Three New Cholinesterase-Inhibiting *cis*-Clerodane Diterpenoids from *Otostegia limbata*

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Three new tricyclic *cis*-clerodane type diterpenoids trivially named as limbatolide A (1), limbatolide B (2) and limbatolide C (3) have been isolated from the roots of *Otostegia limbata* along with two known compounds; oleanic acid and β-sitosterol. The structure elucidation of the new compounds was based primarily on two-dimensional (2D) NMR techniques. Compounds 1—3 displayed inhibitory potential in a concentration-dependent manner against acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) enzymes, respectively.

Key words Lamiaceae; *Otostegia limbata*; clerodane diterpenoid; cholinesterase inhibitory assay

The genus *Otostegia* (Lamiaceae) comprises *ca.* 33 species, mainly occurring in the Mediterranean region.[1] In Pakistan, only two species have been found, namely *Otostegia aucheri* Boiss. and *Otostegia limbata* (Bth.) Boiss. (Syn. *Ballota limbata* Bth.; Labiatae). *Otostegia limbata* is locally called "Bui" or "Phut kandu".[2] *Otostegia limbata* is widely distributed in the North-West 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 genus *Otostegia* 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 three new *cis*-clerodane diterpenoids (Fig. 1), which have shown inhibitory potential against AChE and BChE.

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.[5] According to the cholinergic hypothesis, memory impairments in patients with this senile dementia disease are due to a selective and irreversible deficiency in the cholinergic functions in brain.[6] This serves as a rationale for the use of AChE inhibitors for the symptomatic treatment of Alzheimer's disease (AD) in its early stages. The role of BChE (EC 3.1.1.8) in normal ageing and brain diseases is still elusive. It has been found that BChE is found in significantly higher quantities in Alzheimer's plaques than in plaques of normal age related non-demented brains.[7]

Results and Discussion

The $CHCl_3$ fraction of the air dried roots of *Otostegia limbata* was subjected to silica gel chromatography to give three new *cis*-clerodane type tricyclic diterpenoids trivially named as limbatolide A (**1**), limbatolide B (**2**) and limbatolide C (**3**) along with two known compounds; oleanic acid and β-sitosterol. The structures of compounds **1**—**3** were mainly established by ^{1}H-, ^{13}C-NMR, UV, IR and, supported by heteronuclear multiple bond correlation (HMBC) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments.

Compound **1** was isolated as gummy solid. The molecular formula, $C_{21}H_{28}O_5$, of compound **1** was deduced from accurate mass measurement of the highest peak at *m*/*z* 342 $[M-H_2O]^+$[8] corresponding to a molecular composition of $C_{21}H_{26}O_4$. The IR of compound **1** indicated the presence of five-membered γ-lactone (1760 cm^{-1}).[9] The UV spectrum of compound **1** showed absorption at λ_{max} 212 nm. The HMBC experiment was very informative in the structure elucidation of compound **1**. It strongly supported different connectivities in the compound. The presence of two γ-lactone moieties in the molecule was confirmed from ^{1}H–^{1}H correlation spectroscopy (COSY). In the HMBC experiment of compound **1** (Fig. 2), the olefinic proton at δ_H 6.43 showed correlations to the C-4 (δ_C 140.3), C-18 (δ_C 170.8), C-5 (δ_C 40.5) and C-2 (δ_C 28.5). Similarly, the olefinic proton at δ_H 6.70 showed cross peak correlations to the C-13 (δ_C 139.7), C-16 (δ_C 173.6), C-15 (δ_C 100.8) and C-12 (δ_C 21.3). The β-oriented Me-19 (δ_H 1.30) was connected to the C-10 (δ_C 45.6), C-6

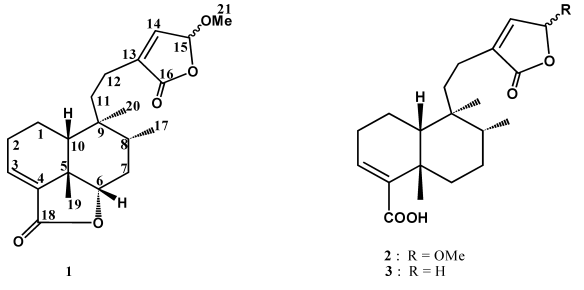

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Fig. 1. Structures of Compounds **1**—**3**

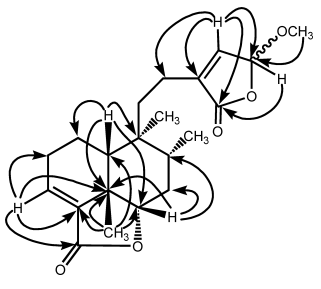

Fig. 2. Important HMBC Correlations of **1**

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Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds **1**—**3** in $CDCl_3$[a)]

Position	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	19.4	1.57—1.65 m	18.2	0.72 m	18.1	0.76 m
2	28.5	2.50 m, ovlp	28.3	2.31—2.40 m	27.3	2.37—2.40 m
3	132.1	6.43 br s	142.1	6.80 br t (3.5)	142.3	6.79 br t (4.1)
4	140.3	—	139.5	—	139.1	—
5	40.5	—	38.2	—	38.5	—
6	85.2	3.78 dd (4.2, 8.3)	36.1	2.40 m	36.0	2.30 m
7	30.3	2.05 m, ovlp	27.2	1.43—1.54 m, ovlp	26.5	1.50—1.57 m
8	37.5	1.90 m	37.1	1.70 m	38.3	1.65—1.69 m
9	40.9	—	39.9	—	40.1	—
10	45.6	1.48 dd (5.1, 11.7)	45.2	1.55 br d (10.5)	45.4	1.60 br d (11.0)
11	37.8	1.50—1.55 m	35.7	1.47—1.58 m, ovlp	36.9	1.45—1.50 m, ovlp
12	21.3	2.20—2.27 m	20.3	2.20—2.25 m	21.3	2.15—2.21 m
13	139.7	—	133.3	—	136.1	—
14	142.3	6.70 br s	142.3	6.78 br s	144.4	6.70 br s
15	100.8	5.75 br s	103.4	5.75 br s	71.4	—
16	173.6	—	173.1	—	173.7	—
17	15.9	0.94 d (7.3)	16.3	0.84 d (7.0)	16.8	0.83 d (7.5)
18	170.8	—	171.8	—	171.5	—
19	31.7	1.30 s	32.3	1.28 s	33.1	1.30 s
20	22.5	0.80 s	23.1	0.70 s	16.8	0.75 s
21	55.7	3.57 s	57.6	3.50 s	—	—

a) Chemical shifts (δ) in ppm relative to TMS; coupling constants (J in Hz) are given in parentheses.

(δ_C 85.2) and C-4 which established the presence of a lactone moiety joining the A/B rings of *cis*-clerodane through C-4 and C-6 carbons.[9,10)] The ^{1}H-NMR spectrum of compound **1** (Table 1) exhibited typical signals for a tricyclic *cis*-clerodane carbon skeleton supported by ^{13}C-NMR spectroscopy, which disclosed the presence of two tertiary and one secondary methyl carbons, two olefinic carbons and six quaternary carbons (which include two carbonyl carbons). In the ^{1}H-NMR spectrum, the two tertiary methyl carbons appeared as singlets at δ_H 1.30 and δ_H 0.8, respectively, and a secondary methyl as a doublet at δ_H 0.94 (J=7.3 Hz). The downfield region showed two olefinic methine signals at δ_H 6.43 and δ_H 6.70 as broad singlets which are characteristic for clerodane class of diterpenoids. Similarly, the methine signal of H-6 was centered at δ_H 3.78 as a doublet of doublet with coupling constant 4.2 and 8.3 Hz. The signal which appeared at δ_H 1.48 as dd (5.1, 11.7 Hz) was assigned to H-10. The up field OCH_3 appeared at δ_H 3.57 (s) and downfield H-15 resonated at δ_H 5.75 (s). In the ^{13}C-NMR spectrum (Table 1), the typical signals at δ_C 173.6 and δ_C 170.6 were assigned to two carbonyl carbons C-16 and C-18, respectively. Similarly, signals appeared at δ_C 31.7, 22.5 and 15.9 due to methyls which are characteristic for *cis*-clerodanes. The olefinic carbons' signals observed at δ_C 132.1 and δ_C 142.3 were ascribed to C-3 and C-14, respectively. The stereo-orientation of this lactone ring was deduced on the basis of 2D-NOESY experiment. Since the irradiated signals of H-10 and H_3-19 showed strong correlation between each other, hence A/B ring junction was deduced to be *cis*. The stereochemistry of compound **1** was established from the combined evidence of its spectral data in comparison with those of clerodane diterpenoids.[10,11)] In ^{13}C-NMR, the chemical shift value showed that we are dealing with an A/B *cis*-fused clerodane. The observed NOE interactions (Fig. 3) were those of H-10 with H_3-19 and H-6. The strong NOE correlations were also observed between H_3-17 and H_3-20. The lactone ring at C-4

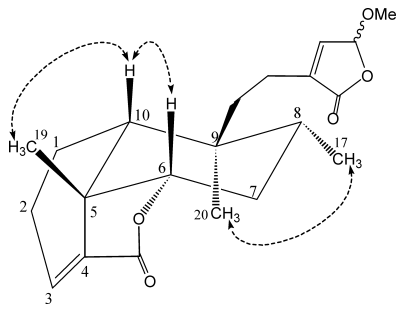

Fig. 3. Important NOE Correlations of **1**

and C-6 was deduced to be oriented equatorially. These results, and the fact that the irradiation of H-10 caused increase in the intensity of H_3-19 and H-6 but did not cause any increase in the intensity of H_3-20, confirmed the *cis* stereochemistry of A and B rings of decalin system.[12)] A *cis* A/B ring junction was also evident from the ^{13}C-NMR chemical shifts of C-19 methyl carbon (δ_C 31.7) and C-20 methyl carbon (δ_C 18.7). Hence the C-17 and C-20 methyls were disposed *trans* to C-19 methyl, *i.e.* these methyls are in two planes.[10,11)] By comparing spectral data with literature; Me-19 was confirmed to be β-oriented.[13,14)] So we are dealing with *cis*-clerodanes. In *cis*-clerodanes, C-19 carbon resonates at about δ_C 25, whereas in *trans*-clerodanes it appears at δ_C 11—19. Moreover, C-20 in *cis*-clerodanes resonates at lower field (δ_C 21—29) than in *trans*-clerodanes (δ_C 17—19).[15)]

Compound **2**, another new tricyclic *cis*-clerodane diterpene, was isolated as gummy solid from $CHCl_3$ fraction. It was found to have the molecular formula $C_{21}H_{30}O_5$ derived from accurate mass measurement of the molecular ion peak at m/z 344.5223 $[M-H_2O]^+$ accompanied by the loss of H_2O molecule, like in compound **1**. It lacked the five-membered lactone ring on C-4 and C-6 position. Compound **2** contained free α,β-unsaturated acid rather than lactone on C-4, de-

duced from the IR absorption. The IR spectrum of compound **2** showed absorption at 1685 cm^{-1} for α,β-unsaturated acid and at 1755 cm^{-1} which is characteristic for γ-lactones. The UV spectrum showed an absorption band at λ_{max} 212 nm. In ^{1}H-NMR spectrum the typical olefinic signal appeared at δ_H 6.80 as a broad triplet with coupling constant 3.5 Hz. This signal showed cross peak analysis to the carbonyl carbon C-18 (δ_C 171.8), C-19 (δ_C 32.3), C-4 (δ_C 139.5), C-5 (δ_C 38.2) and C-2 (δ_C 28.3) which supported the presence of free acid on C-4. The two tertiary methyl signals were observed at δ_H 1.28 and δ_H 0.70, and one secondary methyl appeared as doublet at δ_H 0.84 with coupling constant 7.0 Hz. Similarly, the olefinic proton resonated at δ_H 6.78 as a broad singlet which confirmed the lactone ring attached to C-12 position. This olefinic proton showed correlations to the C-16 (δ_C 173.1), C-15 (δ_C 103.4), C-13 (δ_C 133.3) and C-12 (δ_C 20.3). The methylene signal which appeared at δ_H 2.40 also supported the lack of lactone ring on C-6 position. The H_3-21, which appeared at δ_H 3.50, showed correlations to the C-15 (δ_C 103.4) and C-16 (δ_C 173.1). In the ^{13}C-NMR of compound **2**, like in compound **1**, Me-19 signal at δ_C 32.3 was also confirmed from the literature to be β-oriented.[13,14] The stereochemistry was confirmed by 2D-NOESY and NOE experiments. When H-10 was irradiated, there was an increase in the intensity of H_3-19. It means that we are dealing with *cis*-clerodanes. The NOE interactions were also observed between H_3-20 and H_3-17. Hence the C-17 and C-20 methyls were disposed *trans* to C-19 methyl, *i.e.* these methyls are in two planes.

Compound **3**, also a new tricyclic *cis*-clerodane diterpene, was isolated as gummy solid from the $CHCl_3$ fraction. It was found to have the molecular formula $C_{20}H_{28}O_4$ derived from accurate mass measurement of the molecular ion peak at *m/z* 314.2377 $[M-H_2O]^+$ accompanied by the loss of H_2O molecule, like in compound **1** and **2**. There was the lack of OCH_3 group on C-15 in compound **3**, rest of the compound was similar to compound **2**, confirmed by the high resolution electron impact mass spectrometry (HR-EI-MS) and ^{13}C-NMR spectra. The IR spectrum showed absorption at 1750 and 1678 cm^{-1} for γ-lactone and α,β-unsaturated acid, respectively. The UV spectrum showed an absorption band at λ_{max} 213 nm characteristic for clerodanes. According to ^{1}H- and ^{13}C-NMR spectra, compound **3** contained one secondary and two tertiary methyls, having chemical shift values similar to compound **1** and **2**. Two olefinic signals appeared at δ_H 6.79 and δ_H 6.70, which were assigned to H-3 and H-14 protons, respectively. In compound **3**, Me-19 resonated at δ_C 33.1 ppm which was assigned to be β-oriented; hence the A/B ring junction was *cis*-oriented. The olefinic proton at δ_H 6.70 showed HMBC correlations to the C-16 (δ_C 173.7), C-15 (δ_C 71.4), C-13 (δ_C 136.1) and C-12 (δ_C 21.3). Like the previous two compounds, the stereochemistry of compound **3** was confirmed on the basis of NOE interactions.

Against AChE, compound **1** displayed highest inhibitory potential (IC_{50}=38.5 μM) while compound **2** showed comparatively less inhibitory activity (IC_{50}=47.2 μM). The higher activity (Table 2) of compound **1** may be assumed due to the presence of lactone ring at C-4/C-6 position. Such type of arrangement may be more favorable for compound **1** to show higher inhibitory potential against AChE. Substitution of methoxy group with H-atom on C-15 further reduced the activity as shown by compound **3** (IC_{50}=103.7 μM). Against BChE, compound **3** was found most active (IC_{50}=14.2 μM) while compound **2** (IC_{50}=17.5 μM) and compound **1** (IC_{50}=22.3 μM) showed relatively less inhibitory potential, indicating that the absence of methoxy group at C-15 was favorable for the inhibition of BChE. Similarly the presence of lactone ring at C-4/C-6 position further reduced the activity of compound **1**.

Table 2. *In Vitro* Quantitative Inhibition of AChE and BChE by Compounds **1**—**3**

Compounds	IC_{50}±S.E.M.[a] [μM]	
	AChE	BChE
1	38.5±0.20	22.3±0.50
2	47.2±0.30	17.5±0.60
3	103.7±0.50	14.2±0.30
Galanthamine[b]	0.5±0.01	8.5±0.05

a) Standard mean error of 3—5 assays, *b*) positive control used in assays.

Experimental

General Experimental Procedures UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR were recorded on a Bruker AM-400 spectrophotometer with tetramethylsilane (TMS) as an external standard. 2D NMR spectra were recorded on a Bruker AMX 500 NMR spectrophotometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm cell tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 or MAT 312 spectrophotometers, ions are given in *m/z* (%). FAB-MS were measured on a JEOL HX 11 mass spectrometer. TLC was performed with pre-coated silica gel G-25-UV_{254} plates and detection was done at 254 nm, and by ceric sulphate in 10% H_2SO_4 solution. Silica gel (E. Merck, 230—400 mesh) was used for column chromatography.

Plant Material The root parts of *Otostegia limbata* (Lamiaceae) were collected in July 2002 from Abbottabad, Pakistan, and authenticated by Dr. Manzoor Ahmad (Taxonomist) at the Department of Botany, Post-Graduate College, Abbottabad, Pakistan. A voucher specimen (No. 6872) has been deposited in the herbarium of the Botany Department of Post-Graduate College, Abbottabad, Pakistan.

Extraction and Purification The air-dried roots of *Otostegia limbata* (30 kg) were exhaustively extracted with methanol (50 l×3) at room temperature. The extract was evaporated to yield the residue (450 g), which was partitioned between hexane (50 g) chloroform (80 g), ethyl acetate (120 g), butanol (170 g) and water (30 g). The chloroform extract was subjected to silica gel chromatography using hexane with a gradient on $CHCl_3$ up to 100% and followed by methanol. Eleven fractions were collected. Fraction no. 7 (5 g) of the first column was loaded on silica gel (230—400 mesh) and eluted with EtOAc : hexane (22 : 78) to purify compound **1** (3.4 mg). Fraction no. 8 (9.3 g) was subjected to column chromatography and eluted with EtOAc : hexane (27 : 73) to purify compound **2** (13 mg). Similarly, fraction no. 10 (8.5 g) was subjected to column chromatography and eluted with EtOAc : hexane (35 : 65) to purify compound **3** (11.4 mg). Fractions no. 4 (14 g) and 5 (20 g) were loaded on silica gel (flash silica 230—400 mesh) and eluted with EtOAc : hexane (18 : 82) to purify two known compounds; oleanic acid and β-sitosterol. The purity of the compounds was checked on TLC and HPTLC plates.

Compound **1**: Gummy solid. ^{1}H-NMR ($CDCl_3$, 400 MHz), see Table 1. ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1. IR ν_{max} ($CHCl_3$) cm^{-1}: 2990, 1760. UV λ_{max} (MeOH) nm (log ε): 212 (3.9). EI-MS *m/z* (rel. int.): 342 $[M-H_2O]^+$ (100), 329 (70), 313 (56), 299 (48), 269 (40), 219 (30), 203 (45), 175 (60). HR-EI-MS *m/z*: 342.2138 (Calcd for $C_{21}H_{26}O_4$–H_2O: 342.2821). $[\alpha]_D^{23}$ −85.79° (*c*=0.25, $CHCl_3$).

Compound **2**: Gummy solid. ^{1}H-NMR ($CDCl_3$, 400 MHz), see Table 1. ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1. IR ν_{max} ($CHCl_3$) cm^{-1}: 2890, 1755, 1685. UV λ_{max} (MeOH) nm (log ε): 212 (5.2). EI-MS *m/z* (rel. int.): 344 $[M-H_2O]^+$ (20), 328 (40), 314 (29), 282 (30), 256 (25), 173 (60). HR-EI-MS *m/z*: 344.5223 (Calcd for $C_{21}H_{28}O_4$–H_2O: 344.7023). $[\alpha]_D^{23}$ −32.80° (*c*=0.16, $CHCl_3$).

Compound **3**: Gummy solid. ^{1}H-NMR ($CDCl_3$, 400 MHz), see Table 1.

^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1. IR ν_{max} ($CHCl_3$) cm^{-1}: 2956, 1750, 1678. UV λ_{max} (MeOH) nm (log ε): 213 (3.8). EI-MS *m*/*z* (rel. int.): 314 $[M-H_2O]^-$ (99), 299 (50), 273 (35), 212 (38), 203 (70), 173 (55). HR-EI-MS *m*/*z*: 314.2373 (Calcd for $C_{20}H_{26}O_3$–H_2O: 314.3858). $[\alpha]_D^{23}$ −98.231° (*c*=0.063, $CHCl_3$).

***In Vitro* Cholinesterase Inhibition Assay** Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB) and galanthamine were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. AChE and BChE inhibiting activities were measured by the spectrophotometric method developed by Ellman *et al.*[16] Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay AChE and BChE, respectively. The reaction mixture contained 150 μl of (100 mM) sodium phosphate buffer (pH 8.0), 10 μl of DTNB, 10 μl of test-compound solution and 20 μl of AChE or BChE solution, which were mixed and incubated for 15 min at 25 °C. The reaction was then initiated by 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 as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively at a wavelength of 412 nm (15 min). Test compounds and the positive control (galanthamine) were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro-plate in SpectraMax 340 (Molecular Devices, U.S.A.). The percentage (%) inhibition was calculated as follows $(E-S)/E\times100$, where *E* is the activity of the enzyme without test compound and *S* is the activity of enzyme with test compound.

Determination 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 using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

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