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 was subjected to silica gel chromatography to give 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.3) 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.4) Here, we report the isolation and structure elucidation of three new cis-clerodane diterpenoids (Fig. 1), which have shown significant inhibitory activity against AChE and BChE.

Cholinesterases are widely used by the traditional practitioners against various diseases, mainly occurring in the Mediterranean region.1) In the 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 anti-inflammatory, antispasmodic, antidepressant, anxiolytic and sedative activities.4) The presence of two g-tocopherol. The structures of compounds 1–3 were mainly established by 1H-, 13C-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, C33H50O5, of compound 1 was deduced from accurate mass measurement of the highest peak at m/z 342 [M−H2O]+ correspondingly molecular composition of C33H52O5. The IR of compound 1 indicated the presence of five-membered γ-lactone (1760 cm−1).5) The UV spectrum of compound 1 showed absorption at λmax 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 1H−1H correlation spectroscopy (COSY). In the HMBC experiment of compound 1 (Fig. 2), the olefinic proton at δH 6.43 showed correlations to the C-4 (δC 140.3), C-18 (δC 170.8), C-5 (δC 40.5) and C-2 (δC 28.5). Similarly, the olefinic proton at δH 6.70 showed cross peak correlations to the C-14 (δC 139.7), C-11 (δC 173.6), C-15 (δC 100.8) and C-12 (δC 21.3). The β-oriented Me-19 (δH 1.30) was connected to the C-10 (δC 45.6), C-6 (δC 45.6), C-7 (δC 45.6), C-8 (δC 45.6), C-9 (δC 45.6) and C-13 (δC 45.6) protons.

Fig. 1. Structures of Compounds 1—3

Fig. 2. Important HMBC Correlations of 1

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The 1H-NMR spectrum of compound I (Table 1) exhibited typical signals for a tricyclic cis-clerodane carbon skeleton supported by 13C-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 1H-NMR spectrum, the two tertiary methyl carbons appeared as singlets at $\delta_{\text{H}} 1.30$ and $\delta_{\text{H}} 0.8$, respectively, and a secondary methyl as a doublet at $\delta_{\text{H}} 0.94$ (J=7.3 Hz). The downfield region showed two olefinic methine signals at $\delta_{\text{C}} 6.43$ and $\delta_{\text{H}} 6.70$ as broad singlets which are characteristic for clerodane class of diterpenoids. Similarly, the methine signal of H-6 was centered at $\delta_{\text{H}} 3.78$ as a doublet of doublet with coupling constant 4.2 and 8.3 Hz. The signal which appeared at $\delta_{\text{H}} 1.48$ as dd (5.1, 11.7 Hz) was assigned to H-10.

The upfield OCH$_3$ appeared at $\delta_{\text{C}}$ 39.9 and $\delta_{\text{H}}$ 3.78 as a doublet of doublet ($\delta_{\text{C}}$ 132.1 and $\delta_{\text{H}}$ 6.43 br s) in ppm relative to TMS; coupling constants (J in Hz) are given in parentheses.

The NOESY experiment (Fig. 3) showed that we are dealing with an A/B diterpenoid. 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. A cis A/B ring junction was also evident from the 13C-NMR chemical shifts of C-19 methyl carbon ($\delta_{\text{C}}$ 31.7) and C-20 methyl carbon ($\delta_{\text{C}}$ 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. By comparing spectral data with literature; Me$_3$-19 was confirmed to be $\beta$-oriented. So we are dealing with cis-clerodanes. In cis-clerodanes, C-19 carbon resonates at about $\delta_{\text{C}}$ 25, whereas in trans-clerodanes it appears at $\delta_{\text{C}}$ 11—19. Moreover, C-20 in cis-clerodanes resonates at lower field ($\delta_{\text{C}}$ 21—29) than in trans-clerodanes ($\delta_{\text{C}}$ 17—19).

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$_2$O]$^+$ accompanied by the loss of H$_2$O molecule, like in compound 1. It lacked the five-membered lactone ring on C-4 and C-6 position. Compound 2 contained free $\alpha,\beta$-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 $\alpha,\beta$-unsaturated acid and at 1755 cm$^{-1}$ which is characteristic for $\gamma$-lactones. The UV spectrum showed an absorption band at $\lambda_{\text{max}}$ 212 nm. In $^1$H-NMR spectrum the typical olefinic signal appeared at $\delta_H$ 6.80 as a broad triplet with coupling constant 3.5 Hz. This signal showed cross peak analysis to the carbonyl carbon C-18 ($\delta_C$ 171.8), C-19 ($\delta_C$ 32.3), C-4 ($\delta_C$ 139.5), C-5 ($\delta_C$ 38.2) and C-2 ($\delta_C$ 28.3) which supported the presence of free acid on C-4. The two tertiary methyl signals were observed at $\delta_H$ 1.28 and $\delta_H$ 0.70, and one secondary methyl appeared as doublet at $\delta_H$ 0.84 with coupling constant 7.0 Hz. Similarly, the olefinic proton resonated at $\delta_H$ 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 ($\delta_C$ 173.1), C-15 ($\delta_C$ 103.4), C-13 ($\delta_C$ 133.3) and C-12 ($\delta_C$ 20.3). The methylene signal which appeared at $\delta_H$ 2.40 also supported the lack of lactone ring on C-6 position. The H$_3$-15 (1H) was found to have the molecular formula C$_{20}$H$_{28}$O$_4$ derived from electron impact mass spectrometry (HR-EI-MS) and $^{13}$C-NMR spectra, compound 2 showed absorption at 1685 cm$^{-1}$ which is characteristic for $\gamma$-lactones. The UV spectrum showed an absorption band at 1685 cm$^{-1}$ which is characteristic for $\gamma$-lactones. The IR spectrum of compound 2 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.

### 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 spectrometer with tetramethylsilane (TMS) as an external standard. 2D NMR spectra 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 accompanied by the loss of H$_2$O molecule.

#### Extraction and Purification

The air-dried roots of *Otostegia limbata* (Lamiaceae) were exhaustively extracted with methanol (501±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 as a gradient. Eleven fractions were collected. Fraction no. 2 (5.4 g) was subjected to column chromatography and eluted with EtOAc: hexane (7:3) to purify compound 1 (3.4 mg). Fraction no. 9 (9.3 g) was subjected to column chromatography and eluted with EtOAc: hexane (23:73) 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; oleic acid and $\beta$-sitosterol. The purity of the compounds was checked on TLC and HPTLC plates.

### In Vitro Quantitative Inhibition of AchE and BChE by Compounds 1–3

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$±S.E.M. ($\mu$M)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>AchE</td>
</tr>
<tr>
<td>1</td>
<td>38.5±0.20</td>
</tr>
<tr>
<td>2</td>
<td>47.2±0.30</td>
</tr>
<tr>
<td>3</td>
<td>103.7±0.50</td>
</tr>
<tr>
<td>Galanthamine*</td>
<td>0.5±0.01</td>
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</tbody>
</table>

*Standard mean error of 3–5 assays, *a* positive control used in assays.

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### Table 2.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$±S.E.M.* ($\mu$M)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>385±0.20</td>
</tr>
<tr>
<td>2</td>
<td>472±0.30</td>
</tr>
<tr>
<td>3</td>
<td>1037±0.50</td>
</tr>
<tr>
<td>Galanthamine*</td>
<td>0.5±0.01</td>
</tr>
</tbody>
</table>

*Standard mean error of 3–5 assays, *a* positive control used in assays.
13C-NMR (CDCl₃, 100 MHz), see Table 1. IR ν max (CHCl₃) cm⁻¹: 2956, 1750, 1678. UV λ max (MeOH) nm (log ε): 213 (3.8). EI-MS m/z (rel. int.): 314 [M⁺H₂O]⁺ (99), 299 (50), 273 (35), 212 (38), 203 (70), 173 (55). HR-EI-MS m/z: 314.2373 (Calcd for C₂₀H₂₆O₃·H₂O: 314.3858). [α]D₂₃° 98.231° (c/CHCl₃ 0.063, CHCl₃).

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. 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 thiocarbamoyl, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively. The percentage (%) inhibition was calculated as follows (E/S = E×100, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

Determination of IC₅₀ Values The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC₅₀) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC₅₀ values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

References and Notes