractions 1–12*. *Fr. 7* (6.8 g; with $CHCl_3$/hexane 2:1) was resubmitted to CC (silica gel; AcOEt/hexane of increasing polarity): **1** (9.1 mg) with AcOEt/hexane 1:1 and **2** (19 mg) with AcOEt/hexane 2:3. The combined less-polar fractions of this separation were resubmitted to CC (silica gel; AcOEt/hexane 1:2 isocratic): **3** (13 mg). Compounds **4**–**6** were obtained from *Fr. 18* (7.9 g; with $CHCl_3$/hexane: 4:1) by CC (silica gel; acetone/hexane gradient): **4** (13 mg) with acetone/hexane 4:5, **5** (8 mg) with acetone/hexane 5.5:4.5, and **6** (10 mg) with acetone/hexane 7:3. The purity of the compounds was checked on TLC and HPTLC plates.

*(5a*R*,6*S*,7*R*,8a*R*,8b*R*)-6-[2-(2,5-Dihydro-5-methoxy-2-oxofuran-3-yl)ethyl]-4,5,5a,6,7,8,8a,8b-octahydro-6,7,8b-trimethyl-2*H*-naphtho[1,8-*bc*]furan-2-one* (**1**). Colorless oil. $[\alpha]_D^{23} = -47.05$ ($c = 0.051$, $CHCl_3$). UV (MeOH): 210 (4.3). IR ($CHCl_3$): 2926, 1766, 1678. ^{1}H- (500 MHz) and ^{13}C-NMR: *Tables 1* and *2*. EI-MS: 342 ($[M - H_2O]^+$), 328 (14), 314 (23), 282 (18), 256 (12), 217 (34), 162 (52). HR-EI-MS: 342.1830 ($[C_{21}H_{26}O_4 - H_2O]^+$; calc. 342.4388).

*(4a*R*,5*S*,6*R*,8a*R*)-5-[2-(2,5-Dihydro-5-methoxy-2-oxofuran-3-yl)ethyl]-3,4,4a,5,6,7,8,8a-octahydro-5,6,8a-trimethylnaphthalene-1-carboxylic Acid* (**2**). Colorless oil. $[\alpha]_D^{23} = -76.59$ ($c = 0.376$, $CHCl_3$). UV (MeOH): 210 (4.2). IR ($CHCl_3$): 2960, 1769, 1681. ^{1}H- (500 MHz) and ^{13}C-NMR: *Tables 1* and *2*. EI-MS: 344 (39, $[M - H_2O]^+$), 329 (56), 313 (26), 297 (45), 269 (27), 243 (23), 221 (33), 203 (49), 173 (50). HR-EI-MS: 344.1987 ($[C_{21}H_{28}O_4 - H_2O]^+$; calc. 344.4548).

*(4a*R*,5*S*,6*R*,8a*R*)-5-[2-(2,5-Dihydro-2-oxofuran-3-yl)ethyl]-3,4,4a,5,6,7,8,8a-octahydro-5,6,8a-trimethylnaphthalene-1-carboxylic Acid* (**3**). Colorless oil. $[\alpha]_D^{23} = -53.731$ ($c = 0.0367$, $CHCl_3$). UV (MeOH): 210 (4.8). IR ($CHCl_3$): 2959, 1752, 1680. ^{1}H- (400 MHz) and ^{13}C-NMR: *Tables 1* and *2*. EI-MS: 314 (100, $[M - H_2O]^+$), 299 (34), 271 (40), 221 (33), 203 (54), 175 (59). HR-EI-MS: 314.1987 ($[C_{20}H_{26}O_3 - H_2O]^+$; calc. 314.4283). FAB-MS (pos.): 425 ($[M + \text{glycerol} + 1]^+$), 315, 277, 241.

*(3a*S*,5a*R*,12b*S*,12c*R*)-2,3,3a,4,5a,6,11,12,12b,12c-Decahydro-3a,12b-dimethylfuro[3'',2'':5',6']cyclohepta[1',2':3,4]naphtho[1,8-*bc*]furan-7(1*H*)-one* (**4**). Colorless oil. $[\alpha]_D^{23} = 102.4$ ($c = 0.25$, $CHCl_3$). UV (MeOH): 311 (4.5), 285 (4.2), 272 (4.3), 405 (5.4). IR ($CHCl_3$): 1616, 871. ^{1}H- (400 MHz) and ^{13}C-NMR: *Tables 1* and *2*. EI-MS: 312 (49, M^+, $C_{20}H_{24}O_3^+$), 297 (45), 282 (42), 267 (33), 213 (56), 177 (57), 150 (72).

*(3a*S*,5a*R*,12b*S*,12c*R*)-2,3,3a,4,5a,6,11,12,12b,12c-Decahydro-3a,12b-dimethylfuro[3'',2'':5',6']cyclohepta[1',2':3,4]naphtho[1,8-*bc*]furan-9(1*H*)-one* (**5**). Colorless oil. $[\alpha]_D^{23} = 46.25$ ($c = 0.16$, $CHCl_3$). UV (MeOH): 349 (4.1), 304 (3.9), 255 (4.1), 230 (4.3). IR ($CHCl_3$): 1743. ^{1}H- (400 MHz) and ^{13}C-NMR: *Tables 1* and *2*. EI-MS: 312 (33, M^+, $C_{20}H_{24}O_3^+$), 296 (2), 239 (21), 282 (9), 175 (100).

*(4*S*,4a*R*,9*E*,11b*S*)-9-(Carboxymethylidene)-2,3,4,4a,5,6,9,10,11,11b-decahydro-8-hydroxy-4,11b-dimethyl-1*H*-cyclohepta[*a*]naphthalene-4-carboxylic Acid* (**6**). Colorless oil. $[\alpha]_D^{23} = 67.37$ ($c = 0.063$, $CHCl_3$). UV (MeOH): 250 (3.5), 225 (3.6), 203 (4.2). IR ($CHCl_3$): 3381, 3259, 2932, 1710, 1656. ^{1}H- (400 MHz) and ^{13}C-NMR: *Tables 1* and *2*. EI-MS: 346 (8), 328 (56), 313 (46), 300 (112), 282 (22), 267 (25), 211 (28), 175 (99). HR-EI-MS: 346 ($C_{20}H_{26}O_5^+$; calc. 346.4272).

In vitro *Cholinesterase-Inhibition Assay.* Electric-eel acetylcholinesterase (EC 3.1.1.7), horse-serum butyrylcholinesterase (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 anal. grade. Acetylcholinesterase- and butyrylcholinesterase-inhibiting activities were measured by a slightly modified spectrophotometric method developed by *Ellman et al.* [14]. Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcho-

linesterase, respectively. The mixture contained 140 µl of (100 mM) sodium phosphate buffer (pH 8.0), 10 µl of DTNB, 10 µl of test-compound soln., and 20 µl of acetylcholinesterase or butyrylcholinesterase soln., which were mixed and incubated for 15 min (25°). The reaction was then initiated by the addition of 10 µl of acetylthiocholine or butyrylthiocholine, resp. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 2-nitro-5-thiobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, resp., at a wavelength of 412 nm (15 min). Test compounds and the control were dissolved in EtOH. All the reactions were performed in triplicate on a 96-well microplate in *SpectraMax 340* (*Molecular Devices*, USA). The percentage of inhibition was calculated as follows: $(E-S)/E \cdot 100$, where E is the activity of the enzyme without test compound, and S is the activity of the enzyme with test compound.

Estimation of IC_{50} *Values.* The concentrations of test compounds that 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 by using the EZ-Fit enzyme kinetics program (*Perrella Scientific Inc.*, Amherst, USA).

REFERENCES

[1] G. Citoglu, M. Tanker, B. Sever, J. Englest, R. Anton, N. Altanlar, *Planta Med*. **1998**, *64*, 484.
[2] E. Nasir, S. I. Ali, 'Flora of West Pakistan', Fakhri Printing Press, Karachi, 1972, p. 627.
[3] R. N. Chopra, S. L. Nayar, I. C. Chopra, 'Glossary of Indian Medicinal Plants', Indian Council of Scientific & Industrial Research, India, 1956, p. 183.
[4] K. Vural, N. Ezer, K. Erol, F. P. Shahin, *J. Fac. Pharm*. **1996**, *13*, 29.
[5] E. K. Perry, *Br. Med. Bull*. **1986**, *42*, 63.
[6] V. Tougu, *Curr. Med. Chem*. **2001**, *1*, 155.
[7] S. Q. Yu, H. W. Holloway, T. Utsuki, A. Brossi, N. H. Greig, *J. Med. Chem*. **1999**, *42*, 1855.
[8] A. Rivera, F. Patricio, M. Castillo, *J. Nat. Prod*. **1988**, *51*, 155.
[9] V. Krishna, P. Singh, *Phytochemistry* **1999**, *52*, 1341.
[10] W. Vichnewski, R. Murar, W. Herz, *Phytochemistry* **1979**, *18*, 129.
[11] I. Kitagawa, P. Simanjuntak, K. Hori, N. Nagami, T. Mahmud, H. Shibuya, M. Kobayashi, *Chem. Pharm. Bull*. **1994**, *42*, 1050.
[12] B. Rodriguez, G. Savona, F. Piozzi, *J. Org. Chem*. **1979**, *44*, 2219.
[13] J. Lopez de Lerma, S. Garcia Blanco, *Tetrahedron Lett*. **1980**, *81*, 1273.
[14] G. L. Ellman, K. D. Courtney, V. Andres, R. M. Featherstone, *Biochem. Pharmacol*. **1961**, *7*, 88.

Received September 5, 2003